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Identification of Two New Cell Division Genes That Affect a High-Molecular-Weight Penicillin-Binding Protein in *Caulobacter crescentus*

PETER NATHAN† AND AUSTIN NEWTON*

Departments of Biology and Molecular Biology, Princeton University, Princeton, New Jersey 08544

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Penicillin-binding proteins (PBPs) are membrane proteins associated with the synthesis of the bacterial cell wall. We report the characterization of 14 PBPs in *Caulobacter crescentus*, using in vivo and in vitro penicillin-binding assays and experiments to determine their possible role in cell division. New conditional cell cycle mutants were isolated by selecting cephalosporin-C-resistant mutants of the β-lactamase strain SC1107 at 30°C that are also defective in cell division at 37°C. They fall into two classes, represented by strains PC8002 and PC8003. Strain PC8002 produced short cells arrested at all stages of cell division at 37°C and was found to contain a high-molecular-weight PBP 1B which was temperature sensitive when assayed in vivo and in vitro. Strain PC8003 was blocked at an early stage of cell division and formed tightly coiled, unpinched filaments. This cephalosporin-C-resistant strain was also defective in PBP 1B, but only when assayed in vivo. PBP 1B behaved like a high-affinity PBP, and in competition assays, β-lactams that induced filamentation bound preferentially to PBP 1B. These results and the phenotype of mutant PC8002 suggest that PBP 1B is required for cell division, as well as for cell growth, in *C. crescentus*. The behavior of strain PC8003 suggests that it contains a conditionally defective gene product that interacts in some way with PBP 1B at an early stage of cell division. We have also examined the PBP patterns of a set of temperature-sensitive cell cycle mutants of *C. crescentus* (M. A. Osley and A. Newton, J. Mol. Biol. 138:109–128, 1980) that are blocked at characteristic stages of cell division. None of the mutants showed an allele-specific PBP pattern when assayed in vitro at the nonpermissive temperature, but all of them displayed temperature-sensitive PBP 1C (102 kilodaltons) activity. Thus, it appears that PBP 1C activity is inhibited at 37°C as a consequence of filamentous growth.

Penicillin-binding proteins (PBPs), located in the bacterial membrane, are defined as penicillin-sensitive enzymes that covalently bind penicillins and related β-lactam antibiotics (for a review, see reference 35). A convenient autoradiographic method for the detection and separation of PBPs (30) established that the bacterium *Escherichia coli* contains at least seven major PBPs, ranging in size from 40 to 92 kilodaltons (kDa). Distinct roles in cell division (PBP 3), cell elongation (PBP 1A and PBP 1B), and maintenance of cell shape (PBP 2) have been assigned to these multiple penicillin-sensitive enzymes through a series of genetic and biochemical studies. PBPs generally belong to one of two broad classes, the essential high-molecular-weight PBPs and the nonessential low-molecular-weight PBPs (for a review, see reference 28).

Bacteria typically contain between three and eight PBPs (8, 33), and there is some evidence that they are important in the process of cell differentiation; thus, in *Bacillus subtilis*, sporulation-specific PBPs have been identified (24), and more recently the location of these proteins has been correlated with their proposed function in forespore formation (3). In *Caulobacter crescentus* at least five major PBPs, ranging in molecular mass from 50 to 132 kDa, were previously described (13, 14), but no functions have been assigned for these proteins.

C. crescentus divides asymmetrically to produce two morphologically distinct cell types: a motile swarmer cell possessing a set of differentiated structures, including a polar flagellum, pili, and holdfast, and a nonmotile stalked cell. The stalked cell divides repeatedly like a stem cell to produce the stalked cell plus a new swarmer cell at each division, while the swarmer cell loses motility, sheds the flagellum, and finally develops into a stalked cell before dividing (for a review of cell division in *C. crescentus*, see reference 20). Temperature-sensitive cell cycle mutants of *C. crescentus* have been isolated that grow normally at 30°C (permissive temperature) and 37°C (nonpermissive temperature) but that are blocked at characteristic stages of the cell cycle at 37°C (21). Reciprocal shift experiments have shown that *C. crescentus* cell cycle genes are organized in at least two dependent pathways: a DNA synthesis pathway, described by the dependent sequence of events DNAi (DNA initiation) → DNAe (DNA elongation) → DNAc (completion of chromosome replication), and a cell division pathway, described by the dependent sequence DIVi (initiation of cell division) → DIVp (progression of division) → CS (cell separation) (Fig. 1) (20, 22). Relatively low concentrations of penicillin G block DIVi (32), and a *divA*-dependent step required for DIVi is interdependent with penicillin G (22). These results suggest that specific PBPs are required for cell division, which is required in turn for differentiation in *C. crescentus*.

To extend our understanding of cell division and development in *C. crescentus*, we have initiated an analysis of PBP function in these cells. In this report, we describe the effect of conditional cell cycle mutations on the PBPs of *C. crescentus*. We also describe the isolation of mutations in two genes that confer resistance to the β-lactam cephalosporin C and conditional defects in cell growth and division.

* Corresponding author.
† Present address: New England BioLabs, Inc., Beverly, MA 01915-9990.

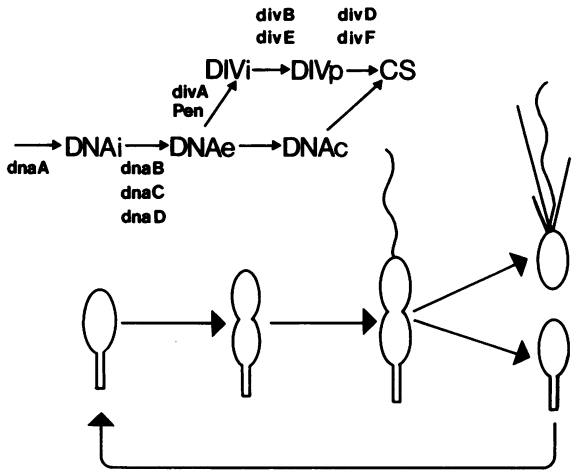

FIG. 1. Organization of DNA synthesis and cell division pathways in *C. crescentus*. Asymmetric cell division produces the swarmer cell and the stalked cell (see the text). The temporal order of events in the cell cycle is determined by the functional dependence of gene-mediated steps. The gene designations for temperature-sensitive cell cycle mutations used to define these steps are indicated (22). See the text for definitions of cell cycle events (for a review, see reference 20).

These strains are also temperature sensitive for the high-molecular-weight PBP 1B.

