Properties of the Penicillin-binding Proteins of *Caulobacter*

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Properties of the *Caulobacter* penicillin-binding proteins (PBPs) were studied. Several *Caulobacter* strains possessed at least five major PBPs, as shown previously for *C. crescentus* CB15, namely PBP1A, PBP1Bs, PBP2, PBP3 and PBP4. PBP4 was not detected in the *C. crescentus* strain CB13. None of the strains examined possessed the major PBPs of low molecular weight which have been found in other species of bacteria. The biochemical properties of the *Caulobacter* PBPs were studied further with two strains, *C. crescentus* CB13 and *C. crescentus* CB15. In these strains, PBPs of similar molecular weight were similar in properties such as affinity for β -lactam antibiotics and heat sensitivity. These properties were markedly different from those of PBPs of *Escherichia coli* and other bacteria. To elucidate the functions of the *Caulobacter* PBPs, the effects of several β -lactam antibiotics on cell morphology and their affinities for different PBPs were examined. The relationship between the antibiotic effect and its affinity for PBPs suggests that PBP3 is involved in the process of cell division.

INTRODUCTION

Penicillin-binding proteins (PBPs) have been described in many bacterial species, both Gram-negative and Gram-positive (Spratt & Pardee, 1975; Buchanan & Strominger, 1976; Shepherd *et al.*, 1977; Chase *et al.*, 1978; Curtis *et al.*, 1979*b*; Noguchi *et al.*, 1979; Ohya *et al.*, 1979). It is thought that PBPs are involved in the formation of bacterial cell wall, specifically in peptidoglycan biosynthesis, and play essential roles in cell elongation, maintenance of cell shape and septum formation (Blumberg & Strominger, 1974; Spratt, 1975; Ghuysen, 1977; Tamaki *et al.*, 1977; Suzuki *et al.*, 1978). In *Escherichia coli*, there are at least seven major PBPs in the cell envelope and their functions have been studied in detail both biochemically and genetically (Spratt, 1975, 1977*a, b*; Spratt *et al.*, 1977; Tamaki *et al.*, 1977; Iwaya *et al.*, 1978; Suzuki *et al.*, 1978).

The stalked Gram-negative bacterium *Caulobacter* divides asymmetrically into a swarmer and a stalked cell. These daughter cells of different morphology follow a defined sequence of events in the cell cycle (Poindexter, 1964; Fukuda *et al.*, 1976; Shapiro, 1976; Osley & Newton, 1977). It is thus of interest to examine the properties of PBPs of this organism and to investigate how the PBPs are involved in the cell morphology of *Caulobacter*. We reported previously (Koyasu *et al.*, 1980) that *C. crescentus* CB15 possessed at least five major PBPs in the cell envelope; namely PBP1A (mol. wt 132 000), PBP1Bs (98 000), PBP2 (77 000), PBP3 (64 000) and PBP4 (50 000). In *C. crescentus* CB15, PBPs other than PBP4 were present mostly in the inner membrane, as found in other bacteria. An additional feature of this strain was the lack of major low molecular weight PBPs corresponding to PBP5/6 of *E. coli*.

In this paper, we extend the studies on several biochemical properties of PBPs in *C. crescentus* strains CB13 and CB15, and survey PBPs in related species of stalked bacteria.

METHODS

Bacterial strains and growth conditions. *Caulobacter crescentus* strains CB1, CB2, CB13 and CB15, *Caulobacter vibrioides* strain CB51 and *Asticcacaulis excentricus* strain AC48 were grown in polypeptone/yeast extract (PYE) nutrient broth at 30 °C. *Escherichia coli* K12 strain J62 was grown in Penassay broth at 37 °C.

