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Coordination between Chromosome Replication, Segregation, and Cell Division in *Caulobacter crescentus*

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Progression through the *Caulobacter crescentus* cell cycle is coupled to a cellular differentiation program. The swarmer cell is replicationally quiescent, and DNA replication initiates at the swarmer-to-stalked cell transition. There is a very short delay between initiation of DNA replication and movement of one of the newly replicated origins to the opposite pole of the cell, indicating the absence of cohesion between the newly replicated origin-proximal parts of the *Caulobacter* chromosome. The terminus region of the chromosome becomes located at the invaginating septum in predivisional cells, and the completely replicated terminus regions stay associated with each other after chromosome replication is completed, disassociating very late in the cell cycle shortly before the final cell division event. Invagination of the cytoplasmic membrane occurs earlier than separation of the replicated terminus regions and formation of separate nucleoids, which results in trapping of a chromosome on either side of the cell division septum, indicating that there is not a nucleoid exclusion phenotype.

Reliable transmission of genetic material to cellular offspring is a fundamental challenge for all organisms. It requires DNA replication, disentanglement of the replicated DNA molecules, efficient segregation of sister chromosomes into the two daughter cells, and coordination of these processes with other cell cycle events, like cell division. Most bacteria possess a single circular chromosome, and DNA replication is normally initiated at a single origin of replication (*ori*) (33). DNA replication proceeds bidirectionally toward the terminus (*ter*) region, where the two replication forks meet, resulting in termination of DNA replication. Several determinants involved in sister chromosome separation are located in the terminus region (31). Studies of the subcellular localization of specific chromosomal regions and components of the replication machinery have shown that bacterial chromosomes are spatially organized within the cell in a dynamic fashion that changes during DNA replication and segregation (13–15, 17, 19, 25, 28, 29, 36, 37, 48–51). Individual loci on the circular chromosome occupy specific positions within the cell and move rapidly and with direction during the DNA segregation process (37, 48). The specific localization patterns are different for different genera and growth conditions, but the fact that bacteria localize their origin and terminus regions of the chromosome at specific positions indicates that there are mechanisms that ensure correct organization and subcellular placement of the chromosome.

The spatial organization of the chromosome and the DNA replication apparatus has been well characterized in *Caulobacter crescentus* (19, 22, 48). *Caulobacter* exists as morphologically and functionally distinct cell types, and cellular differentiation is an integral part of its cell cycle (21, 44, 45). Asymmetrical cell division results in two different progeny cells, a replicative stalked cell and a nonreplicative swarmer cell. In the G_1-arrested swarmer cell, the origin-proximal region of the chromosome is located near the flagellated pole, the terminus-proximal part of the chromosome is located near the opposite pole of the cell, and regions in between are located in the cell with a linear correlation between their positions on the chromosome and their locations in the cell (19, 22, 48). Thus, regions close to the origin on the genetic map are also located close to the origin within the cell. After a specific time, the swarmer cell differentiates into a stalked cell by shedding the flagellum, synthesizing a stalk at the same pole, and initiating chromosome replication. One of the replicated origins moves to the opposite pole of the cell, while the other stays at the original pole (19, 48). Loci along the arms of the chromosomes are sequentially moved in the order that they are replicated to their final destinations in the predivisional cell (22, 48). Cell division results in formation of a replicationally quiescent swarmer cell and a stalked cell, which initiates a new round of DNA replication immediately after cell division. The temporal coordination between DNA replication and separation of the origin and terminus regions of the chromosome in *Caulobacter* has not been characterized.

In *Escherichia coli*, separation and movement of the origin regions may be delayed compared to when they are replicated, suggesting that newly replicated regions of the chromosome cohere to each other (2, 18, 28, 46). Some studies have indicated that cohesion is absent or the period of cohesion is brief (8, 25, 28, 43), but other studies have shown that the period of cohesion is quite long and is observed during most of the S phase (2, 18, 46). Separation of the fully replicated terminus regions occurs late in the cell cycle, shortly before cell division (2, 25, 28, 29). Thus, separation of the replicated terminus regions is delayed significantly compared to completion of DNA replication.

Both *E. coli* and *Bacillus subtilis* use a mechanism called nucleoid occlusion to ensure that cell division does not take place until chromosome segregation has been completed, thereby avoiding catastrophic breakage of the chromosome by division through the nucleoid (35). In both organisms, a DNA-binding protein associated with the bulk of the chromosomal DNA inhibits assembly of the cell division apparatus in the vicinity of the nucleoid (3, 54). This prevents initiation of cell division until the daughter chromosomes are separated from

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each other, forming a DNA-free region or a region with a low concentration of DNA in the middle of the cell. The *Caulobacter* genome does not contain obvious homologs of the previously characterized nucleoid occlusion proteins, and it is not known if nucleoid occlusion actually takes place in *Caulobacter*.

In this work, I examined the coordination between chromosome segregation and cell cycle events like DNA replication and cell division in *Caulobacter* by simultaneously characterizing the DNA replication status using flow cytometry and cellular events like origin and terminus localization or cell division using fluorescence microscopy in cells progressing synchronously through the cell cycle. The results showed that there was not cohesion of the newly replicated origin-proximal regions of the chromosome but there was a significant delay in separation of the replicated terminus regions. Cell division is initiated before final separation of the daughter chromosomes in *Caulobacter*, implying that there is not nucleoid occlusion.

MATERIALS AND METHODS

Strains. To create strains with specific regions of the chromosome tagged with cyan fluorescent protein (CFP)-LacI, the *xylX*::pHPV472 allele expressing the LacI-CFP chimeric protein from a xylose-inducible promoter (48) was moved into the synchronizable wild-type strain CB15N (also designated NA1000) (12) by bacteriophage ΦCr30-mediated general transduction (9) with a lysate prepared from strain MO6 (48), resulting in strain RBJ176. A *lacO* array cassette (25) inserted at position 3,973,878 in the *Caulobacter* chromosome was transduced into the RBJ176 strain with a ΦCr30 lysate prepared from strain MPO17 (48), resulting in strain RBJ185. This strain has the origin-proximal part of the chromosome tagged with CFP-LacI. A *lacO* array cassette inserted into the terminus region of the *Caulobacter* chromosome (position 1,968,285) was transduced into RBJ176 with a ΦCr30 lysate prepared from strain ML133 (48), resulting in strain RBJ200. In this strain the terminus region of the chromosome is tagged with CFP-LacI.