MATERIALS AND METHODS

Strains and growth conditions. *C. crescentus* CB15 (ATCC 19089), β-lactamase mutant SC1107 (6), and conditional cell cycle mutants (21) were routinely grown in peptone-yeast extract (PYE) (23) at either 30 or 37°C. Growth was monitored at 650 nm (A_{650}). Cell division was determined by monitoring numbers of cells with a Coulter Model ZBI particle counter (Coulter Electronics, Inc.) after sample dilution in 0.9% saline–3.7% formaldehyde. SC strains containing Tn*5* insertions were first described by Ely and Croft (6). The cephalosporin-C-resistant strains were derived from strain SC1107 and were isolated as spontaneous mutants resistant to 20 μg of cephalosporin C per ml (Sigma Chemical Co.). The strains used in this study are described in Table 1.

Isolation of cephalosporin-C-resistant mutants. Spontaneous cephalosporin-C-resistant mutants were isolated, at a frequency of 5×10^{-7}, by plating 10^{10} cells of strain SC1107 on PYE plates containing 20 μg of cephalosporin C per ml. Strain SC1107 carries a Tn*5* transposon in the β-lactamase gene (6) and therefore is extremely sensitive to β-lactam antibiotics (see below). Colonies were screened for temperature-sensitive growth by replica printing to PYE plates containing cephalosporin C at 30 and 37°C. Temperature-sensitive cell division was confirmed by the presence of filamentous cells in liquid PYE cultures grown at 37°C.

Preparation of membranes and in vitro PBP assay. The in vitro assays for PBP activity were based on modifications of the procedures described by Spratt and Pardee (30) and Koyasu et al. (15). Membranes were prepared by suspending cells in lysis buffer (0.1 M Tris hydrochloride [pH 7.8], 1.5 mM EDTA) containing 50 μg of lysozyme (Organon Teknika) per ml, as described by Huguenel and Newton (11). Membrane protein was determined by the method of Lowry et al. (18) and adjusted to 5 mg/ml in 50 mM sodium phosphate buffer (pH 7.0), and 30 μl of the preparation was incubated with 3 μl (0.05 mCi; 27 μg of penicillin per ml) of [^{3}H]penicillin G (specific activity, 22 Ci/mmol) (gift from A. Rosegay, Merck Sharp & Dohme) at 30°C for 15 min. A 3-μl portion of a 3.4% sarcosyl–3% penicillin G solution was added, and the mixture was incubated for an additional 15 min at 20°C. Samples were then centrifuged at 13,000 × *g* for 30 min at 20°C, and 30 μl of supernatant was mixed with 15 μl of gel sample buffer (0.2 M Tris hydrochloride [pH 6.8], 3% sodium dodecyl sulfate [SDS], 30% [wt/vol] glycerol, 0.002% bromophenol blue) containing 5 μl of β-mercaptoethanol (17). Samples were then boiled for 3 min and separated by electrophoresis in either a 7.5% SDS–polyacrylamide gel at 35 V or a 7.5 to 15% SDS–polyacrylamide gel at 100 V. The disruption of cells by using a lysis buffer containing lysozyme and EDTA (11) instead of lysing cells by sonication has significantly improved the reproducibility of results with the in vitro assay.

In vivo PBP assay. *C. crescentus* cells (0.3 ml at an A_{650} of 0.9) were centrifuged and suspended in 30 μl of 10 mM sodium phosphate buffer (pH 7.0). Membrane protein was determined to be approximately 110 μg/30-μl sample (data not shown). The cells were incubated at 30°C for 15 min in the presence of 0.05 mCi of [^{3}H]penicillin G (27 μg/ml). Membrane proteins were then solubilized by the addition of 3 μl of a 3.4% sarcosyl–6% penicillin G solution, and the mixture was incubated for 15 min at 20°C. Samples were centrifuged at 15,000 × *g* for 15 min at 4°C, and 30 μl of supernatant was mixed with 15 μl of gel sample buffer, as described above, and separated by SDS-polyacrylamide gel electrophoresis. We have found that PBPs detected in vitro and in vivo are better resolved in 7.5 to 15% gradient SDS–polyacrylamide gels than in the 7.5% SDS–polyacrylamide gels used previously (13).

Electron microscopy. Samples (0.5 ml) were fixed for 1 h by the addition of an equal volume of 0.8% glutaraldehyde in 50 mM cacodylic acid (pH 7.4), and cells were collected by centrifugation and then gently suspended in an equivalent volume of filtered (pore size, 0.22 μm; Millipore Corp.) distilled water. Samples (5 μl) were spotted onto Formvar-coated copper electron microscope grids (200 mesh) and allowed to stand for 45 min. One drop of 0.5% phosphotungstic acid prepared in 0.5% ammonium acetate (pH 7.0) was then added. The grids were immediately blotted dry and examined at 80 kV in a JEOL 100C transmission electron microscope (10).

RESULTS

Characterization of *C. crescentus* PBP patterns. Koyasu et al. (13) previously described five major PBPs in the cell envelope of *C. crescentus*, detected in an in vitro assay: PBP 1A (132 kDa), PBP 1B (98 kDa), PBP 2 (77 kDa), PBP 3 (64 kDa), and PBP 4 (50 kDa). We were able to resolve several of these bands into multiple proteins and to detect additional PBPs by in vitro (30) and in vivo (13) assays. At least 14 PBPs were identified in the assays (Fig. 2). Our in vivo and in vitro assays detected the five major PBPs (see above) and nine additional PBPs: PBP 1C, PBP 1D, PBP 2B, PBP 3B, PBP 3C, PBP 5A, PBP 5B, PBP 5C, and PBP 5D. PBP 1C, PBP 4, and PBP 5D were usually detected only in the in vivo assay, and PBP 1A, PBP 1D, PBP 2B, PBP 3A, PBP 3B, and PBP 5A were detected only in the in vitro assay. These differences indicate that certain PBPs are not accessible to exogenously added penicillin at the concentrations used in the standard in vivo assay, whereas other PBPs may be either lost or inactivated in the cell membrane preparation