Preparation of cell envelopes. Cell envelopes were prepared by the method described previously (Spratt, 1977*a*; Koyasu *et al.*, 1980). Cells were harvested from a 2 l culture in the late exponential phase of growth, washed with 10 mM-sodium phosphate buffer, pH 7.0, suspended in 20 ml of the same buffer and sonicated with a Branson Sonifier at setting 5 for 10 min at 0 °C. Unbroken cells were removed by centrifugation at 9000 *g* for 10 min at 4 °C. The supernatant fluid was centrifuged at 100000 *g* for 30 min at 4 °C in a Hitachi RP50 rotor. The sediments (cell envelopes) were washed once with 10 mM-sodium phosphate buffer, pH 7.0, suspended in 1 ml 50 mM-sodium phosphate buffer, pH 7.0 and stored at -70 °C.

Binding of [¹⁴C]penicillin G to cell envelopes and release of bound [¹⁴C]penicillin G. The cell envelopes, 30 µl in volume or 300 µg protein-equivalent, were incubated with 3 µl [¹⁴C]penicillin G at 30 °C for 10 min, then mixed with 3 µl of a solution containing 3.4% (w/v) Sarkosyl and 3% (w/v) non-radioactive penicillin G (for *Caulobacter* and *Asticcacaulis*) or 13.3% Sarkosyl and 3% non-radioactive penicillin G (for *E. coli*) and further incubated at 20 °C for at least 20 min. The mixture was then centrifuged at 15000 *g* for 15 min. To 30 µl of the supernatant were added 15 µl gel sample buffer [0.2 M-Tris/HCl, pH 6.8, 3% (w/v) sodium dodecyl sulphate (SDS), 30% (w/v) glycerol, 0.002% (w/v) bromophenol blue] and 5 µl β-mercaptoethanol. The mixture was heated at 90 °C for 2 min before electrophoresis.

To measure the rate of release of bound [¹⁴C]penicillin G, 30 µl of cell envelopes were first incubated with 100 µg [¹⁴C]penicillin G ml⁻¹ at 30 °C for 10 min, and then mixed with 5 µl non-radioactive penicillin G (90 mg ml⁻¹) and further incubated at 30 °C. After different times, 2 µl portions of 5% Sarkosyl were added and the remaining radioactivity of bound [¹⁴C]penicillin G was assayed by fluorography of the gel.

Inhibition of [¹⁴C]penicillin G binding by various β-lactam antibiotics. The pre-addition method (Blumberg & Strominger, 1972; Spratt, 1977*a*; Curtis *et al.*, 1979*a, b*) was used. The cell envelopes, 20 µl in volume or 300 µg protein-equivalent, were incubated with 10 µl of a β-lactam antibiotic at various concentrations at 30 °C for 10 min, then mixed with 3 µl [¹⁴C]penicillin G (final concentration 100 µg ml⁻¹) and further incubated at 30 °C for 10 min. The binding was terminated by the addition of 3 µl of a solution containing 3.4% Sarkosyl and 3% non-radioactive penicillin G.

Slab gel electrophoresis and fluorography. The gel was 2 mm thick. The running gel was 7.5% (w/v) acrylamide, 0.1% (w/v) *N,N'*-methylenebisacrylamide, 0.1% (w/v) SDS in 0.375 M-Tris/HCl, pH 8.9, and the stacking gel was 3.75% acrylamide, 0.05% *N,N'*-methylenebisacrylamide, 0.1% SDS in 0.125 M-Tris/HCl, pH 6.8. The running buffer was 0.05 M-Tris/0.384 M-glycine/0.1% SDS. Electrophoresis was carried out at 25 mA for 6 h at room temperature.

Fluorography was carried out as described by Bonner & Laskey (1974) and Laskey & Mills (1975). The gel impregnated with 2,5-diphenyloxazole was dried on a filter paper and exposed to a pre-fogged Kodak X Omat R X-ray film at -80 °C for at least 3 weeks. For quantitative analysis, the film was cut into strips and scanned by a Gilford gel scanner. The peak area was measured and corrections were made for differences in width of the gel slots when necessary.

Minimum inhibitory concentrations and morphological effects of β-lactam antibiotics. Bacteria (about 10⁷ ml⁻¹) were grown in PYE broth (*Caulobacter*) or Penassay broth (*E. coli*) in the presence of various concentrations of an antibiotic. After six to seven generations, turbidity was measured. At the same time, cell morphology was observed under a phase-contrast microscope. The MIC₅₀ value was defined as the concentration at which the antibiotic inhibited cell growth by 50%.

