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Detection of Penicillin–Binding Proteins in *Bacillus Cereus* by Using Biotinylated β–Lactams

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A new method for detection of penicillin binding proteins from bacterial membranes has been developed in this study. The method, that employed biotin–ampicillin conjugate (Bio–PCA), is very rapid and can be a significant alternative of hazardous and time consuming conventional radiometric method for detection of PBPs in cell membranes. PBPs from *B. cereus* were examined by this technique. In vegetative and sporulating cells, 8 major PBPs were detected. Comparing with standard marker proteins, these PBPs were estimated for their molecular weight as 106, 83, 75, 72, 63, 46, 40, and 32 kDa. Affinity of cephalexin, cefoxitin, and cefotaxime to PBPs was measured indirectly by competition for subsequent binding of Bio–PCA. PBPs 2, 3, 4, and 7 were decreased or disappeared in the electrophoregram by prebinding with these β-lactams. Two PBPs, PBP 3 and PBP 4, which were predominant in vegetative and sporulating cells, respectively, showed strong affinity for cephalexin.

INTRODUCTION

The penicillin–binding proteins (PBPs) are a family of membrane–bound enzymes that are active in the metabolism of prokaryotic cell walls. They are evolutionarily related to some of the soluble β–lactamases and D, D–peptidases secreted by bacteria and also have some structural features in common with a penicillin–binding transmembrane protein involved in signal transduction (Joris *et al.*, 1990). Peptidoglycan is the major structural element of cell wall in eubacteria. Peptidoglycan synthesis is necessary not only for the maintenance of cell morphology but also for survival. PBPs function in peptidoglycan synthesis with their dual enzymatic activities, a transglycosylase activity that polymerizes the glycan strands and a transpeptidase activity that cross–links them via their peptide side chains. All bacteria have multiple PBPs. Some PBPs seem dispensable whereas others are essential for peptidoglycan syntheses. Each bacterial species has its own assortment of PBPs.

In *Escherichia coli*, there are eight well–characterized PBPs. These PBPs are generally divided into two classes, the higher molecular mass PBPs (PBP 1a/1b, PBP 2, and PBP 3) and the lower molecular mass PBPs (PBP 4, PBP 5, PBP 6, and PBP 7), numerically numbered in the order of their decreasing mobility in sodium dodecyl sulfate (SDS)–polyacrylamide gels (Waxman and Strominger, 1983). The higher molecular mass

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PBPs are bifunctional enzymes. They are generally D, D-transpeptidases, but in addition they have transglycosylase activity. These enzyme activities are essential for murein metabolism during bacterial growth and division, and they are the targets of the β-lactam antibiotics (Suzuki *et al.*, 1978). The lower molecular mass PBPs are generally monofunctional D, D-carboxypeptidases, and they do not seem to be essential for the growth and division of *E. coli*.

In *Bacillus subtilis*, seven high molecular mass PBPs, and four low-molecular mass PBPs have been identified (Blumberg and Strominger, 1972; Buchanan and Gustafson, 1992; Daniel *et al.*, 1994; Kleppe and Strominger, 1979; Sowell and Buchanan, 1983; Todd *et al.* 1983; Wu and Piggot, 1992). While most of these PBPs are present in vegetatively growing cells, some are specific to sporulation. Synthesis of cell-wall component peptidoglycan is important for two major morphological events of sporulation. Soon after the induction of sporulation of *B. subtilis*, the prespore and mother cell are separated by an asymmetric septum, which contains a thin layer of peptidoglycan. Around two hours after induction, a modified form of peptidoglycan is laid down between the membranes separating the prespore and mother cell to form a layer known as cortex.

Bacillus cereus is one of the food poisoning bacteria and produces heat resistant enterotoxin and spores. The presence of its spore in food is one of the biggest problems in food industry, since the spore is resistant to heating, ultra violet light and drugs. We have shown that cephalexin effectively inhibited sporulation of *B. cereus*. By using [^{3}H] benzylpenicillin, eight PBPs were detected from the cells of *B. cereus*. We have found two PBPs in *B. cereus* ts-4, with molecular masses of 75 kDa and 72 kDa, have high affinity for cephalexin (Miyamoto *et al.*, 1997).