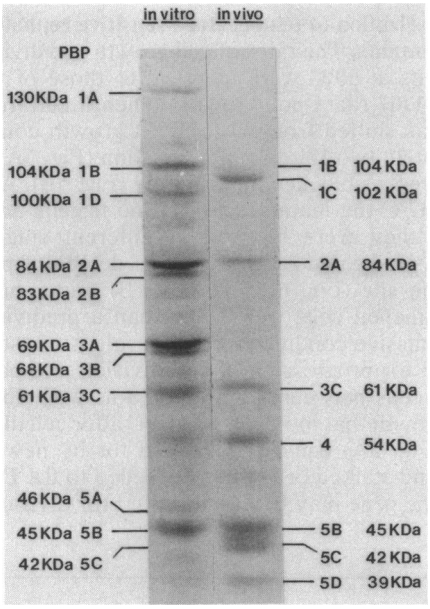

FIG. 2. In vivo and in vitro assays of *C. crescentus* PBPs. Membrane or whole cells of strain SC1107 were assayed at 30°C with 0.05 mCi of [^{3}H]penicillin G (27 μg of penicillin G per ml; specific activity, 22 Ci/mmol). Membrane proteins were separated by electrophoresis in a 7.5% SDS–polyacrylamide gel. PBP identities were designated by the method of Koyasu et al. (13); however, we detected several additional bands (see the text); 11 PBPs were routinely detected in vitro, and 8 were routinely detected in vivo. Molecular masses are indicated. The difference in molecular masses between PBPs detected in our analysis and that of Koyasu et al. (13) are probably due to the different lysis and gel-running conditions. A comparison can be made between PBPs detected in vitro and in vivo. Although PBP 1B appears faint in the in vivo autoradiogram, this protein was usually seen in vivo and is clearly visible in Fig. 3.

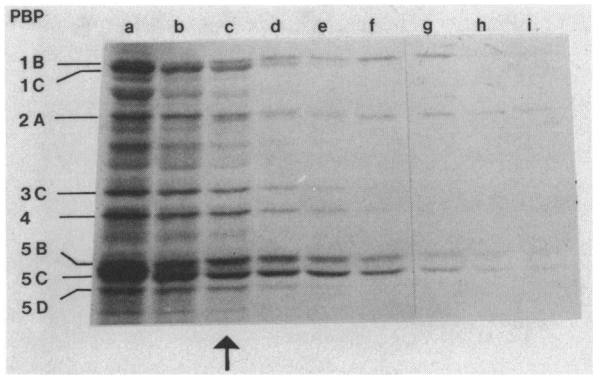

FIG. 3. Binding of [^{3}H]penicillin G to PBPs of *C. crescentus* in whole cells. Wild-type strain CB15 was incubated at 30°C with twofold decreasing concentrations of [^{3}H]penicillin G (specific activity, 61 mCi/mg or 22 Ci/mmol) in each assay. The amount of radiolabeled penicillin G added in assays a to i (0.2 to 7.8 × 10^{-3} mCi) was 110, 55, 27.3, 13.7, 6.8, 3.4, 1.7, 0.85, and 0.43 μg/ml, respectively. Sarcosyl-soluble proteins were separated on a 7.5 to 15% SDS–polyacrylamide gel. The standard assay concentration of [^{3}H]penicillin G (0.05 mCi; 27 μg/ml) is indicated by the arrow (lane C). PBPs 1B, 2A, 5B, and 5C were detected at the lowest concentration of [^{3}H]penicillin G (7.8 × 10^{-3} mCi; 0.43 μg of penicillin G per ml) and are considered to behave like high-affinity binding proteins.

and not detected in the in vitro assay. Although PBP 1B appeared faint in this particular in vivo assay (Fig. 2), this PBP was normally present as a strong band (Fig. 3).

PBPs that display a higher affinity for the labeled penicillin were identified by adding different levels of [^{3}H]penicillin G to the in vivo PBP assay of wild-type strain CB15 (Fig. 3). Comparative binding of the labeled penicillin to the PBPs in whole cells is shown in Fig. 3. PBPs 1B, 2A, 5B, and 5C, which were still detected at 7.8 × 10^{-3} mCi of [^{3}H]penicillin G (0.43 μg of penicillin G per ml), behaved like high-affinity PBPs. Comparable results were obtained with the β-lactamase mutant SC1107 and the cephalosporin-C-resistant mutants described below (data not shown). Genetic evidence presented in the following sections suggests that high-affinity PBP 1B is involved in cell growth and division.

PBP patterns in cell cycle mutants of *C. crescentus*. When conditional cell cycle mutants (Table 1) (21) were incubated either at 30 or 37°C and then assayed for PBPs in vivo, PBP 1C was not detected at either temperature (data not shown). When cell cycle mutations were transferred to new genetic backgrounds by the construction of isogenic strains (A. Newton and L. Kulick, unpublished data) and assayed for PBPs in vivo, however, all the recombinant, conditionally defective cell division strains were temperature sensitive for PBP 1C activity (Fig. 4). This suggests that PBP 1C activity is inhibited as a consequence of filamentous growth and that none of the original conditional cell cycle mutants is specifically altered in a PBP. Control experiments demonstrated that PBP 1C is not temperature sensitive in normally dividing cells of the wild-type strain CB15 (data not shown). Although some variability in the PBP patterns was observed at 37°C, only PBP 5D was found to be temperature sensitive in all division-defective mutants and in the wild-type strain (Fig. 4). Since wild-type cells grow and divide at 37°C, the low-molecular-weight PBP 5D appears to be a nonessential binding protein for cell growth and division.

Isolation of cephalosporin-C-resistant mutant strains. A direct approach to the identification of PBPs required for cell division is to isolate β-lactam-resistant mutants at 30°C which are also defective in cell division at 37°C (27). This type of experiment was not initially successful with *C. crescentus*, using several β-lactams, including penicillin G, because of an inducible β-lactamase produced in the wild-

TABLE 1. *C. crescentus* strains used

Strain	Cell cycle event blocked	Genotype or characteristic	Source or isolation
CB15		Wild type	ATCC 19089
CB15F		Density variant	Evinger and Agabian (7)
PC1029	CS	*divF*	This laboratory
PC1040	CS	*divC*	This laboratory
PC1042	DNAe	*dnaB*	This laboratory
PC1049	DIVp	*divE*	This laboratory
PC1053	DIVi	*divA*	This laboratory
PC2116	DIVp	*divO*	This laboratory
PC2179	DNAe	*dnaC*	This laboratory
PC2244	DIVp	*divB*	This laboratory
PC2269	CS	*divD*	This laboratory
PC8002	DIVp	*amp-101*::Tn*5 str-152 divH*	Spontaneous, from SC1107
PC8003	DIVi	*amp-101*::Tn*5 str-152 divI*	Spontaneous, from SC1107
PC8005	DIVi	*amp-101*::Tn*5 str-152 divI*	Spontaneous, from SC1107
SC1107		*amp-101*::Tn*5 str-152*	Ely and Croft (6)