Growth conditions. Strains were grown in peptone-yeast extract (PYE) medium (9) at 30°C until they reached an optical density at 450 nm of 0.1 to 0.2. When relevant, expression of the LacI-CFP protein was induced by adding 0.2% xylose, and 50 μM isopropyl-β-D thiogalactoside (IPTG) was added to reduce binding of the repressor protein. After 30 to 45 min of induction, the cells were synchronized as described previously (47). The isolated swarmer cells were resuspended in PYE (containing 50 μM IPTG for the LacI-CFP-expressing strains), incubated with shaking at 30°C, and allowed to synchronously proceed through the cell cycle. Samples were collected every 10 min for a total of 100 min and fixed for either flow cytometry or microscopy.

Flow cytometry analysis of DNA replication. Samples used for flow cytometry were fixed using cold 70% ethanol as described previously (52) at the same times and from the same cultures as the samples used for fluorescence microscopy. Batch cultures were fixed either without any additional treatment or after addition of 60 μg/ml rifampin and 36 μg/ml cephalexin, followed by incubation at 30°C for 3 h to allow ongoing rounds of replication to be completed. Staining using mithramycin and ethidium bromide and flow cytometry was performed as described previously (30), using an A10-Bryte flow cytometer (Apogee Flow Systems Ltd.). Data from synchronized cultures were analyzed using the programs AFSCtrl (version 1.61; Apogee Flow Systems Ltd.) and Microsoft Excel 2002 (Microsoft Corporation). The percentage of the chromosome replicated was determined based on the average channel number of the flow cytometry DNA signal. To determine the percentage of cells that had initiated DNA replication, a normal distribution was fitted to the DNA histogram of isolated swarmer cells, where ~98% of the cells had a single nonreplicating chromosome. This normal distribution was fitted to the left parts of the one-chromosome peaks in the DNA histograms from the early times during the synchrony. The number of cells in the fitted one-chromosome peak and the total number of cells were used to calculate the percentage of cells that had initiated DNA replication. Similarly, a normal distribution was fitted to the two-chromosome peak in a DNA histogram from a sample taken at 90 min, where gating based on the cell size (light scatter) was used to remove the DNA signals from divided cells. This two-chromosome peak normal distribution was fitted to the right parts of the two-chromosome peaks in the DNA histograms from the late times of the synchrony. Control experiments showed that the flow cytometer gave a signal during data acquisition for all the samples from a single experiment that was sufficiently stable to make this type of comparison between different samples valid. The percentage of cells that divided was determined by gating the light scatter and DNA fluorescence flow cytometry data in order to determine the percentage of the cells that were small and had a low DNA content at the late times during the synchrony. Cytograms were displayed, and DNA histograms for mixed cultures of exponentially growing cells were analyzed as described previously (34), using the program WinFlow (version 3.0; Flemming G. Hansen, The Technical University of Denmark, Lyngby, Denmark).

Microscopy. Synchronized *Caulobacter* cells were fixed by addition of 2.5% paraformaldehyde and 30 mM $NaPO_4$ buffer (pH 7.5) and incubation for at least 1 h at 0°C. The fixation solution was removed by gently spinning the cells. Identical localization patterns were obtained using live and fixed cells. The cells were immobilized using a thin layer of agarose as described previously (16). When relevant, 2 μg/ml *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)-phenyl)hexatrienyl)pyridinium dibromide (FM4-64) was added to the agarose to fluorescently label the cytoplasmic membrane, or 1 μg/ml 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) was added to stain the chromosomal DNA. Similar nucleoid staining patterns were obtained using live cells and cells fixed using formaldehyde, methanol, or osmium tetroxide, as well as with cells stained with the DNA-specific dyes DAPI, propidium iodide, and SYTO16. Phase-contrast and fluorescence images were acquired using a Leica DM5000B microscope with a ×100 HCX PL APO NA 1.4 objective and a Leica DFC350FX cooled charge-coupled device camera controlled through FW4000 software (version 1.2.1; Leica Microsystems). Images were processed using ImageJ (Wayne S. Rasband, National Institutes of Health, Bethesda, MD) and Photoshop CS (Adobe). The different cell types and localization patterns were counted manually using ImageJ and the Cell Counter plug-in (Kurt De Vos, Columbia University, New York, NY). At least 300 cells were counted for each data point.

RESULTS

Cytological tools for characterizing chromosome organization. The coordination between DNA replication, chromosome movement, and cellular morphology in synchronized *Caulobacter* cells was characterized using LacI-CFP-expressing strains with a cassette containing tandem *lac* operator arrays integrated in the origin- or terminus-proximal region of the chromosome (48). The intracellular location of the tagged region of the chromosome was visualized based on the fluorescence signal from the LacI-CFP chimera binding to the *lacO* cassette. Cell morphology was examined both by phase-contrast microscopy and by staining the cytoplasmic membrane using the fluorescent lipophilic styryl dye FM4-64 (39). The chromosome replication status and size of the cells were simultaneously characterized using flow cytometry. Only a single operator cassette was integrated in the strain, the expression of the fluorescent repressor was kept at the minimum level required to detect the signal, and repressor binding was reduced by adding an intermediate amount of the LacI inducer IPTG. Under these conditions and with the cassette inserted 43 kbp anticlockwise from the replication origin or in the terminus region of the chromosome, no effect on cell cycle progression, DNA contents, or cell size was observed using flow cytometry (Fig. 1A to D). High-level production of the fluorescent repressor protein, use of a cassette insertion site closer to the origin, or simultaneous use of two different operator cassettes resulted in detectable cell cycle defects (data not shown), so these conditions were not used in this work.