PBPs are conventionally identified by [^{125}I]ampicillin or [^{3}H]benzylpenicillin. However, radiometric method is hazardous and rather time consuming. Recently, two promising methods for the non-radiometric identification of PBPs from bacterial membranes have been reported, one employed digoxigenin labeled ampicillin, whereas the other one exploited fluorescein-labeled penicillins to detect PBPs upon illumination with UV light or a laser beam (Weigel *et al.*, 1994; Galleni *et al.*, 1993).

In this paper, we have tried to develop a new and simple method using biotinylated β-lactams for the detection of bacterial PBPs and investigated the PBP profile of vegetative and sporulating cells of *B. cereus*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Escherichia coli IFO 3301 and *Bacillus cereus* JCM 2152 were obtained from Institute of Fermentation, Osaka and Japan Collection of Microorganisms, Wako, Saitama, respectively. *Bacillus cereus* ts-4 was a temperature sensitive mutant of *B. cereus* JCM 2152 isolated in our laboratory. *E. coli* was cultivated in LB broth at 30 °C with shaking. *B. cereus* was cultivated as described previously (Miyamoto *et al.*, 1997).

Preparation of bacterial membrane

Membrane fractions were prepared according to the method of Buchanan (1979) with little modifications. Vegetative and sporulating cells were harvested by centrifugation at

1,400×*g* for 5 min at 4°C, washed once with a membrane preparation buffer (MP buffer; 50 mM Tris–HCl buffer, pH 7.6, containing 1 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride) at 4°C and all further steps were carried out at 4°C. The cells were disrupted by ten 30–sec pulses for vegetative cells and twenty 30–sec pulses for sporulating cells with a Branson Sonifier model 185 at 60 W. Unbroken cells were removed by centrifugation at 8,500×*g* for 15 min. Membranes were pelleted by ultracentrifugation at 100,000×*g* for 1 h and washed once with MP buffer. Membrane fractions were then dissolved in a small volume of MP buffer. Proteins were measured by the dye binding method of Bradford (1976).

Biotinylation of β–lactams

Two milligrams of β–lactams (ampicillin, cephalexin, cefoxitin, and cefotaxime) in 1 ml of phosphate–buffered saline, pH 8.5 (PBS) was incubated for 2 h at room temperature with 1 mg of D–biotinylated–ε–aminocaproic acid–N–hydroxysuccinimide ester (Biotin–X–NHS ester, Boehringer Mannheim GmbH, Mannheim, Germany) dissolved in 100 μl of dimethyl sulfoxide. Unconjugated Biotin–X–NHS ester was trapped with glycine in a final concentration of 5 mM. Biotinylated antibiotic was purified by gel filtration on a Bio–Gel P2 column (Bio–Rad, CA, USA) equilibrated with PBS (pH 8.5). The concentrations of biotinylated β–lactams were calculated to be 1.5 mg/ml. The biotinylated ampicillin, cephalexin, cefoxitin, and cefotaxime were abbreviated as Bio–PCA, –CEX, –CFT, and –CTX, respectively. Structures of β–lactams and biotinylated ones used are shown in Fig. 1.

SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a 10% acrylamide gel according to the method of Laemmli (1970). Gels were stained with 0.1% coomassie brilliant blue R–250 dissolved in 20% methanol solution containing 10% acetic acid and proteins were visualized by destaining the gel with 50% methanol solution containing 5% acetic acid.