Chemicals. [¹⁴C]Penicillin G (50 to 60 Ci mol⁻¹; 1.8 to 2.2 TBq mol⁻¹) was purchased from Amersham. Other β-lactam antibiotics were generous gifts from the following sources: apalcillin, Sumitomo Chemical Co., Osaka; cefazolin, Fujisawa Pharmaceutical Co., Osaka; cephalixin and cephaloridine, Takeda Pharmaceutical Industries Co., Osaka; cephaloglycin, Shionogi Pharmaceutical Co., Osaka; penicillin G and cloxacillin, Meiji Seika Co., Tokyo; mecillinam, W. O. Godtfredsen, Leo Pharmaceutical Products, Denmark.

RESULTS

Electrophoretic patterns of PBPs of Caulobacter species

It was reported previously (Koyasu *et al.*, 1980) that *C. crescentus* CB15 possessed at least five major PBPs and some minor ones. We first examined whether or not similar patterns of PBPs are observed among other species of stalked bacteria. Cell envelopes were incubated with 100 µg [¹⁴C]penicillin G ml⁻¹, which was a concentration sufficient to saturate most PBPs (see below). *Caulobacter crescentus* CB1, *C. crescentus* CB2 and *C. vibrioides* CB51

Fig. 1. Gel electrophoresis of PBPs. Envelopes of *C. crescentus* CB1 (a), *C. crescentus* CB2 (b), *C. crescentus* CB13 (c), *C. crescentus* CB15 (d), *C. vibrioides* CB51 (e), *A. excrucians* AC48 (f) or *E. coli* K12 (g) were incubated with 100 μg [^{14}C]penicillin G ml^{-1} at 30 $^{\circ}\text{C}$ for 10 min and then mixed with Sarkosyl and non-radioactive penicillin G as described in Methods. Samples were analysed by SDS-polyacrylamide gel electrophoresis and fluorography. The numbers on the left are the designations of the *Caulobacter* PBPs (Koyasu *et al.*, 1980); those on the right are the designations of the *E. coli* PBPs (Spratt *et al.*, 1977; Tamaki *et al.*, 1977).

were shown to have at least five major and some minor PBPs as also found in *C. crescentus* CB15 (Fig. 1). In all the *Caulobacter* strains examined, PBP1A, PBP1Bs and PBP 2 formed major bands in the gel. However, PBP4 could not be detected in *C. crescentus* CB13; evidently this strain does not possess PBP4 in its cell envelope. PBP1Bs often formed multiple gel bands that migrated close together and appeared to contain at least two components (Fig. 1). Many species of bacteria have been shown to have major bands of PBPs in a low molecular weight region, such as PBP5/6 in *E. coli* (Spratt & Pardee, 1975; Shepherd *et al.*, 1977; Chase *et al.*, 1978; Curtis *et al.*, 1979; Noguchi *et al.*, 1979; Ohya *et al.*, 1979). Such PBPs were not observed in the low molecular weight region of any of the *Caulobacter* strains examined (Fig. 1). We conclude that *Caulobacter* species possess five major PBPs (PBP1A, PBP1Bs, PBP2, PBP3 and PBP4).

Asticcacaulis is a Gram-negative bacterium with a subpolar stalk and is closely related to *Caulobacter*, with similar cell cycle events and morphogenesis (Poindexter, 1964). This organism exhibited a widely different pattern of PBPs from those of both *Caulobacter* and *E. coli* and did not possess PBPs of low molecular weight (Fig. 1).