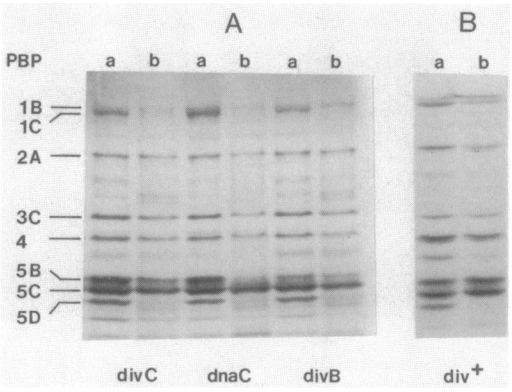

FIG. 4. In vivo PBP patterns in recombinant cell division mutants of *C. crescentus*. (A) Recombinant PC strains temperature sensitive for *divC* (CS), *dnaC* (DNAe), and *divB* (DIVp) were incubated with 0.05 mCi of [^{3}H]penicillin G at 30°C (lanes a) or 37°C (lanes b). Mutations were transferred to isogenic backgrounds by conjugation (A. Newton and L. Kulick, unpublished data). Cells were incubated for 13 h at 30 or 37°C and assayed for PBPs in vivo. Sarcosyl-soluble proteins were separated on a 7.5 to 15% SDS–polyacrylamide gel. All the recombinant cell division mutants were temperature sensitive for PBP 1C. (B) Strain SC1107 is included from a different gel for reference as a *div*$^+$ control and has a pattern identical to that for strains C1 and CB15. Although there was some variation in the levels of PBPs detected at 30 and 37°C, only PBP 5D was missing at 37°C and is thus considered a nonessential binding protein.

type strain (12). As an alternative approach we used as the parent strain SC1107, containing a Tn*5* insertion mutation in the penicillinase gene (6). This strain was 10- to 100-fold more sensitive to all β-lactams examined (Table 2), and its PBP pattern was identical to that in wild-type cells (data not shown).

Spontaneous mutants of strain SC1107 resistant to cephalosporin C were isolated at a frequency of 5×10^{-7}. Cephalosporin C was selected for the isolation of resistant mutants because it was the most potent of the drugs that caused filamentation (see Table 4). A screen of 650 drug-resistant mutants for growth at 30 and 37°C identified 11 temperature-sensitive mutants, which were assigned to one of two classes based on their morphology at 37°C. By light microscopy, one class, represented by strain PC8002, was cell division defective but did not produce filaments. The second class of mutants, represented by strain PC8003, was cell division defective and formed unpinched coiled filaments (Table 1; data not shown). Strains PC8002 and PC8003 are defective in new cell cycle genes designated here as *divH* and *divI*, respectively.

TABLE 2. Sensitivity of strain SC1107 to β-lactam antibiotics

Drug	Amt (μg/ml) required for lysis[a]	
	CB15	SC1107
Apalcillin	>1,000	150
Cephalosporin C	600	20
Cephalexin	30	4.5
Cloxacillin	>1,500	200
Mecillinam	250	40–50
Monobactam	300	300
Penicillin G	3,000	40–60

[a] Cells were grown for 18 h in liquid PYE. The quantity of drug indicated is for complete lysis. The levels of penicillin G are in units per milliliter.

Characterization of temperature-sensitive cephalosporin-C-resistant mutants. The rates of cell growth and division of the two mutants at 30°C were identical to those of the parent strain, SC1107 (data not shown). When a culture of strain PC8002 was shifted from 30 to 37°C, growth continued for approximately two-thirds of a generation (Fig. 5A), whereas cell division was blocked immediately (Fig. 5B). After 3 h or longer at 37°C the mutant cells had no flagella and a single stalk and they were blocked at different stages of cell division, as indicated by the variable degree of pinching at the division site (Fig. 6B and Table 3). Since none of the partially pinched cells was larger than a predivisional cell under permissive conditions, these results suggest that *divH* is required for progression of cell division and possibly for growth of cells between DIVi and CS as well (Fig. 1). The residual growth that took place at 37°C after cell division had stopped (Fig. 5A) can be accounted for by newly divided swarmer and stalked cells that proceeded to the DIVi stage, at which the gene may be first required for division and cell

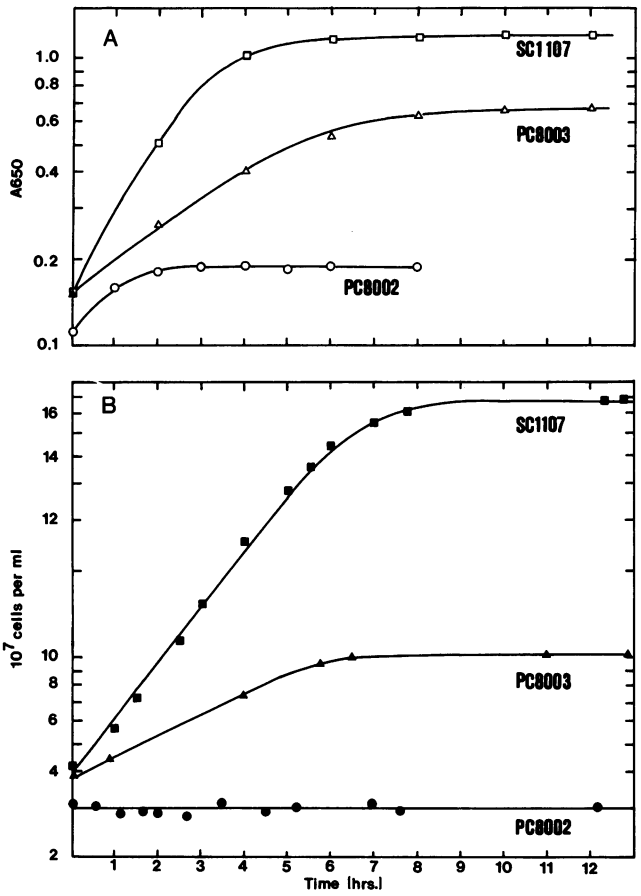

FIG. 5. Cell growth and cell division of temperature-sensitive mutants PC8002 and PC8003 at 30 and 37°C. Cells grown in PYE medium at 30°C were diluted into fresh PYE and incubated at 30 or 37°C for the times indicated. (A) Growth of strain PC8002 (○) continued for approximately two-thirds of a generation at 37°C, whereas growth of strain PC8003 (△) continued for approximately 11 to 13 h. SC1107 (□) reached a saturation level in 5.5 to 6 h. (B) Cell division was measured with a Coulter Model ZBI particle counter. Division stopped immediately when PC8002 (●) was incubated at 37°C, whereas PC8003 (▲) continued to divide for approximately 6 h and then formed coiled filaments. Division of SC1107 (■) is also shown.