Detection of PBPs with biotinylated β–lactams

Labeling of PBPs with biotinylated ampicillin (Bio–PCA) and other biotinylated β–lactams was principally same as that with [^{3}H]benzylpenicillin (Miyamoto *et al.*, 1997). Two microliter of biotinylated β–lactam (0.03, 0.3, or 3 μg as unlabeled β–lactam) was added to 500 μg of membrane protein in a final volume of 30 μl of 100 mM Tris–HCl buffer (pH 7.6) and incubated for 30 min at 25°C. The labeled proteins were solubilized and separated in a 10% acrylamide gel as described above. The proteins were electroblotted onto a nitrocellulose membrane. The membrane was washed with water for 3 min and blocked in Tris–buffered saline (TBS: 100 mM Tris–HCl buffer, pH 7.5, containing 0.9% NaCl) containing 0.1% Tween 20 for 15 min and was treated for 30 min with streptavidin–horse radish peroxidase conjugate (Amersham Inter. plc. Amersham, UK) diluted 1,000–fold with TBS containing 0.01% Tween 20 (TTBS). Excess peroxidase was removed by washing the membrane 4 times with TTBS and the washed membrane was equilibrated in 50 mM Tris–HCl buffer (pH 7.5) for 10 min. For visualization of PBPs, the membrane was treated for 1 min in visualization solution (50 mM Tris–HCl buffer, pH 7.5,

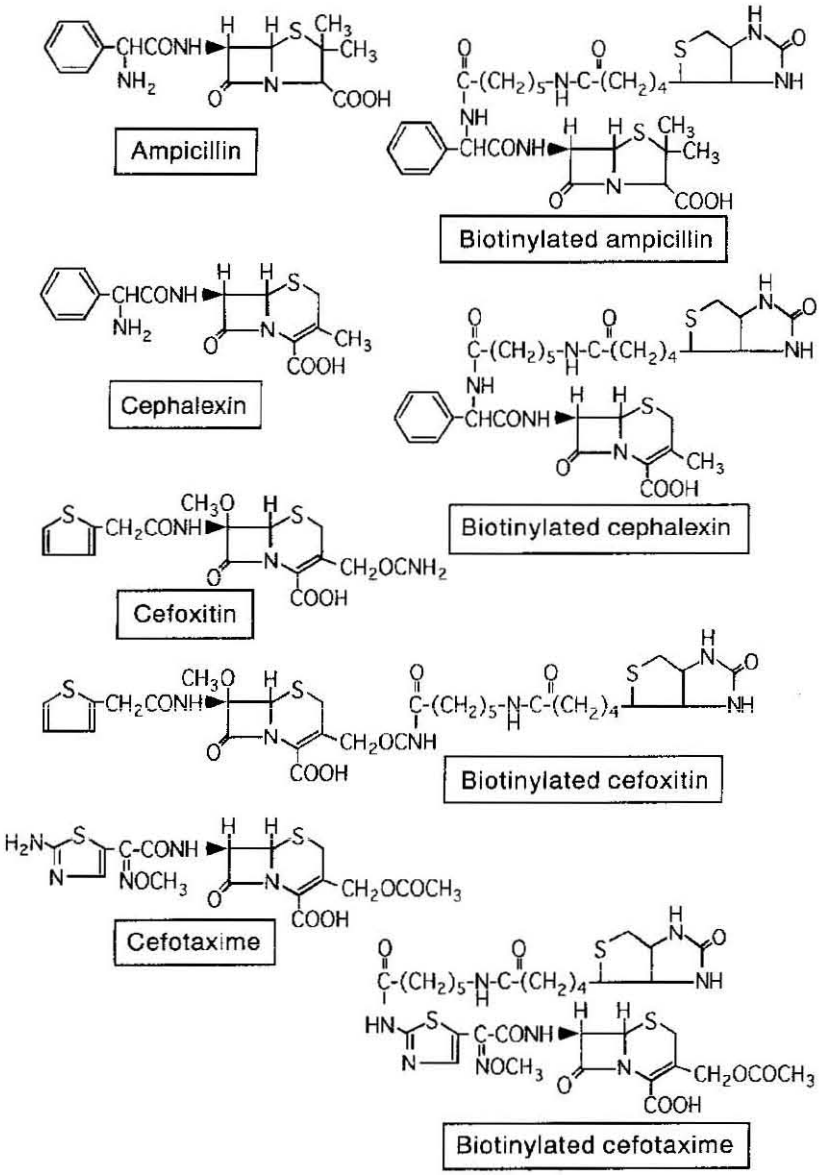

Fig. 1. Structures of β-lactams labeled with biotin.

containing 0.4 mg/ml luminol, 0.1 mg/ml *p*-iodophenol and 0.02% hydrogen peroxide) and then immediately placed in a plastic bag and exposed to a Fuji RX X-ray film for a minimal 20 min to overnight.