Binding of [^{14}C]penicillin G to PBPs and release of bound [^{14}C]penicillin G

The extent of penicillin G binding is a criterion used to characterize PBPs and is considered to reflect their affinity for the antibiotic. For penicillin G binding to the *Caulobacter* PBPs, cell envelopes were incubated with [^{14}C]penicillin G at final concentrations of 0.003 to 300 μg ml^{-1} at 30 $^{\circ}\text{C}$ for 10 min and the extent of binding of [^{14}C]penicillin G to the different PBPs was estimated as described in Methods. In Fig. 2, the radioactivity associated with each PBP is presented as a percentage of that observed at the highest concentration of [^{14}C]penicillin G tested (300 μg ml^{-1}).

PBP1Bs of both *C. crescentus* CB13 and *C. crescentus* CB15 were saturated with [^{14}C]penicillin G at the lowest concentration (compared with the saturation concentrations for other PBPs) and PBP1A was saturated at the next lowest concentration. PBP1Bs and PBP1A were 50% saturated in the presence of 0.1 to 2.0 μg [^{14}C]penicillin G ml^{-1} in the binding mixture. Other PBPs required much higher concentrations (10 to 50 μg ml^{-1}) for 50% saturation. PBP4 was not saturated even at 100 μg ml^{-1} , and seemed to bind more

Fig. 2. $[^{14}\text{C}]$ Penicillin G binding to PBPs. Envelopes of *C. crescentus* CB13 (a) or *C. crescentus* CB15 (b) were incubated with $[^{14}\text{C}]$ penicillin G at final concentrations of 0.003 to 300 $\mu\text{g ml}^{-1}$ at 30 °C for 10 min. The radioactivity bound to PBPs was estimated by densitometry of X-ray films and is plotted as a percentage of the radioactivity bound with 300 μg $[^{14}\text{C}]$ penicillin G ml^{-1} . $\circ$, PBP1A; $\bullet$, PBP1B; $\square$, PBP2; $\blacksquare$, PBP3; $\triangle$, PBP4.

$[^{14}\text{C}]$ penicillin G when the concentration of the antibiotic was increased above 300 $\mu\text{g ml}^{-1}$. It is noteworthy that in *C. crescentus* CB13 and *C. crescentus* CB15, PBPs of similar molecular weight showed similar affinities for $[^{14}\text{C}]$ penicillin G, except for PBP3 of *C. crescentus* CB13 which, unlike PBP3 of *C. crescentus* CB15, showed lower affinity for $[^{14}\text{C}]$ penicillin G than PBP2 (Fig. 2). On the basis of the above results, $[^{14}\text{C}]$ penicillin G was used in the subsequent experiments at 100 $\mu\text{g ml}^{-1}$, a concentration at which most PBPs were saturated with the antibiotic.

PBPs bind penicillin G covalently (Blumberg & Strominger, 1974). The release of penicillin G therefore indicates the presence of an enzymic activity in PBPs. In *E. coli*, PBP5/6 has been identified as D-alanine carboxypeptidase IA (Spratt & Strominger, 1976; Matsuhashi *et al.*, 1978). It contains weak penicillinase activity and rapidly releases bound $[^{14}\text{C}]$ penicillin G (Tamura *et al.*, 1976; Spratt, 1977a): 50% release from the PBP5 and PBP6 bands was observed after 5 min and 19 min incubation, respectively (Spratt, 1977a). In order to examine the release of bound $[^{14}\text{C}]$ penicillin G from the *Caulobacter* PBPs, cell envelopes were first incubated with 100 μg $[^{14}\text{C}]$ penicillin G ml^{-1} , and then mixed and further incubated with excess non-radioactive penicillin G. The radioactivity remaining bound was measured at intervals. The release of $[^{14}\text{C}]$ radioactivity was slow for all the PBPs and at least 50% of the radioactivity remained bound to each of the PBPs after 40 min incubation (Fig. 3). None of the PBPs of the *Caulobacter* envelope released bound $[^{14}\text{C}]$ penicillin G as rapidly as PBP5/6 of *E. coli*.

Effect of temperature on PBPs

Another criterion used to characterize PBPs is the differential effect of temperature on their ability to bind penicillin G (Nakagawa *et al.*, 1979; Noguchi *et al.*, 1979; Ohya *et al.*, 1979).

