PENICILLIN-BINDING PROTEINS IN *BACILLUS SUBTILIS* THE EFFECTS ON PENICILLIN-BINDING PROTEINS AND THE ANTIBACTERIAL ACTIVITIES OF β-LACTAMS

SUSUMU HORIKAWA and HIROSHI OGAWARA*

Second Department of Biochemistry, Meiji College of Pharmacy,
35-23, Nozawa-1, Setagaya-ku, Tokyo 154, Japan

(Received for publication December 1, 1979)

Several β-lactams were investigated on the affinity for the penicillin-binding proteins (PBPs) and the antibacterial activity in *Bacillus subtilis*. The β-lactams such as ampicillin, PS-5, methicillin and SCE-963, which had high affinities for PBP-2 showed strong antibacterial activities and the β-lactams such as cephamycin C, Y-G19Z-GG and Y-G19Z-G, which had high affinities for PBP-1 but low affinities for PBP-2, showed weak antibacterial activities. Clavulanic acid and nocardicin A, which had almost no affinities for all the PBPs detected, showed very low antibacterial activities. These results suggest that PBP-2 in *Bacillus subtilis* is the lethal target of these β-lactam antibiotics.

It has been reported that the β-lactam antibiotics kill bacteria by inhibiting transpeptidase in the synthesis of the bacterial peptidoglycan[1]. However, the demonstration of multiple penicillin-binding proteins in the bacterial membrane indicates the complex mechanism of β-lactam antibiotics on bacteria[2].

Among Gram-positive bacteria, the PBPs in *Bacillus* species have been investigated by STROMINGER and co-workers, who indicated that *Bacillus subtilis* had at least seven PBPs in membrane[3], PBP-2 was the most likely target for killing by β-lactam antibiotics[4], and that PBP-5 had D-alanine carboxypeptidase activity[5].

We have studied PBPs of *Streptomyces* species in relation to β-lactam biosynthesis[6] and β-lactamase[7,8]. As the basis of studying the physiological roles of the PBPs in *Streptomyces*, we have compared the effects of several β-lactams on the PBPs and the antibacterial activities in *Streptomyces* and those in *Bacillus subtilis*. This paper describes the results with *Bacillus subtilis* and the companion paper describes those with *Streptomyces cacaoi*.[19]

Materials and Methods

Bacterial strain

Bacillus subtilis PCI 219 was supplied by the Department of Antibiotics, the NIH of Japan.

Chemicals

Sodium dodecylsulfate (SDS), acrylamide, N,N,N′,N′,-tetramethylethylenediamine (TEMED) were purchased from Nakarai Chemicals Ltd., N,N′-methylene bis(acrylamide) and diphenyloxazole from Wako Pure Chemicals, tris(hydroxymethyl) aminomethane from Sigma Chemicals Co., [^{14}C] benzylpenicillin (56 mCi/mmol) from Radiochemical Centre, Amersham, crystalline bovine serum albumin from Nutritional Biochemical Co. Ovalbumin and bovine serum albumin used for the molecular weight standards were obtained from Schwarz/Mann.

PS-5[9] was a kind gift of Dr. T. ISHIKURA of the Sanraku Ocean Co., Ltd., SCE-963[10] and mecillinam from Dr. K. NARA of the Takeda Chemicals Co., Ltd., Y-G19Z-GG[11], Y-G19Z-G[12] and timoxi-