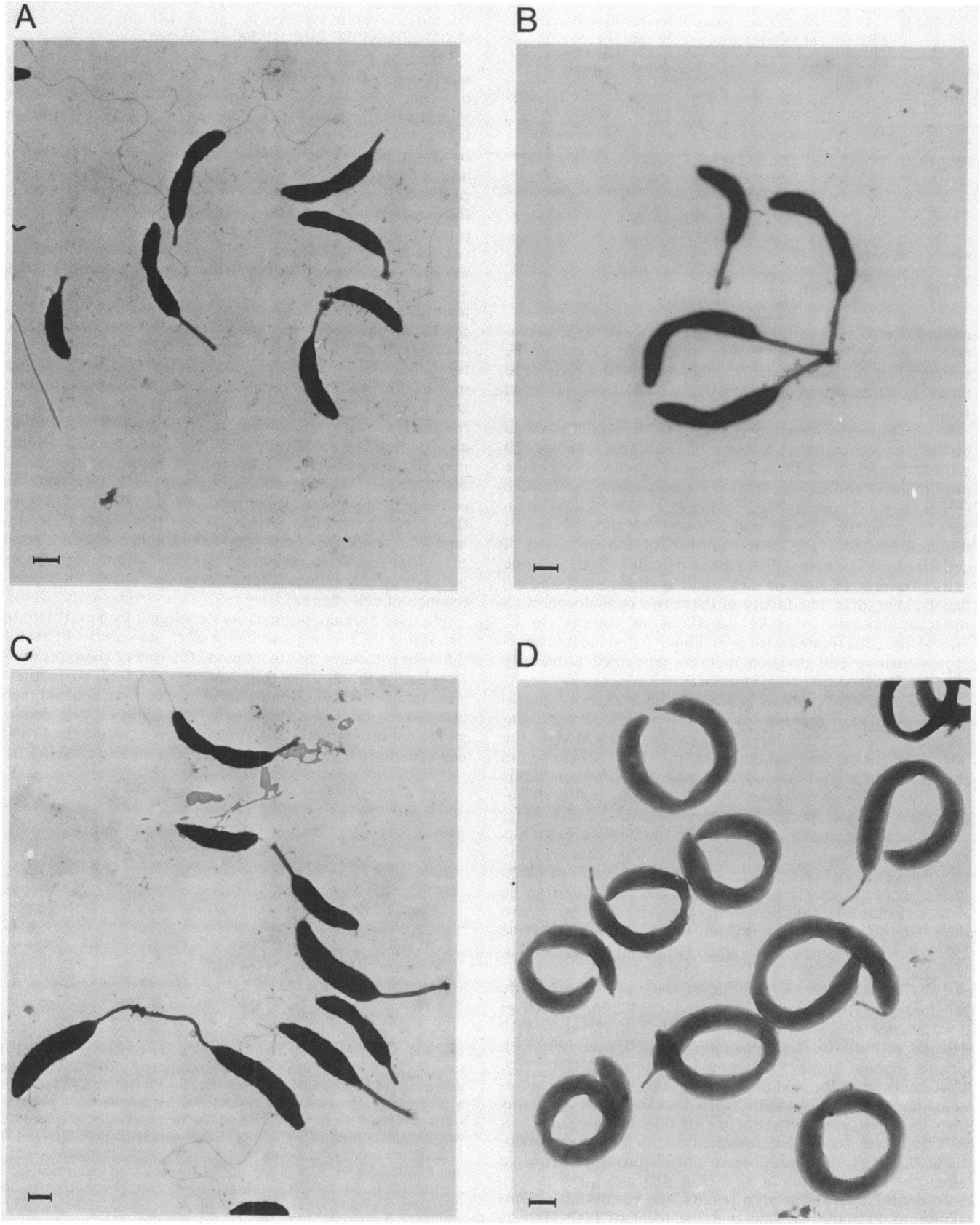

FIG. 6. Electron micrographs of temperature-sensitive strains PC8002 and PC8003 after growth at 30 and 37°C. PC8002 cells were incubated at 30°C (A) or for 3 h at 37°C (B). PC8003 cells were incubated at 30°C (C) or for 13 h at 37°C (D). The cells were fixed and stained as described in the text. More than 85% of the PC8002 cells were blocked in the division pathway at 37°C (Table 4). All of these cells were nonflagellated. PC8003 formed coiled filaments at 37°C that were also nonflagellated. Bar = 0.5 μm.

TABLE 3. Distribution of cell types of temperature-sensitive PBP mutant PC8002 grown at 30 and 37°C

Cell type[a]	Fraction (%) of cells at:	
	30°C	37°C
Swarmer	17	0.5
Stalked[b]	40	8
Predivisional[c]	43	92[d]

[a] Cells were observed and counted in the electron microscope and scored into three groups, each containing cells from a portion of the cell cycle.
[b] These cells had not yet entered the division pathway.
[c] Elongated stalked cells and partially pinched predivisional cells at various stages of cell division.
[d] Of these cells, 57% were blocked at DIVi, as judged by slight or no pinching at the division site.

growth. Consistent with this interpretation are the observations that very few swarmer cells were present 3 h after the temperature shift and approximately 60% of the cells were unpinched and presumably arrested at DIVi (Table 4 and Fig. 6).

Cephalosporin-C-resistant mutant PC8003 continued to divide for approximately 6 h at 37°C (Fig. 5B), whereas cell growth continued for 11 to 13 h (Fig. 5A) and resulted in the production of short filaments that were unpinched and coiled (Fig. 6D). These results suggest that PC8003 is blocked at an early stage in the division pathway and that the *divI* gene is required for DIVi (Fig. 1) but not for subsequent stages of cell division. The coiled filaments of mutant PC8003, like the cells of strain PC8002 at 37°C, had a single stalk and no flagella (Fig. 6D). The failure of these two cephalosporin-C-resistant mutants to make flagella is in contrast to *C. crescentus* cells treated with penicillin (32) and most temperature-sensitive cell division mutants described previously (21; see Discussion).

Analysis of PBP patterns in temperature-sensitive cephalosporin-C-resistant mutants. When strain PC8002 was incubated at 37°C for 1 to 3 h and assayed for PBPs in vivo, the 104-kDa PBP 1B was not detected (Fig. 7A). No change in the PBP 1B pattern was observed under the same conditions in either the parent strain, SC1107, or another division-defective cephalosporin-C-resistant strain, PC8011 (Fig. 7A). The temperature-sensitive effect on the PBP pattern is reversible, because when cells were shifted back to the permissive temperature (30°C), PBP 1B was again detected after 3 h (data not shown). PBP 1B was also found to be defective in this mutant by in vitro analysis: as shown in two different PBP assays (Fig. 8A and B), the amount of active PBP 1B was more than 90% depleted in mutant cells incubated at 37°C for 3 h.