To compare the durations of the G_1, S, and G_2/M phases of the cell cycle observed using synchronized tagged cells with the durations in batch cultures of nonsynchronized exponentially growing wild-type cells, computer simulations of DNA histograms (34) were performed (Fig. 1E and F). The same growth

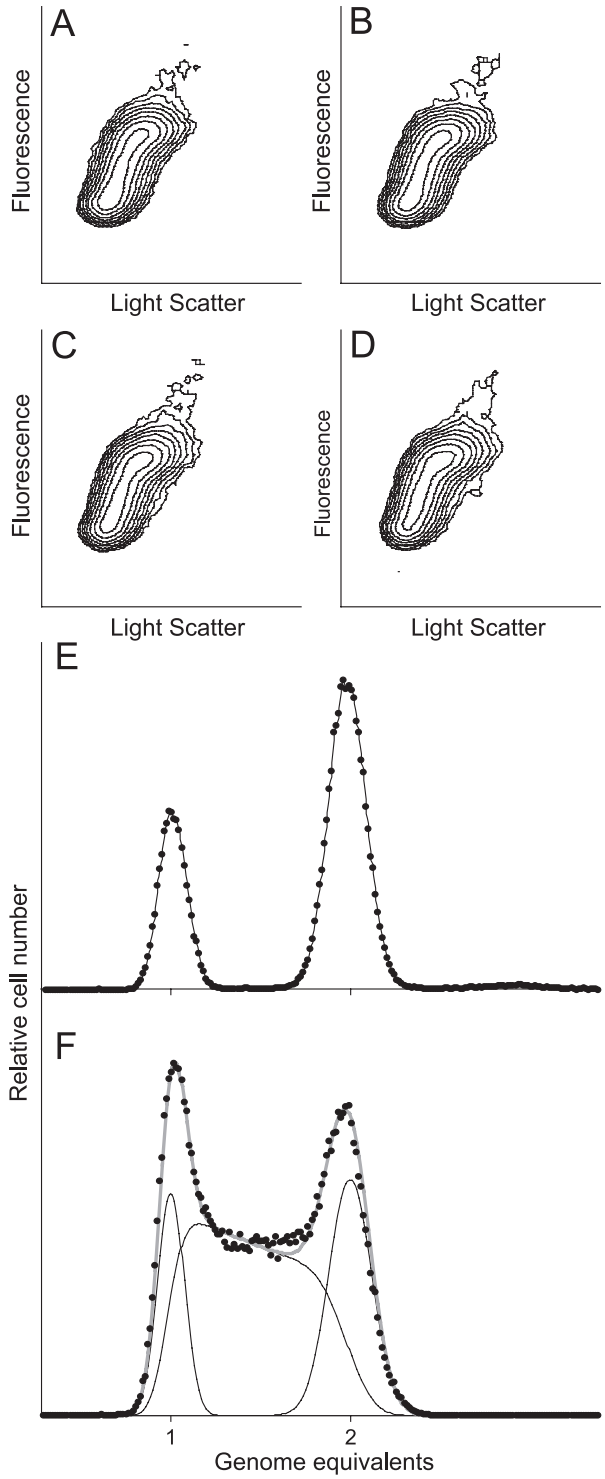

FIG. 1. Analysis of the cell cycle in tagged and wild-type *Caulobacter* cells using flow cytometry. (A to D) Cytograms showing the sizes and DNA contents of *Caulobacter* cells with specific regions of the chromosome labeled using CFP-LacI. Orthogonal light scatter is proportional to cell size, and fluorescence is proportional to the DNA content of the cells. (A and B) Cytograms of strain RBJ185 in which a cassette containing tandem repeated *lac* operators is integrated near the origin. (C and D) Cytograms of strain RBJ200 in which the *lac* operator cassette is integrated in the terminus region of the chromosome. (A and C) Strains in the absence of expression of the CFP-LacI protein. (B and D) Cytograms of the same cultures after induction of CFP-LacI as described in Materials and Methods. Approximately 100,000 cells were analyzed. (E and F) Analysis of the cell cycle in exponentially growing wild-type *Caulobacter* cells using computer simulations of DNA histograms obtained by flow cytometry. (E) DNA distribution pattern (solid circles) of wild-type strain CB15N treated with rifampin to block initiation of replication and cephalexin to block cell division. Ongoing rounds of DNA replication continue, resulting in accumulation of cells with either one or two fully replicated chromosomes. The data were used to determine the means and standard deviations of normal distributions fitted to the one- and two-chromosome peaks. (F) DNA distribution pattern (solid circles) of a mixed untreated exponentially growing CB15N culture. Simulations of the cells in the G_1, S, and G_2/M phases are indicated by thin black lines. The thick gray curve represents the sum of the simulated cells in different stages of the cell cycle. The means and standard deviations for the one- and two-chromosome peaks obtained from rifampin-treated cells (A) were used in the simulation to determine the proportions of cells in the different cell cycle stages.

conditions were used for the synchronized cultures and the exponentially growing cultures. Simulation of the cell cycle in exponentially growing nonsynchronized cells resulted in an 18- ± 2-min G_1 phase; the length of the S phase was 50 ± 2 min, and the G_2/M phase was 27 ± 3 min long. The cell cycles in synchronized cultures of tagged strains were characterized by performing a flow cytometry analysis of samples taken at different times during the cell cycle (see Materials and Methods). The time of occurrence of an event in a synchronized culture is the time at which 50% of the cells have entered the corresponding stage (2). Initiation of DNA replication took place ~16 min into the cell cycle of synchronized tagged strains, replication proceeded at approximately the same rate until the entire chromosome had been replicated at ~64 min, and the cells divided approximately 95 min into the cell cycle (Fig. 2B, 3B, and 4B). Thus, the cell cycle timing of the tagged strains in synchronized cultures was essentially the same as the cell cycle timing in a batch-grown culture of the parent wild-type strain. Therefore, the CFP-LacI tagging of the chromosome and the synchronization procedure used in this work did not appear to significantly affect cell physiology.