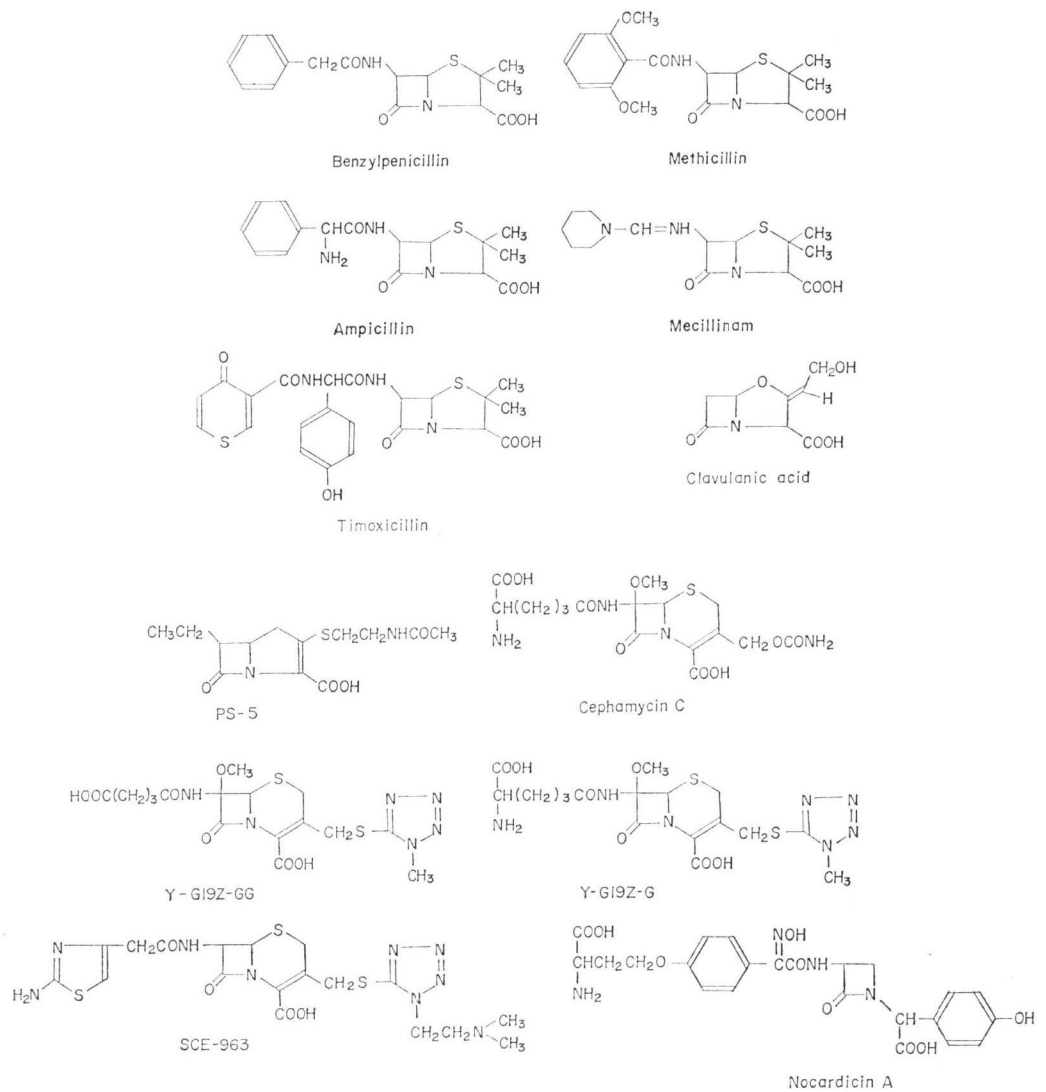
* To whom the reprint order should be addressed.

cillin[13] from Dr. S. WATANABE of the Yamanouchi Pharmaceutical Co., Ltd., cephamycin C from Dr. T. HIRAOKA of the Sankyo Co., Ltd., and nocardicin A[14] from Dr. H. IMANAKA of the Fujisawa Pharmaceutical Co., Ltd. Benzylpenicillin, ampicillin and methicillin were obtained from the commercial sources. The chemical structures of all the β-lactams used in this and the accompanying papers are shown in Fig. 1.

Membrane preparation

Membranes of *B. subtilis* were prepared as follows. *B. subtilis* was grown in 3 liters of nutrient broth at 37°C on a reciprocal shaking machine at 110 rpm. After 3.5-hour cultivation (late-log phase), cells (21 g, wet weight) were harvested, washed and resuspended in 70 ml of 0.01 M sodium phosphate buffer (pH 7.0) at 4°C. Cells were broken by sonication (Ohtake Works, Tokyo) three times for three minutes at 0°C. Unbroken cells were removed by centrifugation at 8,000 $\times g$ for 20 minutes at 4°C and the cell membranes were pelletted out of the supernatant by centrifugation at 100,000 $\times g$ for 40 minutes at 4°C. The membranes were washed and resuspended in 0.01 M sodium phosphate buffer (pH 7.0).

Fig. 1. The chemical structures of β-lactams.