Competition assay was performed for measurement of the binding of cephalexin,

cefoxitin and cefotaxime to PBPs, the membrane protein was incubated with unlabeled β-lactams at 10 and 100 μg/ml for 10 min at 25 °C before incubation with Bio-PCA at 100 μg/ml.

RESULTS

Detection of PBPs of *E. coli* by using biotinylated ampicillin

The PBP pattern of *E. coli* IFO 3301 (K12 strain) detected by Bio-PCA is shown in Fig. 2. Seven PBPs were detected from the membrane preparation from *E. coli*. Their molecular masses were calculated to be 90, 89, 66, 60, 49, 42, and 40 kDa. To examine the concentration dependency of Bio-PCA on binding to *E. coli* PBPs, membrane proteins were incubated with graded concentrations of Bio-PCA. PBPs 3 and 5/6 were labeled at the lowest concentration of Bio-PCA tested, while the full complement of PBPs was detected at 10 μg/ml.

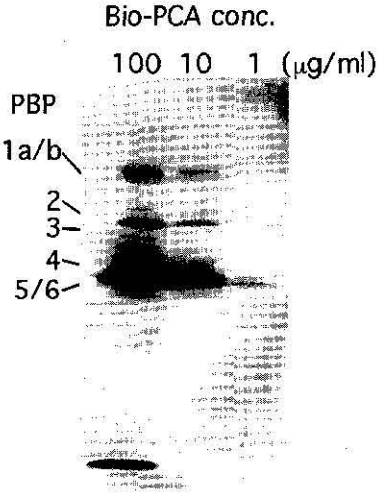

Fig. 2. Binding of *E. coli* PBPs with biotinylated ampicillin. *E. coli* membrane proteins were treated with biotinylated ampicillin (Bio-PCA) at a concentration of 1, 10 and 100 μg/ml.

Detection of PBPs of *B. cereus* by using biotinylated β-lactams

By using Bio-PCA, PBPs were detected from the cells of *B. cereus* ts-4 in mid-exponential growth and from sporulating cells after 3 h of induction. Fig. 3 shows the SDS-PAGE profiles of PBPs of vegetative and sporulating cells of *B. cereus* ts-4 by using biotinylated β-lactams. In vegetative and sporulating cells, 8 major PBPs were detected. In the initial stage of sporulation, PBPs 3, 4, 6, 7, and 8 were detected in the

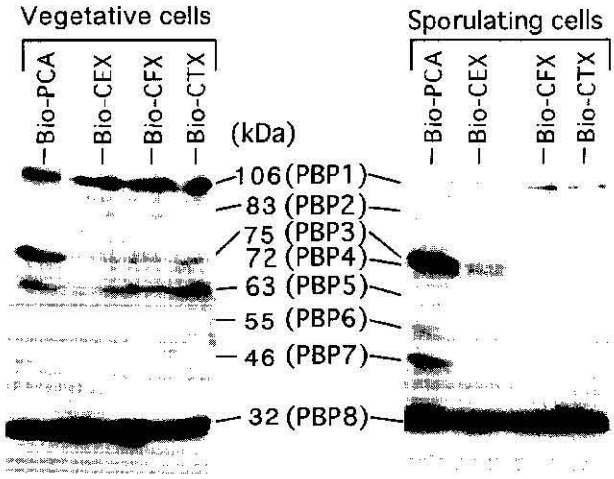

Fig. 3. SDS–PAGE profiles of PBPs of *B. cereus* ts–4 labeled with biotinylated β–lactams. Membrane proteins of *B. cereus* ts–4 were prepared from vegetative and sporulating cells by the method described in the text. The proteins were incubated with 100 μg/ml biotinylated ampicillin (Bio–PCA), cephalexin (Bio–CEX), cefoxitin (Bio–CFX), and cefotaxime (Bio–CTX).