The in vivo PBP pattern in PC8003 was significantly affected only when cells were shifted to 37°C for more than 3 h (data not shown). After 6 to 8 h at 37°C, the levels of PBP 1B and PBP 1C were significantly reduced, and after 13 h neither one of these PBPs could be detected (Fig. 7B). Consistent with the results obtained with the temperature-sensitive cell cycle mutants (Fig. 4), PBP 1C activity was also inhibited as a consequence of filamentous growth at 37°C. Control assays demonstrated that in the parent strain, SC1107, PBPs 1B and 1C were not temperature sensitive after 13 h of incubation at 37°C (Fig. 4B).

In contrast to the in vivo results, the results of in vitro assays of PC8003 revealed that the level of PBP 1B was approximately the same as 37 and 30°C (Fig. 8A and B). Thus, both strains PC8002 and PC8003 exhibited an in vivo defect in PBP 1B at 37°C, but only strain PC8002, which was the most severely blocked in cell growth and cell division, also exhibited the PBP 1B defect in vitro.

Effect of β-lactam antibiotics on *C. crescentus* growth, morphology, and penicillin binding. As observed with other bacteria, β-lactam antibiotics produced characteristic morphological effects on *C. crescentus* (Table 4) (14). Penicillin G at a low concentration (210 U/ml), like conditional mutations in *divA*, blocked an early step in cell division (DIVi) and induced the formation of filaments, whereas higher concentrations of the drug led to rapid cell lysis (32). This is the same effect as that observed in *E. coli* (25). Most of the β-lactams examined produced a similar effect on *C. crescentus*, including ampicillin, apalcillin, cephaloglycin, cephalosporin C, cloxacillin, penicillin G, and penicillin V. A second effect, caused by the β-lactams cefazolin, cephalexin, cephaloridine, and cephalothin and the monobactam SQ26776, was rapid cell lysis (Table 4). In *E. coli*, low concentrations of cephalexin and SQ26776 induce filament formation (25, 31). A third effect, induced by the drug mecillinam, was observed only in the β-lactamase mutant strain SC1107. Mecillinam-treated cells (10 to 20 μg/ml) rounded up with no immediate block in cell division or growth; however, a division block did occur after 3 h. Unlike penicillin-treated cells, which produce long multiflagellated filaments (32), electron microscopy showed that the addition of mecillinam induced large pear-shaped cells with two or three flagella (data not shown). Thus, the effect of mecillinam on *C. crescentus* cell shape was similar to its effect on *E. coli* (19) and implies that, like *E. coli*, *C. crescentus* possesses a mecillinam-sensitive target involved in the maintenance of cell shape (25).

We used a competition assay developed by Spratt (26) to determine the binding specificity of *C. crescentus* PBPs for different β-lactams and to examine the role of these proteins in cell growth, division, and morphology. Cells or extracts were incubated initially with the unlabeled β-lactam and then assayed for binding to the labeled penicillin G. The results show that drugs blocking cell division, e.g., penicillin G and cephalosporin C, bound only to the high-affinity protein PBP

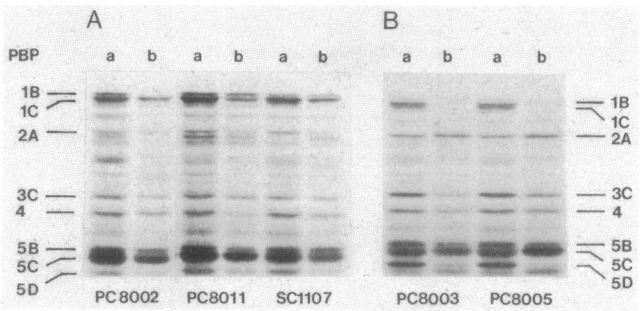

FIG. 7. In vivo PBP patterns in temperature-sensitive cephalosporin-C-resistant strains. (A) Exponential-phase cells were diluted to an A_{650} of 0.25 with fresh PYE and incubated at 30 or 37°C for one generation (3 h). PBPs were then assayed in vivo at 30°C (lanes a) or 37°C (lanes b), as described in the text. Sarcosyl-soluble proteins were separated on a 7.5 to 15% SDS–polyacrylamide gel. PC8002 was the only strain with a temperature-sensitive PBP 1B after 3 h at 37°C. Other strains, including PC8011 (partially filamentous at 37°C) and PC8003 and PC8005 (data not shown), behaved like the SC1107 control. (B) Exponential-phase cells of temperature-sensitive strains PC8003 and PC8005 were diluted to an A_{650} of 0.25 with fresh PYE and incubated at 30 or 37°C for four generations (12 to 13 h). PBPs were then assayed in vivo at 30°C (lanes a) or 37°C (lanes b). Both PC8003 and PC8005 produced coiled filaments at 37°C and were temperature sensitive for PBPs 1B and 1C.

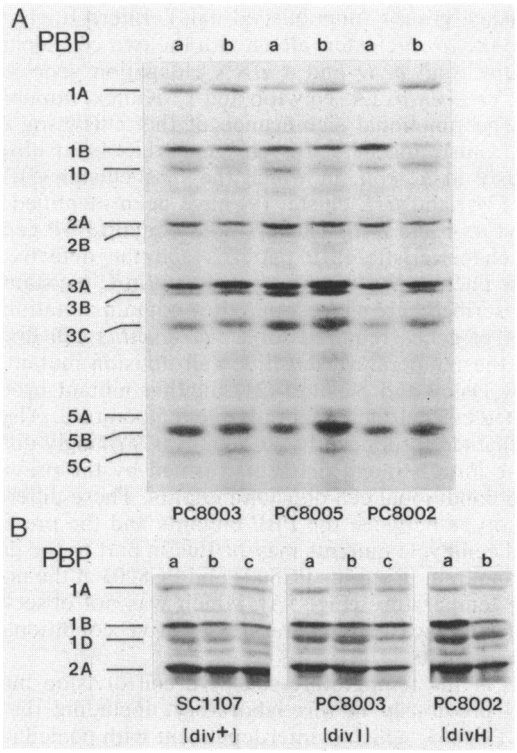

FIG. 8. In vitro PBP patterns in temperature-sensitive cephalosporin-C-resistant strains. (A) Strains PC8003 and PC8005 were diluted with fresh PYE to an A_{650} of 0.20 and incubated at 30 or 37°C for 13 h. Strain PC8002 was diluted and incubated for 3 h at 37°C. PBPs were then assayed in vitro at 30°C (lanes a) or 37°C (lanes b) as described in the text. Sarcosyl-soluble proteins were separated on a 7.5% SDS–polyacrylamide gel. Only strain PC8002 demonstrated temperature-sensitive PBP 1B activity in vitro. (B) In an independent experiment the in vitro penicillin-binding activities in membranes prepared from SC1107 (*div*$^+$ control), PC8003 (*divI*), and PC8002 (*divH*) were assayed after cells were incubated for 0 (lanes a), 3 (lanes b), or 13 (lanes c) h at 37°C. The penicillin-binding activity of PBP 1B was unaffected in both SC1107 and PC8003, whereas there was a considerable reduction in binding activity in PC8002 after 3 h.