Fig. 3. Release of bound ^{14}C penicillin G from PBPs. Envelopes of *C. crescentus* CB13 (a) or *C. crescentus* CB15 (b) were incubated with $100\ \mu\text{g}$ ^{14}C penicillin G ml^{-1} at $30\ ^\circ\text{C}$ for 10 min and then mixed with $5\ \mu\text{l}$ non-radioactive penicillin G ($90\ \text{mg}\ \text{ml}^{-1}$) and incubated further. At the indicated times, $2\ \mu\text{l}$ 5% (w/v) Sarkosyl was added and the radioactivity remaining bound to PBPs was estimated by densitometry of X-ray films; it is plotted as a percentage of the radioactivity bound when Sarkosyl was added simultaneously with non-radioactive penicillin G. $\circ$, PBP1A; $\bullet$, PBP1Bs; $\square$, PBP2; $\blacksquare$, PBP3; $\triangle$, PBP4.

To study the effect of temperature on the *Caulobacter* PBPs, cell envelopes were incubated at various temperatures for 10 min prior to the PBP assay at $30\ ^\circ\text{C}$. As shown in Fig. 4, the major PBPs of *C. crescentus* CB13 showed essentially similar heat sensitivity to the corresponding PBPs of *C. crescentus* CB15. PBP3 was very thermolabile and lost its activity for ^{14}C penicillin G binding after incubation at $40\ ^\circ\text{C}$ for 10 min. The other PBPs were relatively thermostable and retained high binding activities after the same incubation period. PBP1Bs and PBP2 were the most thermostable and partially retained their binding activities even after incubation at $50\ ^\circ\text{C}$ for 10 min.

Effects of related β -lactam antibiotics

As shown above, there are at least five major PBPs in the *Caulobacter* cell envelope. It was of interest to investigate how these proteins are related to the cell morphology. Several β -lactam antibiotics were tested for their MIC_{50} for *Caulobacter* growth, their effect on the cell morphology and their affinity for various PBPs.

The MIC_{50} values varied extensively between the β -lactam antibiotics used, ranging from $5\ \mu\text{g}\ \text{ml}^{-1}$ for cephalexin and cephaloridine to $500\ \mu\text{g}\ \text{ml}^{-1}$ for cloxacillin for *C. crescentus* CB15 (Table 1). This variation of growth inhibition indicated different effects of the β -lactams on cell permeability and/or affinity for the killing target in the cell.

Some antibiotics, namely penicillin G, apalcillin, cloxacillin and cephalexin for *C. crescentus* CB15, and apalcillin for *C. crescentus* CB13, induced, as a primary effect, the formation of filamentous cells at low concentrations and caused lysis at higher concentrations. Other antibiotics induced immediate lysis without prior morphological change, even at lower

Fig. 4. Effect of temperature on PBPs. Envelopes of *C. crescentus* CB13 (a) or *C. crescentus* CB15 (b) were pre-incubated at the indicated temperature for 10 min and then incubated with [^{14}C]penicillin G at 30 °C. The amount of bound [^{14}C]penicillin G was estimated by densitometry of X-ray films and is plotted as a percentage of the amount bound after pre-incubation at 30 °C for 10 min. ○, PBP1A; ●, PBP1Bs; □, PBP2; ■, PBP3; △, PBP4.

Table 1. MIC_{50} values and morphological effects of β -lactam antibiotics

Caulobacter crescentus strains CB13 and CB15 were grown in PYE broth at 30 °C, and *E. coli* K12 was grown in Penassay broth at 37 °C, each in the presence of various concentrations of β -lactam antibiotics. The change of cell morphology was observed under a phase-contrast microscope at each antibiotic concentration. The MIC_{50} value is the concentration at which the antibiotic inhibited cell growth by 50%. The primary effect of the antibiotics on cell morphology was usually observed at antibiotic concentrations that were lower than the MIC_{50} values.