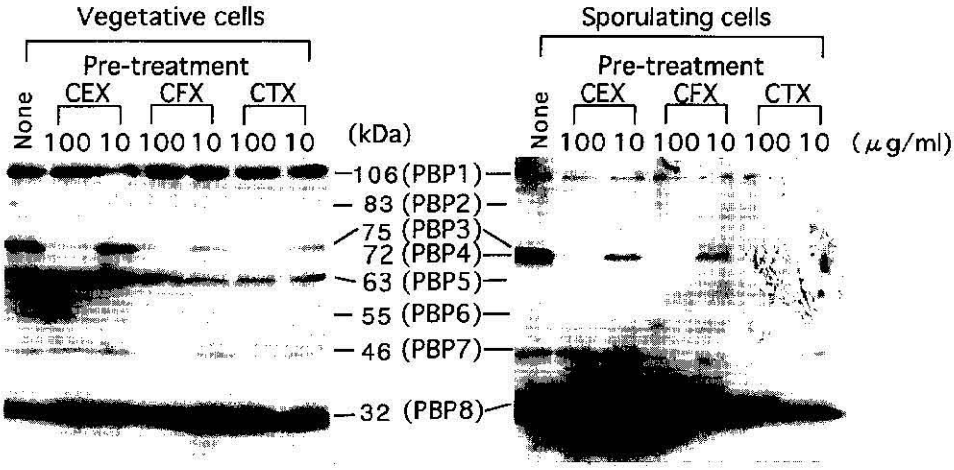

Fig. 4. SDS–PAGE profiles of PBPs of *B. cereus* ts–4 detected by biotinylated ampicillin. Membrane proteins prepared from vegetative and sporulating cells were incubated with Bio–PCA. To measure the bindings of cephalexin (CEX) and cefoxitin (CFX), and cefotaxime (CTX) to PBPs, the membrane proteins were incubated with these β-lactams at 10 and 100 μg/ml before addition of biotinylated ampicillin (Bio–PCA).

membrane fraction of *B. cereus*. Comparing with standard marker proteins, these 8 PBPs were estimated for their molecular weight as 106, 83, 75, 72, 63, 46, 40, and 32 kDa. The PBP pattern was completely same as that detected by [^{3}H]benzylpenicillin (Miyamoto *et al.*, 1997). The sensitivity of Bio–CEX, CFX, and CTX to PBP 3, 4, and 7 in the vegetative and sporulating cells was lower than that of Bio–PCA.

SDS–PAGE profiles of PBPs prebound with cephalexin, cefoxitin and cefotaxime and labeled with Bio–PCA are shown in Fig. 4. PBPs 2, 3, 4, and 7 were decreased or disappeared in the electrophoregram by the prebinding with these β–lactams. PBPs 3 and 4 in sporulating cells were disappeared by the prebinding with all 3 β–lactams at 100 and 10 μg/ml, respectively. PBP 7 was also disappeared by the prebinding with cefoxitin and cefotaxime at 100 μg/ml.

DISCUSSION

A new method for identifying penicillin–binding proteins was used in this study that employed biotin–streptavidin system. Biotin–streptavidin system is widely used in the area of immunoassay, immunocytochemistry, and protein blotting (Guesdon, 1979). The fact that proteins may be directly labeled with biotin via free amino groups suggested that the free amino group of ampicillin, cephalexin, cefoxitin, and cefotaxime might also have the potential for coupling with biotin (Fig. 1).

The *E. coli* PBP pattern detected by our method using Bio–PCA perfectly resembled with the well characterized PBP pattern of it as established by the radiometric method, demonstrating the validity of this new method (Fig. 2). It should be noted here that there are several advantages in the Bio–PCA method over radiometric method. First, the method is much more rapid, it takes only 20 min to overnight exposure after chemiluminescence reaction, compared with at least 20 day exposure for detection with [^{3}H]penicillin. Second, the biotinylation reaction is simple, inexpensive, and free of the hazard of radioactivity. Our present labeling method is applicable to antibiotics, other antimicrobial agents, or drugs possessing free amino groups and thus providing the ease of detection of their target sites, if the amino groups are not necessary for interaction with their target sites.