Timing of origin movement. The timing of origin movement was compared to the timing of initiation of DNA replication by synchronizing a LacI-CFP-expressing strain with a *lac* operator cassette inserted 43 kbp anticlockwise from the replication origin. Four different localization patterns of the origin-proximal part of the chromosome were observed (Fig. 2). Nonreplicating swarmer cells had a single origin focus located in the flagellated-pole-proximal 15% of the cell. Shortly after initiation of DNA replication, one of the newly replicated origins moved to the opposite pole of the cell, while the other origin copy remained at its previous location, resulting in formation of cells with a bipolar origin localization pattern. Transiently, during the part of the cell cycle in which initiation of DNA replication took place, a few cells with a single nonpolar origin focus or two asymmetrically located foci, with at least one of the foci in the central part of the cell, were observed (Fig. 2C). These cells were most likely in the process of moving one of the newly replicated origin regions and therefore were intermediates in the DNA segregation process. The low abundance of cells with origin localization patterns that reflected intermedi-

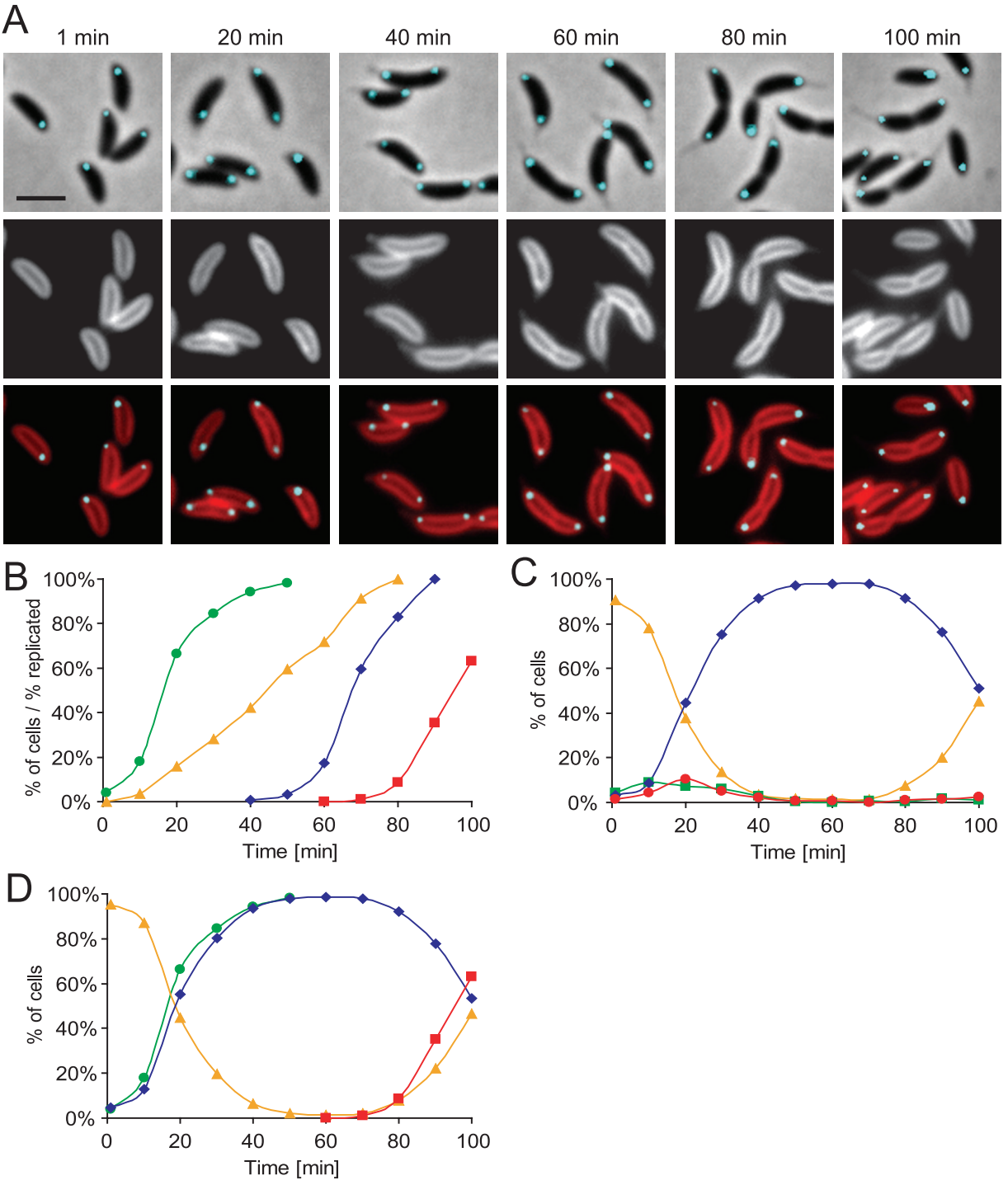

FIG. 2. Intracellular location of the origin-proximal region of the chromosome during the *Caulobacter* cell cycle. (A) Phase-contrast and fluorescence microscopy images of RBJ185 cells with the origin-proximal part of the chromosome tagged using CFP-LacI. The cells were synchronized, and samples were removed at different times after incubation of the isolated swarmer cells in PYE growth medium. The top panel shows overlays of phase-contrast and CFP fluorescence (cyan) images. The middle panel shows fluorescence signals from the dye FM4-64, which specifically binds to the cytoplasmic membrane. The lower panel shows overlays of the fluorescence signals of the cytoplasmic membrane dye (red) and the CFP fluorescence (cyan) marking the position of the origin-proximal region of the chromosome. Scale bar = 2 μm. (B) DNA replication and cell division status of the synchronized culture determined using flow cytometry. The green circles indicate the percentage of cells that have initiated DNA replication, the yellow triangles indicate the progression of DNA replication, the blue diamonds indicate the percentage of cells that have completed chromosome replication, and the red squares indicate the percentage of cells that have divided. (C) Quantification of the abundance of cells with different origin localization patterns. The yellow triangles indicate cells with a single origin focus in the pole-proximal 15% of the cell, the green squares indicate cells with a single origin focus in other parts of the cell, the blue diamonds indicate cells with two bipolarly located origin foci, and the red circles indicate cells with two origin foci with other localization patterns (e.g., one focus at a pole and one focus near the middle of the cell). (D) Data from panels B and C combined. The yellow triangles indicate cells with a single pole-proximal origin focus, the green circles indicate cells that have initiated DNA replication, the blue diamonds indicate cells with two origin foci, and the red squares indicate cells that have divided.

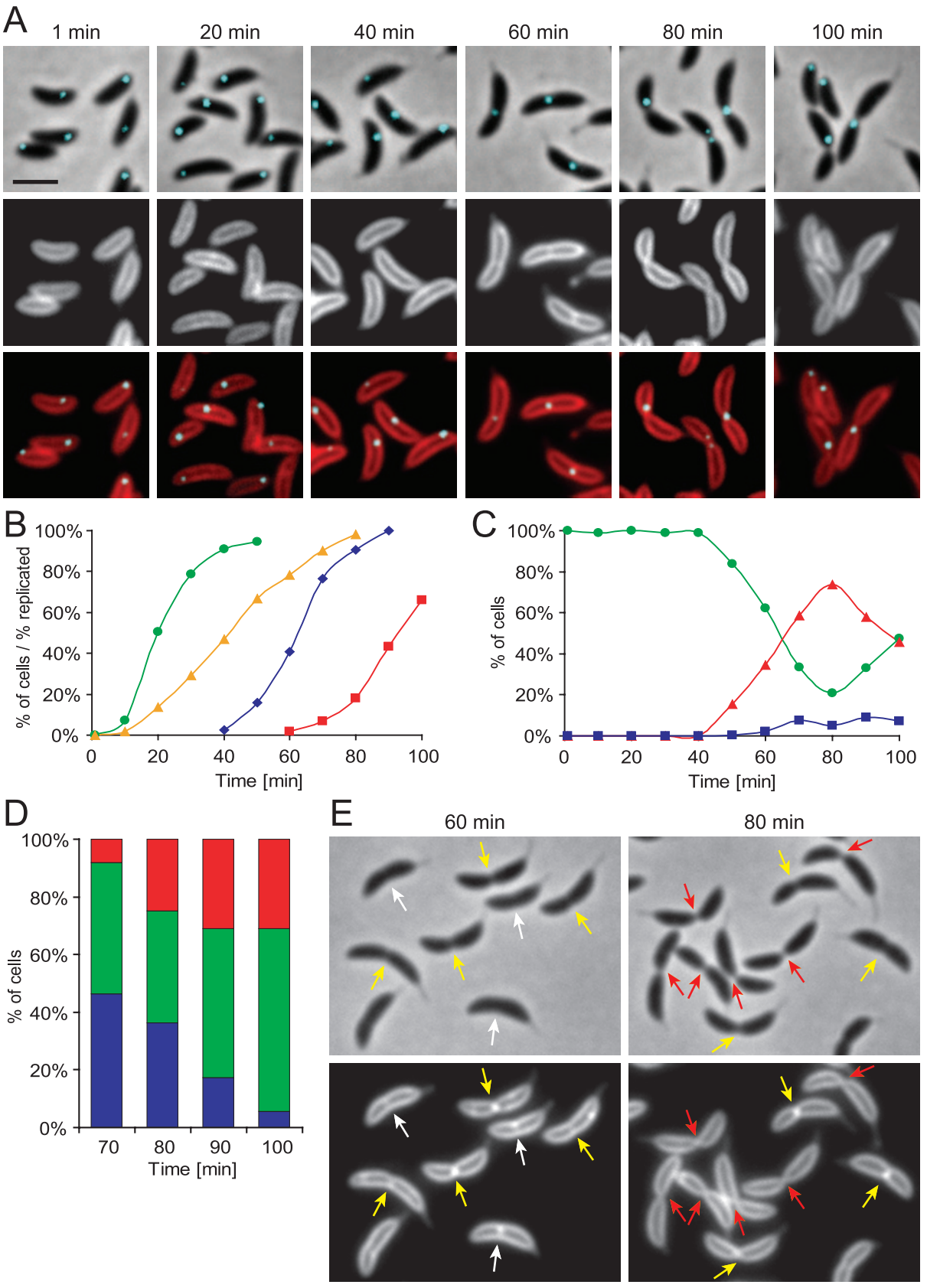

FIG. 3. Intracellular location of the chromosomal terminus region during the *Caulobacter* cell cycle. (A) Fluorescence and phase-contrast microscopy images of RBJ200 cells with the terminus region tagged using CFP-LacI progressing synchronously through the cell cycle. The top panel shows overlays of phase-contrast and CFP fluorescence (cyan) images. The middle panel shows the FM4-64-labeled cytoplasmic membrane. The

ates in the origin segregation process indicated that origin movement was a fast process, as previously observed using time-lapse microscopy (48). When the timing of initiation of DNA replication was compared with the timing of appearance of cells with two separated origin foci (Fig. 2D), a very short 2- to 4-min delay between initiation of DNA replication and origin movement was observed.