Antibiotic	<i>C. crescentus</i> CB15		<i>C. crescentus</i> CB13		<i>E. coli</i> K12	
	MIC_{50} ($\mu\text{g ml}^{-1}$)	Morphology	MIC_{50} ($\mu\text{g ml}^{-1}$)	Morphology	MIC_{50} ($\mu\text{g ml}^{-1}$)	Morphology
Penicillin G	20	Filament	40	Lysis	15	Filament
Apalcillin	50	Filament	70	Filament and lysis	1	Filament
Cloxacillin	500	Filament	250	Lysis	200	Filament
Mecillinam	100	Thickened cell	80	Lysis	0.1	Sphere
Cephaloridine	5	Lysis	15	Lysis	1.5	Lysis
Cephalexin	5	Filament	6	Lysis	5	Filament
Cephaloglycin	15	Lysis	70	Lysis	1	Filament
Cefazolin	30	Lysis	40	Lysis	1.5	Filament

concentrations than the MIC_{50} . Mecillinam, which induces spherical cells in *E. coli* and other Gram-negative rod-shaped bacteria, did not induce such typical spherical forms in *Caulobacter*. This antibiotic induced a thickening of the cell with a stalk at one pole in *C. crescentus* CB15, but induced only lysis in *C. crescentus* CB13. Mecillinam might therefore

Table 2. Concentrations of β -lactam antibiotics for 50% saturation of PBPs estimated by the pre-addition method

Cell envelopes were first incubated with a β -lactam antibiotic at various concentrations at 30 °C for 10 min, and then mixed with [14 C]penicillin G at a final concentration of 100 μ g ml $^{-1}$. The amounts of bound [14 C]penicillin G were assayed by densitometry of X-ray film. The ID $_{50}$ value is the concentration of the antibiotic at which the amount of bound [14 C]penicillin G to each PBP was 50% of that in the absence of the antibiotic.

Antibiotic	PBP ...	ID $_{50}$ (μ g ml $^{-1}$)									
		<i>C. crescentus</i> CB15					<i>C. crescentus</i> CB13				
		1A	1Bs	2	3	4	1A	1Bs	2	3	
Penicillin G		<0.84	<0.84	37	7.4	26	2.1	<0.84	74	84	
Apalcillin		<1.2	<1.2	15	1.2	>1.9 $\times$ 10 4	<1.2	<1.2	52	7.5	
Cloxacillin		1.3	<1.0	46	13	>1.6 $\times$ 10 4	5.2	<1.0	26	26	
Mecillinam		4.6	0.73	180	18	6.4 $\times$ 10 3	9.1	18	270	64	
Cephaloridine		0.93	<0.93	5.8	170	58	4.6	<0.93	170	700	
Cephalexin		29	0.97	5.8	7.8	>2.4 $\times$ 10 3	49	2.4	15	58	
Cephaloglycin		5.1	<0.82	7.1	200	410	7.1	<0.82	51	200	
Cefazolin		2.6	2.0	10	39	20	10	2.0	100	330

Fig. 5. Inhibitory effect of cephalixin on [^{14}C]penicillin G binding. Envelopes of *C. crescentus* CB15 were incubated with cephalixin at the concentrations shown prior to [^{14}C]penicillin G addition. Cephalixin-PBP and [^{14}C]penicillin G-PBP complexes were separated by SDS-polyacrylamide gel electrophoresis and the radioactivity was detected by fluorography. The numbers on the left are the designations of the *Caulobacter* PBPs.

have a different target in *Caulobacter*, especially in *C. crescentus* CB13, compared with other bacteria.