Weigel *et al.* (1994) used digoxigenin ester to label ampicillin for detecting PBPs. The Bio–PCA method may be superior to the method using digoxigenin labeled ampicillin in detecting bacterial PBPs, since streptavidin–conjugated multiple reporter enzymes bind to each Bio–PCA giving a high sensitivity.

Cephalosporins consist of a dihydrothiazine ring fused to a β–lactam ring (Fig. 1). Numbering of the molecule begins with the sulfur moiety, position 1. Substitutions on or in the molecule have been made at positions 1, 2, and 3, by moving the double bond between positions 3 and 4, and by substitution at position 7 or addition of different acyl side chains. These modifications were designed to increase the efficacy of cephalosporins against gram–negative bacteria, which rely mainly on β–lactamases for resistance. Cephalosporins containing amino group, such as cephalexin, cefaclor, cefadroxil and cefotaxime, were highly active against sporulation of *B. cereus* ts–4, whereas ampicillin and cefoxitin, also contain a free amino group in the side chain, were not and less effective, respectively (Miyamoto *et al.*, 1997). The sensitivity of Bio–CEX, –CFX, and

–CTX to PBP 3, 4, and 7 in the vegetative and sporulating cells was lower than that of Bio–PCA. It has been known that the introduction of any substituents (methyl, methoxy, imino or larger side chains) at position 7 of the bicyclic nucleus of cephalosporin reduced PBP affinity of the β–lactam (Neu, 1985). It seems that the free amino group of these cephalosporins somehow has the potential in giving them binding specificity to PBP(s) essential for sporulation in *B. cereus*.

PBPs were detected from vegetative and sporulating cells of *B. cereus* ts–4 in the present study by the Bio–PCA method. PBP pattern of vegetative cells of wild–type JCM 2125 was also identical to that of ts–4 (data not shown). In the initial stage of sporulation, PBPs 3, 4, 6, 7, and 8 were detected in the membrane fraction of *B. cereus* (Fig. 3 and 4). Among them, PBP 4 was the sole PBP that increased in amount during early stages of sporulation and it has high binding activity to cephalexin, cefoxitin, and cefotaxime (Fig. 4). The PBPs of *B. subtilis* have been extensively characterized (Todd *et al.*, 1983; Sowell and Buchanan, 1983; Buchanan and Sowell, 1983). Several distinct sporulation–specific PBPs have been identified (e.g. PBP 4* and PBP 5*), and several more PBPs that are present in vegetative cells have been reported to increase in abundance during sporulation (e.g. PBP 2B and PBP 3). Such increases were not observed during the early stages of sporulation of *B. cereus*, except PBP 4. This could have been the result of induction of sporulation when cells eliminate much of its vegetative proteins except those essential for survival, and synthesize new proteins essential for sporulation process and for dormancy.

We are interested in two PBPs of *B. cereus*, namely PBP 3 and PBP 4, though their functions are not known. Previous study with *E. coli* (Spratt, 1975) has shown that cephalexin inhibited septum formation and it did so by inhibiting PBP 3 of that organism. Amino acid substitutions of *E. coli* PBP 3 have resulted in profound loss of its affinity to cephalexin (Hedge and Spratt, 1985). Two PBPs from *B. subtilis* were reported to be homologous to *E. coli* PBP 3. A homologous protein in *B. subtilis* (PBP 2B) has been shown to be required for septum formation in vegetative and sporulating division of the bacterium (Yanouri *et al.*, 1993). The other homolog, *B. subtilis* SpoVD, seems to be evolutionary related to *E. coli* PBP 3, since their respective genes, *spoVD* and *pbpB*, occupy similar chromosomal locations immediately upstream from a conserved gene called *murE*. Considered from the nature of cephalexin affinity, it seems that *B. cereus* PBP 4 and also PBP 3 probably have the functional similarity with *E. coli* PBP 3 and *B. subtilis* PBP 2B and/or SpoVD. We are trying to determine the genes coding PBP 3 and 4 of *B. cereus* in order to clarify the mechanism of expression and to overproduce these PBPs. These results will be reported shortly.

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