Terminus movement and separation. The coordination between cell cycle progression, terminus separation or movement, and cell division was examined using a strain with the terminus region of the *Caulobacter* chromosome tagged with LacI-CFP. In nonreplicating swarmer cells, the terminus region of the chromosome is initially located near the pole opposite the origin (20, 48). During the DNA replication process, the terminus region moved from the pole opposite the stalk to near midcell (Fig. 3). Even after completion of DNA replication, the majority of the cells had only a single terminus focus (Fig. 3B and C). Only in deeply constricted late predivisional cells were two separated terminus foci observed, and the low frequency (~5%) of cells with two separated terminus foci indicated that the life span of the cellular stage with separated termini was quite short. Similar terminus localization patterns and frequencies of cells with separated termini were observed in wild-type *Caulobacter* cells using fluorescence in situ hybridization (data not shown). Thus, the completely replicated terminus regions of sister chromosomes stayed associated with each other for an extended period (20 to 30 min) after chromosome replication was completed. The terminus regions disassociated shortly before the final cell separation step in the cell division process. The intracellular location of the terminus focus in predivisional cells varied significantly (Fig. 3D). In early predivisional cells, the terminus focus was located primarily in the flagellated part of the cell or at the invaginating septum. In late predivisional cells, the terminus was located primarily at the septum or in the stalked compartment of the cell. The high proportion of predivisional cells with the terminus region located at or very close to the invaginating septum indicated that the terminus was actively trapped or localized at the future division site.

Chromosome trapping by the invaginating septum. Cell division and chromosome segregation have to be coupled in order to avoid the formation of nucleoid-free cells or chromosome breakage at cell division. To investigate the coordination between chromosome segregation and cell division, the number of cells with any sign of constriction at the future division site in phase-contrast microscopy images was determined (Fig. 3). This revealed that initiation of cell constriction, an early step in the cell division process, took place at approximately the same time in the cell cycle as completion of chromosome replication. Simultaneous staining of the cytoplasmic membrane using the fluorescent dye FM4-64 and examination of cell morphology using phase-contrast microscopy revealed that the cell constriction process involves several distinct steps. At 60 min, cells with visible ingrowth of the cytoplasmic membrane but no sign of indentation as determined by phase-contrast microscopy were observed (Fig. 3E). The bright signal from the membrane dye at the invaginating septum reflected the fact that two membranes were located close to each other, which resulted in a more intense signal. Cells with constriction visible both by cytoplasmic membrane staining and phase-contrast microscopy were also observed (Fig. 3E). Late in the cell cycle, very deeply constricted cells were observed (Fig. 3E). Cytoplasmic membrane staining indicated that separate compartments were present in the two halves of the late predivisional cells. The vast majority of the constricted cells had only a single terminus focus (Fig. 3A and C), showing that cytoplasmic membrane invagination took place well before separation of the replicated sister chromosome termini.

To examine coordination between cell division and nucleoid separation, the chromosomal DNA in synchronized wild-type cells were stained using the DNA-specific fluorescent dye DAPI (Fig. 4). Chromosomal DNA was present in the entire cell at all stages during the cell cycle, and no distinct nucleoids and chromosome-free regions, like those observed in *E. coli* and *B. subtilis* (42, 56), were present in *Caulobacter*. Simultaneous staining of the cytoplasmic membrane and the chromosome showed that initiation of invagination of the cytoplasmic membrane in *Caulobacter* takes place over the nucleoid. Pinched cells had a single bilobed nonseparated nucleoid with significant DNA staining at the invaginating septum. Only deeply constricted late predivisional cells had completely separated nucleoids in which no DNA staining was observed at the division site (Fig. 4). Thus, most of the *Caulobacter* cell division processes take place before complete separation of the replicated chromosomes. Only the last step in the cell division process, final cell separation, takes place after

lower panel shows overlays of the fluorescence signals of the cytoplasmic membrane dye (red) and the CFP fluorescence (cyan) marking the position of the chromosome terminus region. Scale bar = 2 μm. (B) DNA replication and cell division status of the synchronized culture. The green circles indicate cells that have initiated DNA replication, the yellow triangles indicate the progression of DNA replication, the blue diamonds indicate cells that have completely replicated the chromosome, and the red squares indicate cells that have divided. (C) Quantification of cells with different terminus localization patterns and the presence of an invagination at the future division site. The green circles indicate smooth cells with a single terminus focus, the red triangles indicate cells with a single terminus focus that have a visible constriction at the future division site, showing that the cell division process has been initiated, and the blue squares indicate constricted cells with two separated terminus foci. No smooth cells with two terminus foci were observed. (D) Quantification of the location of the terminus focus in predivisional cells at different times during the cell cycle. Blue indicates cells in which the terminus focus is located in the flagellated part of the predivisional cell, green indicates predivisional cells in which the terminus focus is located at the invaginating septum, and red indicates predivisional cells with the terminus focus in the stalked part of the cell. (E) Phase-contrast and cytoplasmic membrane staining images of early and late predivisional cells having different membrane invagination patterns. The white arrows indicate cells in which a clear invagination of the cytoplasmic membrane could be seen using the cytoplasmic membrane dye but no invagination of the cell was visible by phase-contrast microscopy. The yellow arrows indicate cells with the cytoplasmic membrane invaginated and a mild indentation at the future division site that could be observed by phase-contrast microscopy. The red arrows indicate late predivisional cells in which phase-contrast microscopy showed a very deep invagination at the future division site and the cytoplasmic membrane stain indicated that separate compartments were formed in the two parts of the cell.