We next examined the extent of inhibition by the various β -lactam antibiotics of penicillin G binding to the *Caulobacter* PBPs, which should reflect their affinities for the PBPs. In recent studies of PBPs in other bacteria, two methods have been used extensively: one is the pre-addition method, which measures the affinity of PBPs for the particular antibiotic that is added first, and the other is the simultaneous-addition (competitive inhibition) method, which measures only the relative affinity of PBPs for the antibiotic compared with [^{14}C]penicillin G. We used the pre-addition method. The results are summarized in Table 2, and the inhibitory effect of cephalixin is shown in Fig. 5. [^{14}C]Penicillin G was used at a concentration of $100\text{ }\mu\text{g ml}^{-1}$, which saturates most PBPs (Fig. 2). The ID_{50} values are the concentrations of the antibiotics required for 50% inhibition of [^{14}C]penicillin G binding to PBPs. None of the β -lactam antibiotics tested exhibited a specific inhibitory effect on a single PBP of *Caulobacter*. Mecillinam, which is specific for PBP2 in *E. coli* and for single PBPs of other bacteria, and induces the formation of spherical cells (Spratt & Pardee, 1975; Curtis *et al.*, 1979b; Noguchi *et al.*, 1979; Ohya *et al.*, 1979), was not specific and showed similar patterns of affinity to those of other β -lactam antibiotics (Table 2). Penicillin G, apalcillin, cloxacillin and cephalixin induced the formation of filaments in *C. crescentus* CB15 (Table 1). The ID_{50} values of these β -lactam antibiotics to PBP3 were low, from 1.2 to $13\text{ }\mu\text{g ml}^{-1}$ (Table 2). In *C. crescentus* CB13, only apalcillin induced the formation of filaments and showed a low ID_{50} value of $7.5\text{ }\mu\text{g ml}^{-1}$ for PBP3.

PBP4 of *C. crescentus* CB15 is remarkable in that it is present in the outer membrane (Koyasu *et al.*, 1980). Penicillin G and other β -lactam antibiotics (except cefazolin), however, showed only weak affinity for this PBP (Table 2). Although the function of PBP4 is not known, the absence of this PBP in *C. crescentus* CB13 (Fig. 1) suggests that PBP4 is dispensable for *Caulobacter*. PBP1Bs could be separated into closely migrating bands in the gel. Some of the β -lactam antibiotics showed a biphasic inhibitory effect on [^{14}C]penicillin G binding to this PBP. For example, when cephalixin was added, [^{14}C]penicillin G binding to PBP1Bs was reduced to a low level, but appreciable binding activity persisted even at a high

concentration of the antibiotic (Fig. 5). PBP2 behaved similarly to PBP1Bs. Although there has been no genetic analysis of the *Caulobacter* PBPs, it seems likely, together with the gel pattern (Fig. 1), that PBP1Bs consists of at least two components of different nature.

DISCUSSION

In this paper, we have demonstrated that *Caulobacter* species possess at least five major penicillin-binding proteins—PBP1A, PBP1Bs, PBP2, PBP3 and PBP4—in the cell envelope. The *Caulobacter* strains examined here did not possess PBPs of low molecular weight corresponding to the *E. coli* PBP 5/6. Such PBPs have been shown to have weak penicillinase activity and, in some bacteria, have been identified with D-alanine carboxypeptidase (Shepherd *et al.*, 1977; Spratt, 1977*a*; Chase *et al.*, 1978; Noguchi *et al.*, 1979).

When [¹⁴C]penicillin G and the envelopes of *C. crescentus* CB15 were incubated at a low pH (5.0), a minor band appeared with a molecular weight similar to the *E. coli* PBP5/6 in the gel (S. Koyasu, unpublished results). If it is a PBP like PBP5/6, perhaps the penicillinase activity is so high that the PBP cannot be detected easily by the usual method. It is also possible that such a low molecular weight PBP exists mostly in the soluble fraction of the cell. It would be necessary to examine the enzymic activity of D-alanine carboxypeptidase in the *Caulobacter* envelope to resolve this problem.