Binding of [^{14}C] benzylpenicillin to membranes

The membrane fraction (20 mg protein/ml) was treated as described previously[6,15] for detecting the PBPs in the membrane, except that the SDS soluble fraction was obtained by centrifugation at 23,000 $\times g$ for 30 minutes at 20°C instead of 100,000 $\times g$ for 40 minutes at 20°C.

Slab gel electrophoresis and fluorography

The slab gel electrophoresis was performed by the method of LAEMMLI and FAVRE[16]. The polyacrylamide gel was composed of 8.25% acrylamide and 0.275% N,N'-methylene bis (acrylamide) and the electrophoresis gel was prepared for fluorography as described by BONNER and LASKEY[17]. The concentration required for the 50% inhibition of [^{14}C] benzylpenicillin (ID_{50}) was estimated from the densitometric analysis.

Antibacterial activity

Antibacterial activities of β-lactams were determined by the disc method. On an agar plate (13.5 $\times$ 9.5 cm) containing nutrient agar (0.5% meat extract, 1% peptone, 0.5% NaCl and 2% agar) and an overnight culture (1.6%) of *Bacillus subtilis* PCI 219, discs (8 mm in diameter) containing 20 μl of β-lactam solutions were placed. For the elimination of the different rate of diffusion of β-lactams in the disc method, the plate was incubated at 37°C after holding at 4°C for 1 hour.

Protein determination

Protein was determined by the method of LOWRY *et al.*[18] using crystalline bovine serum albumin as a standard.

Results

Effects of β-Lactams on the PBPs in *B. subtilis*

Fluorographic patterns in Figs. 2-a and 2-b show the effects of β-lactams on the PBPs in *B. subtilis*. As observed in slot 3 of Fig. 2-b, the PBP-2 was separated into two bands that was a little inconsistent with the data of KLEPPE and STROMINGER[3] which showed three bands (2a, 2b and 2c). Two bands observed between PBP-4 and PBP-5 in both fluorographic patterns, appeared not to bind any β-lactams except [^{14}C] benzylpenicillin in these Figs., but in another experiment they showed affinities for many β-lactams. Thus, their physiological significance was not clear at the present time.

On the basis of their affinities for PBP-1 and PBP-2, the β-lactams used could be classified into three groups.

Group 1: β-Lactams which had their affinities for both PBP-1 and PBP-2 such as ampicillin, PS-5, methicillin, SCE-963 and timoxicillin. The ID_{50} values for PBP-2 of these compounds and mecillinam were under 0.01 mM. Although ampicillin and timoxicillin were included in this same group, they were differentiated in that ampicillin had a higher affinity for PBP-2 than PBP-1 and timoxicillin had a higher affinity for PBP-1 than PBP-2.

Group 2: β-Lactams which had high affinities only for PBP-1 such as cephamycin C, Y-G19Z-G and Y-G19Z-GG. Their ID_{50} values for PBP-2 were about 0.09, 0.04 and 0.16 mM, respectively.

Group 3: β-Lactams which had weak affinities for all the PBPs such as clavulanic acid and nocardicin A. Mecillinam had an affinity for PBP-2 and PBP-4 but not for PBP-1.

The binding of [^{14}C] benzylpenicillin to PBP-3 increased gradually by the addition of clavulanic acid while the binding to other PBPs decreased (slots 4~6 in Fig. 2-a). In addition, all the β-lactams except clavulanic acid and nocardicin A (slots 4~6 and slots 7~9 in Fig. 2-a) had high affinities for PBP-4.