1B in vivo when tested at concentrations that induced filamentation (Fig. 9) but bound to a number of high-molecular-weight PBPs in vitro, including PBP 1B (Fig. 10). Thus, the primary targets of these drugs cannot be correlated by in vitro assay. However, we conclude from the in vivo assay results and those obtained with the temperature-sensitive mutants that PBP 1B may be directly involved in cell division. It is more difficult to identify a specific target involved either in cell shape maintenance or in cell wall elongation since mecillinam, which affected cell shape, did not compete with any of the PBPs in vivo or in vitro. SQ26776, a drug which caused cell lysis in *C. crescentus* (Table 4), competed with several PBPs in vivo, and cephalexin, cephaloridine, and cephalothin, which also caused cell lysis, competed with several PBPs in vitro (data not shown).

DISCUSSION

We report here the first isolation of conditional mutants that may define a role for a PBP in cell division and growth in *C. crescentus*. One class of these cephalosporin-C-resistant mutants, represented by strain PC8002, showed a

TABLE 4. Effects of β-lactam antibiotics on *C. crescentus*

β-Lactam	Primary effect[a]	Concn for primary effect (μg/ml)[b]	Concn for lysis (μg/ml)[c]
Ampicillin	Filamentation	100	400
Apalcillin	Filamentation	250	NE
Cefazolin	Lysis	500	500
Cephalexin	Lysis	30	30
Cephaloglycin	Filamentation	300	1,000
Cephaloridine	Lysis	75	75
Cephalosporin C	Filamentation	150	600
Cephalothin	Lysis	100	100
Cloxacillin	Filamentation	800	NE
Mecillinam[d]	Lysis	250	250
Penicillin G	Filamentation	300[e]	3,000[e]
Penicillin V	Filamentation	100	500
SQ26776[f]	Lysis	300	300

[a] Primary effect of drug established by incubating *C. crescentus* CB15 in 2 ml of PYE in the presence of drug for 8 h.
[b] Concentration of drug at which all cells in the culture displayed the primary effect of the drug.
[c] Concentration of drug at which all cells in the culture were lysed. NE, Not established.
[d] Mecillinam (10 to 20 μg/ml) affected cell shape in the β-lactamase mutant SC1107.
[e] Concentration in units per milliliter.
[f] SQ26776 is a monobactam (gift of E. R. Squibb & Sons, Inc.).

temperature-sensitive defect in PBP 1B when assayed in vivo (Fig. 7A) or in vitro (Fig. 8). The rapid loss of PBP 1B activity from cells incubated at 37°C was accompanied by an immediate block in cell division (Fig. 5B and Table 4) and a

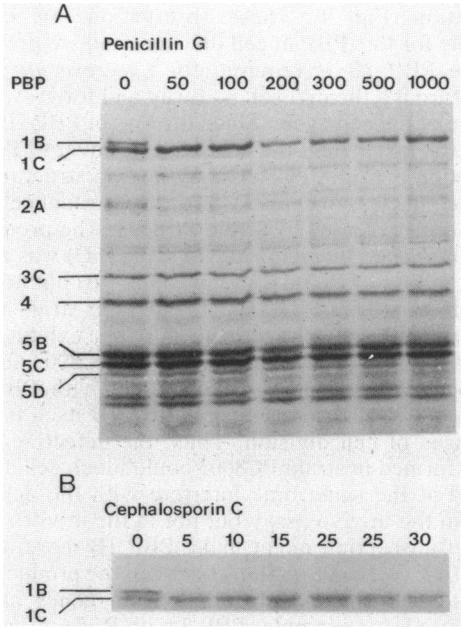

FIG. 9. Competition of β-lactam antibiotics for PBPs in vivo. Strain CB15 (A) or SC1107 (B) was preincubated with 0.0 to 1,000 U of penicillin G per ml (1,595 U = 1 mg) or 0.0 to 30 μg of cephalosporin C per ml, respectively, for 15 min and then assayed in vivo for PBPs. Sarcosyl-soluble proteins were separated on a 7.5 to 15% SDS–polyacrylamide gel. Strains CB15 and SC1107 differ in sensitivity to β-lactams, SC1107 being the most sensitive (Table 2). All the PBPs except PBP 1B were still accessible to [^{3}H]penicillin G. In CB15 the same PBP pattern was detected in competition experiments with up to 3,000 U of penicillin G per ml.

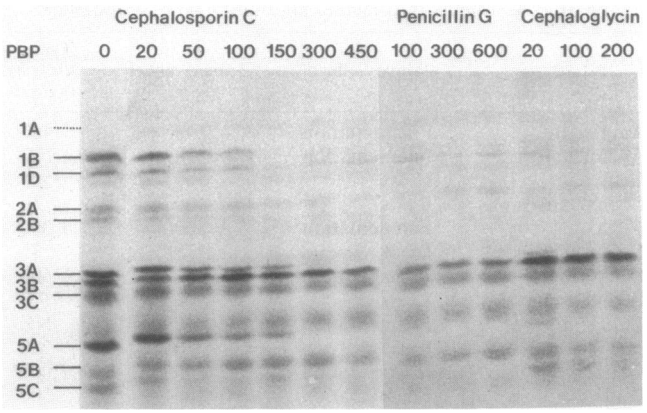

FIG. 10. Competition of β-lactam antibiotics for PBPs in vitro. Membrane protein (150 μg) from strain CB15 was preincubated with 20 to 450 μg of cephalosporin C per ml, 100 to 600 U of penicillin G per ml, or 20 to 200 μg of cephaloglycin per ml. The results of the control PBP binding assay in the absence of drug are shown in the leftmost lane. PBPs were assayed in vitro as described in the text. Sarcosyl-soluble proteins were separated on a 7.5% SDS–polyacrylamide gel. Several PBPs were not accessible to the [^{3}H]penicillin G under these conditions: PBPs 1B, 1D, 2A, 2B, 3A, and 5A. Both the in vivo and in vitro competition analyses showed PBP 1B as a major target for β-lactams.