What are the functions of these PBPs in *Caulobacter*? Which PBP is the primary killing target of β -lactam antibiotics and which PBP is primarily involved in peptidoglycan biosynthesis and the formation of the cell wall? The relationship between effects on cell morphology and affinity of different PBPs for β -lactam antibiotics can be used to answer some of these questions. Mecillinam induces spherical cells in Gram-negative rod-shaped bacteria and has a specific affinity for one PBP (Spratt & Pardee, 1975; Curtis *et al.*, 1979*b*; Noguchi *et al.*, 1979; Ohya *et al.*, 1979). In *E. coli*, PBP2 is specifically affected by mecillinam and PBP2 mutants grow as spherical cells (Spratt & Pardee, 1975; Iwaya *et al.*, 1978). No such specificity of mecillinam was detected in *Caulobacter*. Penicillin G induced the formation of filaments in *C. crescentus* CB15, while it induced cell lysis in *C. crescentus* CB13. Correspondingly, the antibiotic showed higher affinity for PBP3 than for PBP2 in *C. crescentus* CB15, while it showed lower affinity for PBP3 than for PBP2 in *C. crescentus* CB13 (Fig. 2; Table 2). Similar results were obtained with other antibiotics, except for mecillinam (Tables 1 and 2). This type of correlation is also observed with the *E. coli* PBP1Bs and PBP3 (Spratt, 1975; Curtis *et al.*, 1979*a*). Thus, it is likely that PBP3 of *Caulobacter* is involved in cell division, as is PBP3 of *E. coli*, while PBP2 in *Caulobacter* might be involved in cell elongation. PBP1A and PBP1Bs showed low ID₅₀ values for the antibiotics used here and would be affected in vivo by low concentrations of these antibiotics. However, as shown in other bacteria, the PBP which shows the highest affinity for a β -lactam antibiotic is not necessarily the primary killing target of the antibiotic (Buchanan & Strominger, 1976; Curtis *et al.*, 1979*a*; Noguchi *et al.*, 1979). PBP1A and PBP1Bs might be inhibited by the antibiotics without causing any detectable change of cell morphology. Alternatively, the induction of filamentous cells or of lysis by inhibiting, as suggested above, either PBP3 or PBP2 might require the prior inhibition of PBP1A and/or PBP1Bs.

The MIC₅₀ values of the antibiotics were comparable to the ID₅₀ values in the case of PBP2 but not for PBP1Bs and PBP1A (Tables 1 and 2). It should be noted, however, that there are some difficulties in interpreting the MIC₅₀ value, since it is a function of cell permeability, β -lactamase activity and the affinity of the antibiotic for the killing target. The MIC₅₀ values were estimated here in a culture of low bacterial density, while the ID₅₀ values or 50% saturation levels were estimated by using concentrated suspensions of envelopes. Furthermore, it is not known to what extent the killing targets need to be inhibited in order to result in the inhibition of bacterial growth. In addition to the comparison of MIC₅₀ or ID₅₀ values, further studies, including mutational analysis, should serve to confirm the function of PBPs in the cell cycle and morphogenesis of *Caulobacter*.

It was shown previously (Koyasu *et al.*, 1980) that PBP4 of *C. crescentus* CB15 was localized in the outer membrane. Most of the β -lactam antibiotics used in this study had an extremely low affinity for PBP4. In addition, this PBP was not found in *C. crescentus* CB13. Therefore, despite its unique localization, PBP4 may not be essential for the *Caulobacter* cell. [^{14}C]Penicillin G did not completely saturate PBP4 of *C. crescentus* CB15 even at a concentration of $300\ \mu\text{g ml}^{-1}$ (Fig. 2). It is possible, although unlikely, that our failure to detect PBP4 in the *C. crescentus* CB13 envelope may have been due to its extremely low affinity for [^{14}C]penicillin G or an extremely high penicillinase activity.

In recent studies on PBPs in Gram-negative rod-shaped bacteria, the patterns of PBPs and their properties, such as the affinity for β -lactam antibiotics, have been shown to be quite similar to those of *E. coli*. By extrapolation, it may therefore be possible to deduce the function of each PBP of Gram-negative bacteria by comparing, with those of the *E. coli* PBPs, properties such as electrophoretic patterns, heat sensitivities, binding and release of [^{14}C]penicillin G, and effects of β -lactam antibiotics on cell morphology and [^{14}C]penicillin G binding. As noted above, however, comparative analysis of the properties of *Caulobacter* PBPs does not lead to putative PBP functions. The *Caulobacter* PBPs are so different in several properties from *E. coli* PBPs that the elucidation of their functions remains an interesting problem.

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