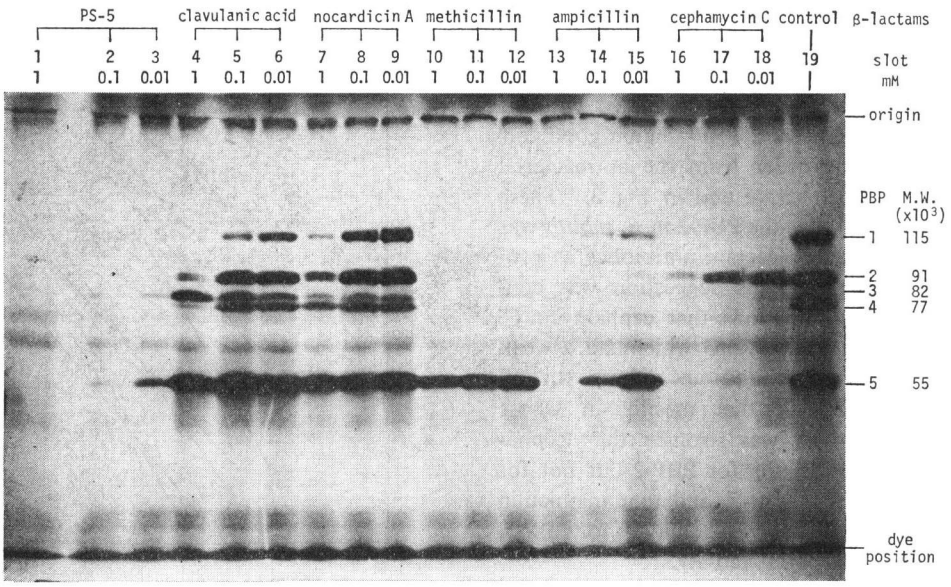
Antibacterial Activity

On the basis of their minimal inhibitory concentration (MIC) by the broth dilution method, the β-

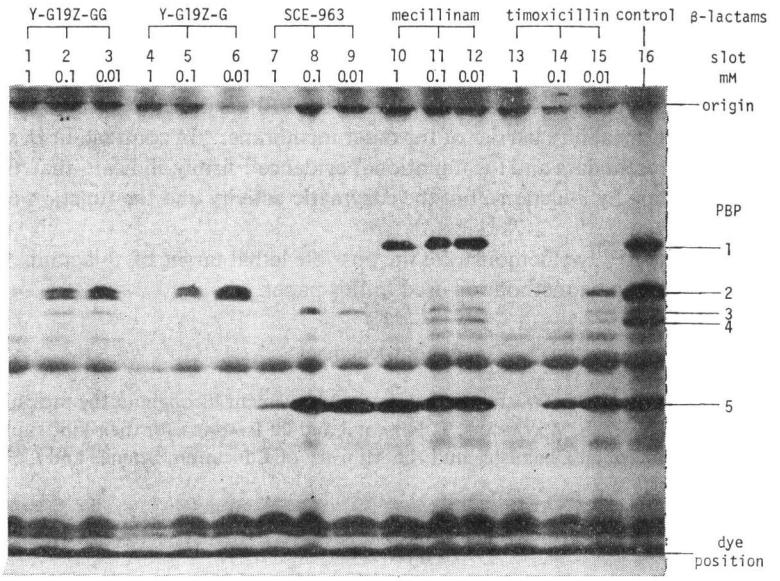
Fig. 2. Fluorographic patterns of PBPs in *B. subtilis*.

Slot 19 in (a) and slot 16 in (b) are control. In other slots, membrane samples were preincubated with β-lactams.

(a)



(b)

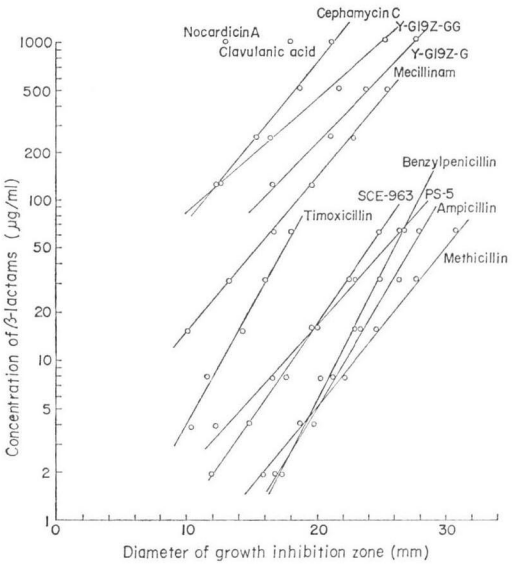


lactams could be grouped into A, B and C. Group A: Benzylpenicillin, ampicillin, methicillin, SCE-963 and timoxicillin, whose MIC values were under 1 μg/ml. Group B: Cephamycin C, Y-G19Z-GG, Y-G19Z-G and mecillinam whose MIC values were between 1 μg/ml and 100 μg/ml. Group C: Nocardicin A which showed MIC value of over 100 μg/ml.

The order of the β-lactams described above had a good correlation with that from their antibacterial activities determined by the disc method (Fig. 3).