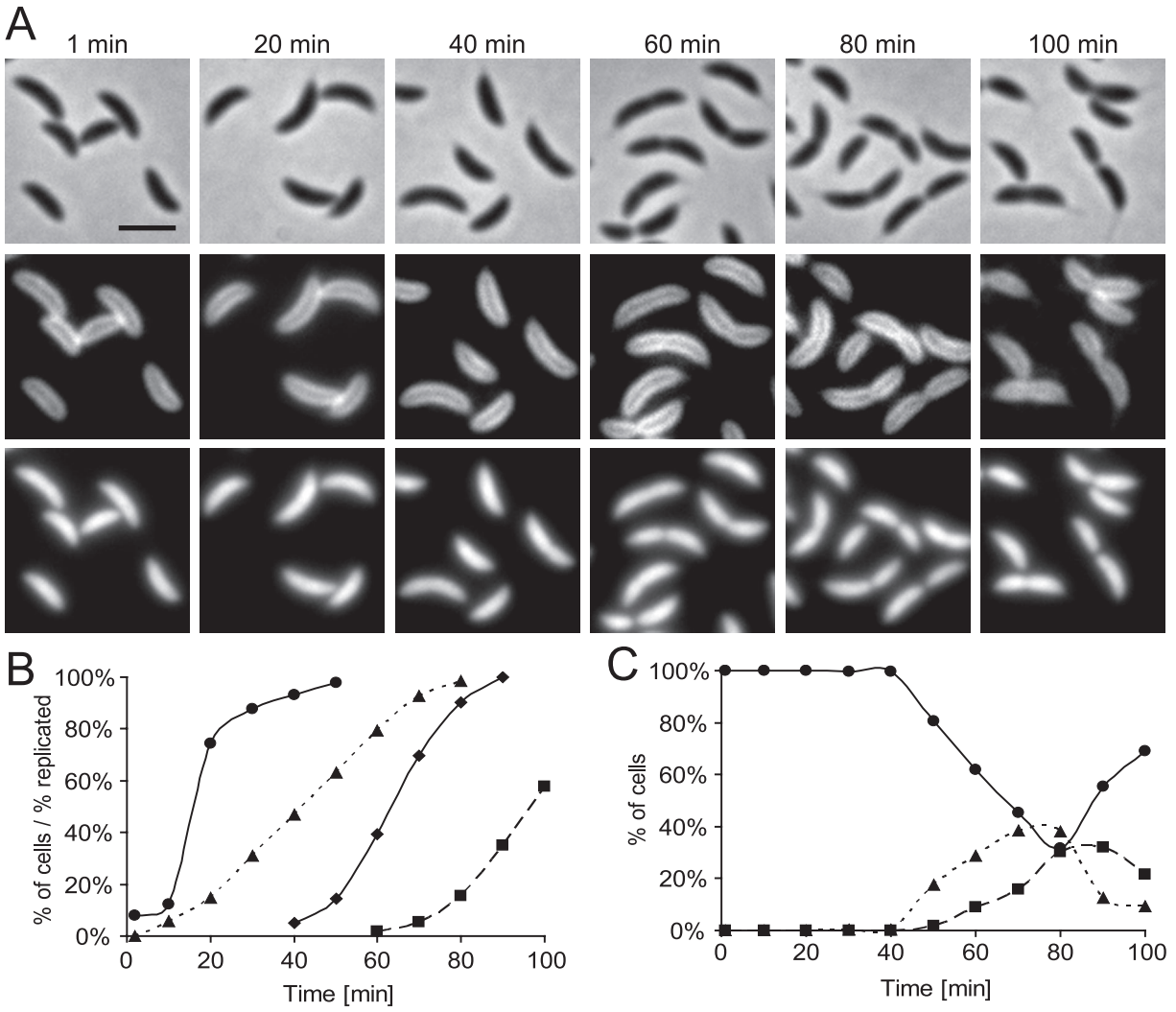

FIG. 4. Nucleoid morphology during the *Caulobacter* cell cycle. (A) Phase-contrast and fluorescence microscopy images of wild-type CB15N cells with the chromosomal DNA stained using DAPI. The cells were synchronized, and cell samples were removed at different times during incubation of the isolated swarmer cells in growth medium. The top panel shows phase-contrast images, and the middle panel shows the FM4-64 signal from the cytoplasmic membrane. The lower panel shows the fluorescence signal from DAPI binding to DNA. Scale bar = 2 μm. (B) DNA replication and cell division status of the synchronized culture. The circles indicate the cells that have initiated DNA replication, the triangles indicate the progression of DNA replication, the diamonds indicate cells that have completely replicated the chromosome, and the squares indicate the cells that have divided. (C) Quantification of cells with different nucleoid morphology patterns and the presence of a constriction at the future division site. The circles indicate smooth cells with a single nucleoid, the triangles indicate cells with a single bilobed nucleoid that have a visible indentation at the future division site, and the squares indicate constricted cells with two separated nucleoids. No smooth cells with a single bilobed nucleoid or two separate nucleoids were observed.

separation of the sister chromosome terminus regions (Fig. 3) and complete segregation of the nucleoids (Fig. 4).

DISCUSSION

Generation of viable offspring is very complicated, involving many different processes that all have to be coordinated with each other both temporally and spatially. Despite recent advances, the mechanisms by which different bacterial cell cycle events are coordinated have not been well characterized (5, 32). *Caulobacter* is particularly well suited for the study of bacterial cell cycle control. The unique characteristics of this organism include asymmetric cell division that results in progeny with distinct morphological features and fates (45), which makes it easier to track the intracellular location and movement of cell cycle proteins and chromosomal regions. Synchronized cell populations can be obtained without perturbing the normal physiology of the cell (Fig. 1), which allows examination of morphology, protein and DNA localization, and gene expression at defined stages of the cell cycle. Additionally, clear G_1, S, and G_2/M phases of the cell cycle can be identified since initiation of replication takes place only once per cell cycle. However, in most of the previous work in which different events during the *Caulobacter* cell cycle were examined, only a single process was examined at a time. When the results of different laboratories or even different papers from the same

research group are compared, very different data for timing of cell cycle events and duration of the entire cell cycle are found, making it difficult to compare the data for timing of different cell cycle events to each other. In this work, growth conditions were standardized to ensure equivalent values for timing of cell cycle events in different strains and for experiments done on different days. Additionally, multiple types of data were simultaneously acquired from synchronized *Caulobacter* cultures to obtain information about several different cell cycle events.