subsequent loss of growth potential within one generation (Fig. 5A). These results suggest that PBP 1B is required at all stages of cell division and for growth during the period of cell division but not for swarmer-to-stalked-cell development. PBP 1B is a protein of 104 kDa with a high affinity for penicillin G (Fig. 3) that is a target for β-lactams that cause filamentation (Fig. 9). These observations are consistent with a role for this PBP in cell division, and we propose that an active PBP 1B is required for *C. crescentus* cells to proceed through the division pathway and for growth during this phase of the cell cycle. Since the loss of PBP 1B in strain PC8002 was faster than the observed turnover of PBPs (16), the mutation in strain PC8002 may be in a structural gene.

The second class of cephalosporin-C-resistant cell division mutants is represented by strain PC8003. The production of coiled, unpinched filaments at 37°C (Fig. 6D) was accompanied by the loss of PBP 1B in vivo (Fig. 7B) but not in vitro (Fig. 8). How are the PBP 1B defects in this strain and strain PC8002 related and why do the mutants exhibit different phenotypes? One explanation is that the PC8003 mutation is in a gene whose product must interact with PBP 1B for initiation of cell division, whereas PBP 1B itself is required at all stages of cell division. Thus, the defective initiation complex formed in strain PC8003 could block cell division at DIVi and at the same time interfere with the detection of PBP 1B in the in vivo assay but not in the in vitro assay, in which cell disruption might make PBP 1B more accessible (Fig. 8). In *E. coli*, interactions between the products of PBP genes and cell shape and cell division genes have been demonstrated, for example, PBP-3 with RodA (2) and FtsA, as well as interactions between FtsQ, FtsA, and FtsZ (4).

By using Tn*5* insertions linked to these PBP genes, it has been possible to construct isogenic *amp*$^+$ recombinants of the mutants, and they display the same temperature-sensitive cell division phenotypes as the ampicillin-sensitive parent strains. Preliminary genetic mapping of the cell division mutations isolated here also supports the conclusion that the two division genes *divH* and *divI* defined by strains PC8002 and PC8003, respectively, map to different transduction linkage groups (unpublished data). Interestingly, *divH* maps to the *aroF* cluster, along with the two cell separation genes *divC* and *divD* and a DNA elongation gene, *dnaC*, from *C. crescentus* (A. Newton and L. Kulick, unpublished data). The functional significance of this clustering is unknown. Clustering of cell cycle genes has been observed previously in *E. coli*, in which the *ftsA* cluster (5), *rodA* cluster (29), and *ftsE* cluster (9) have been identified. Further, the *ftsA* and *rodA* clusters both contain PBP genes.

The characteristic PBP patterns and the defective cell division phenotypes of the cephalosporin-C-resistant mutants described here suggest that they contain mutations in a new set of genes required for *C. crescentus* cell division. Unlike the temperature-sensitive cell division mutants isolated by Osley and Newton (21), neither mutant produced flagellated cells at the nonpermissive temperature. Also, the coiled filament produced by PC8003 was strikingly different from the long, straight filaments formed by the previously isolated conditional cell division mutants. These differences in phenotype between the PBP mutants and the previously isolated cell cycle mutants may be due in part to the growth defect apparent in strains PC8002 and PC8003 at the nonpermissive temperature (Fig. 5A), which was not observed in the other classes of temperature-sensitive conditional cell division mutants.

None of the temperature-sensitive cell division mutants isolated previously in this laboratory, including the *divA* mutant PC1053, which is interdependent with penicillin G in reciprocal shift experiments (22), display specific defects in their PBP patterns. A temperature-sensitive defect was observed in PBP 1C when it was assayed in vivo in isogenic backgrounds (Fig. 4A). This may be a general effect of conditions that lead to filamentation. Support of this conclusion is provided by the behavior of conditional mutants PC8002, which did not form filaments and showed no effect on PBP 1C, and PC8003, which produced coiled filaments at 37°C and lost PBP 1C under these conditions (Fig. 7B). This may have been due to a change in the accessibility or affinity of PBP 1C in the filamenting cells, as suggested by the observation that PBP 1C could be detected in vivo in coiled filaments of strain PC8003 that were incubated in the presence of a threefold higher concentration of [^{3}H]penicillin G (0.15 mCi; data not shown).

Our observation that the 104-kDa PBP 1B was the highest-affinity PBP in vivo (Fig. 3) is consistent with its role in cell division and with the observation that it has the highest affinity in vitro (14). The relatively low affinity previously reported for PBP 4 in vitro is consistent with our in vivo result and suggests that our inability to detect PBP 4 in vitro could be partly a consequence of the specific activity of the labeled penicillin and the reported localization of this protein in the outer membrane (13). Similarly, the detection of PBPs 1A and 3A in our in vitro assay but not in the in vivo assay is consistent with their suggested localization in the inner membrane.

In summary, the cephalosporin-C-resistant mutants described in this report displayed a number of properties that are characteristic of PBP mutants of other bacteria: decreased sensitivity to a β-lactam antibiotic, a greatly diminished quantity or a labile form of a particular PBP at the nonpermissive temperature, and physiological defects under the nonpermissive conditions. The phenotypes of these strains suggest that the high-molecular-weight PBP 1B may be essential throughout the cell division pathway and that it is a lethal target for many β-lactam drugs in *C. crescentus*. Our results also suggest that the mutation in PC8002 (*divH*)

may be in the structural gene for PBP 1B and that the gene defined by PC8003 (*divI*) codes for a product that may interact with PBP 1B. The ability of PBP 1B to affect both growth and division in *C. crescentus* contrasts with the function of PBPs in *E. coli*, in which they appear to have exclusive roles in one of three systems: peptidoglycan synthesis (PBPs 1A and 1B), maintenance of cell shape (PBP 2), or cell division (PBP 3) (for a review, see references 1 and 5). Thus, for an *E. coli* PBP mutant defective in one of these systems, growth of the cell is not affected. The involvement of a single PBP in both growth and division in *C. crescentus* may reflect a fundamental difference in the mechanism of cell division between organisms such as *E. coli* that divide by producing a septum and those such as *C. crescentus* that divide by a progressive pinching at the division site.

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