In *E. coli* newly replicated origin-proximal regions of the chromosome may cohere to each other, resulting in a delay in separation and movement of the origin regions (2, 18, 28, 46). The temporal extent of the period of cohesion is debated; according to some studies the period of cohesion is brief or absent (8, 25, 28, 43), but other studies have shown that the period of cohesion is quite long, taking place during most of S phase (2, 18, 46). Previous studies of chromosome organization in *Caulobacter* did not examine the temporal relationship between DNA replication and movement of the origin-proximal part of the chromosome, since only origin movement was characterized (19, 48). By simultaneously examining DNA replication using flow cytometry and the intracellular location of the origin-proximal part of the chromosome using LacI-CFP binding to a *lac* operator cassette inserted near *ori*, the coordination between these processes was characterized (Fig. 2). Within 2 to 4 min of initiation of DNA replication, cells with two separated origin foci were observed. The *lac* operator cassette used to visualize the intracellular location of the origin-proximal region was inserted 43 kbp from the origin, which was expected to be replicated approximately 1 min after initiation of DNA replication. Additionally, because of the limited resolution of light microscopy, origin foci had to move at least 0.2 μm from each other before separate foci could be observed. With the previously observed rate of origin movement (48), this could result in up to a 1-min delay between initiation of movement and the time when separated origin foci could be observed by light microscopy. Thus, the very short time between initiation of DNA replication and separation of the replicated origins showed that no cohesion between the newly replicated origin-proximal regions of the sister chromosome takes place in *Caulobacter*. Alternatively, the cohesion period is very short, less than 2 min. The newly replicated DNA regions appear to stay associated for only enough time to allow homology-directed repair of replication errors.

In contrast, there appears to be a very significant delay, at least 20 min, between replication of the terminus region of the *Caulobacter* chromosome and the appearance of two separated terminus foci in the cells (Fig. 3). This indicates that the terminus regions of sister chromosomes either cohere to each other or for other reasons remain associated with each other for an extended time. The separation of the replicated terminus regions occurs late in the cell cycle, shortly before the final step in the cell division process. A significant delay between completion of DNA replication and separation of the fully replicated sister chromosome terminus regions has also been observed in *E. coli* (2, 25, 28, 29). The potential causes of the delay in terminus separation include formation of a chromosome dimer by homologous recombination and catenation of the sister chromosomes. XerCD-mediated site-specific recombination at the *dif* recombination site located in the terminus is responsible for converting a chromosome dimer to monomers (26), and topoisomerases, primarily topoisomerase IV, are responsible for decatenation (11). Both of these activities are activated by FtsK, a DNA translocase located at the tip of the invaginating septum that has functions both in transporting DNA and in cell division (1, 7, 10, 55). Directional information provided by short repeated sequences with a biased orientation that change abruptly at *dif* may help FtsK determine the direction that its substrate has to be pumped (4, 6, 38). Directed DNA movement by FtsK may be involved in positioning the terminus at the division site. The multiple functions of FtsK may have a role in coupling DNA segregation and cell division temporally and spatially. The XerCD, topoisomerase IV, and FtsK proteins are highly conserved in *Caulobacter*, making it likely that similar mechanisms are employed in terminus localization and separation.

During vegetative growth *E. coli* and *B. subtilis* ensure that cell division does not take place until chromosome segregation has been completed using a specific protein associated with the bulk of the chromosomal DNA (3, 54). This protein inhibits initiation of cell division, thereby preventing assembly of the cell division machinery in the regions of the cell where the chromosome is located. When the daughter chromosomes are separated from each other, forming a DNA-free region in the middle of the cell, cell division is allowed. This mechanism for preventing cell division until completion of chromosome segregation is termed nucleoid occlusion (35). Assembly of the FtsZ ring at midcell, the earliest known step in the cell division process, takes place during the S phase in *Caulobacter* (41). Invagination of the cytoplasmic membrane in *Caulobacter* is observed at approximately the same time that DNA replication is completed, much earlier than separation of the replicated terminus regions and formation of two separate nucleoids (Fig. 3 and 4). Thus, a chromosome is trapped on either side of the cell division septum, implying that there is not nucleoid occlusion of cell division in *Caulobacter*. A similar trapping of the chromosome by an invaginating septum is observed during sporulation in *B. subtilis*, in which an asymmetric cell division event traps about one-third of the chromosome in the prespore compartment; the rest of the chromosome is then transported into the small prespore compartment by the SpoIIIE protein (53). However, when DNA replication is inhibited in *Caulobacter*, FtsZ rings form primarily at polar regions with little or no chromosomal DNA (40). Thus, assembly of the cell division apparatus in *Caulobacter* may have a preference for, but not an absolute requirement for, assembling in regions of the cell with low DNA contents.

Cytoplasmic membrane invagination takes place approximately 20 min before separation of the progeny cells, the final step of the cell division process (Fig. 3). Simultaneous visualization of ingrowth of the cytoplasmic membrane using the fluorescent dye FM4-64 and invagination of the outer part of the cell wall using phase-contrast microscopy indicated that the two events are uncoupled. Invagination of the cytoplasmic membrane occurs before invagination of the outer parts of the cell wall, and several different stages in the cell constriction process can be observed by light microscopy (Fig. 3E). Faster constriction of the cytoplasmic membrane than of the outer membrane was also recently observed by cryoelectron tomography (23). The *Caulobacter* late predivisional cell is compartmentalized, since cytoplasmic and membrane proteins cannot diffuse between the nascent progeny cell compartments (23, 24). At this stage during the cell cycle, the cytoplasmic mem-

brane is very deeply invaginated, and in some cells there is complete separation into separate compartments (Fig. 3) (23). It is likely that the septum formed by cytoplasmic membrane invagination is responsible for preventing diffusion between the two cell parts. Such compartmentalization by a septum is observed during sporulation in *B. subtilis* (27). Additional high-resolution analysis, using a method like cryoelectron tomography, of cells during different stages of cell division is required to obtain a detailed understanding of how and when the inner and outer membranes are closed off, how this is coupled to separation of the terminus region of the chromosome, and if it is involved in formation of a diffusion barrier between the two parts of the cell.

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