Fig. 3. Antibacterial activities against *B. subtilis* determined by the disc method.

Absolute values of diameter of growth inhibition zone were not the same in another experiment, but the order of antibacterial activity was the same.



Discussion

The groups 1, 2 and 3 determined by their affinities for PBP-1 and PBP-2 had a good correlation with the order from the antibacterial activities described above and in Fig. 3. These results suggested that the PBP-2 in *B. subtilis* was the lethal target of β-lactam antibiotics as proposed previously[4]. This suggestion was confirmed by the observations that cephamycin C, Y-G19Z-GG and Y-G19Z-G which had a weak antibacterial activity, had an affinity for all PBPs except PBP-2, that mecillinam whose antibacterial activity was stronger than cephamycin C, had an affinity for PBP-2 but not for PBP-1, PBP-3 and PBP-5, and that methicillin and SCE-963 which had weak affinities for PBP-5, had strong antibacterial activity.

The effect of clavulanic acid on PBP-3, that is, the addition of clavulanic acid increased the binding of [^{14}C] benzylpenicillin to PBP-3, was interesting. Although the mechanism was completely unknown at the present time, this might have some relationships with the observation by KLEPPE and STROMINGER[3] that an extremely high concentration of [^{14}C] benzylpenicillin was needed in order to bind and saturate PBP-3.

The demonstration of a possible lethal target among the PBPs is difficult especially in Gram-negative bacteria because of the permeability barrier of the outer membrane. In contrast, in *B. subtilis*, a Gram-positive bacterium, the present data and the mutational evidence[4] firmly indicate that the PBP-2 is the most likely target for killing by β-lactams, but the enzymatic activity and the function of the PBP-2 are not known yet.

In the succeeding paper[19], we demonstrate the possible lethal target of β-lactams in the PBPs in *Streptomyces cacaoi* by the same methods as used in this paper.

Acknowledgment

We are grateful to Prof. H. UMEZAWA for his hearty encouragement throughout the present work. We also thank Drs. T. ISHIKURA, K. NARA, S. WATANABE, T. HIRAOKA and H. IMANAKA for their kind supplies of β-lactams. We thank the Institute of Microbial Chemistry and the Ministry of Education, Science and Culture in Japan for the kind support of this work.

References

1) WISE, E. M. & J. T. PARK: Penicillin: Its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. Proc. Natl. Acad. Sci. U.S.A. 54: 75~81, 1965
2) SUGINAKA, H.; P. M. BLUMBERG & J. L. STROMINGER: Multiple penicillin-binding components in *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli*. J. Biol. Chem. 247: 5279~5288, 1972
3) KLEPPE, G. & J. L. STROMINGER: Studies of the high molecular weight penicillin-binding proteins of *Bacillus subtilis*. J. Biol. Chem. 254: 4856~4862, 1979
4) BUCHANAN, C. E. & J. L. STROMINGER: Altered penicillin-binding components in penicillin-resistant mu-

tants of *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. 73: 1816~1820, 1976

5) BLUMBERG, P. M. & J. L. STROMINGER: Isolation by covalent affinity chromatography of the penicillin-binding components from membranes of *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. 69: 3751~3755, 1972

6) OGAWARA, H. & S. HORIKAWA: Penicillin-binding proteins of *Streptomyces cacaoi*, *Streptomyces olivaceus* and *Streptomyces clavuligerus*. Antimicr. Agents & Chemoth: 17: 1~7, 1980

7) OGAWARA, H.: Production and property of β-lactamases in *Streptomyces*. Antimicr. Agents & Chemoth. 8: 402~408, 1975

8) OGAWARA, H.; S. HORIKAWA, S. SHIMADA-MIYOSHI & K. YASUZAWA: Production and property of β-lactamases in *Streptomyces*: Comparison of the strains isolated newly and thirty years ago. Antimicr. Agents & Chemoth. 13: 865~870, 1978

9) OKAMURA, K.; S. HIRATA, Y. OKUMURA, Y. FUKAGAWA, Y. SHIMAUCHI, K. KOUNO & T. ISHIKURA: PS-5, a new β-lactam antibiotic from *Streptomyces*. J. Antibiotics 31: 480~482, 1978

10) NOZAKI, Y.; A. IMADA & M. YONEDA: SCE-963, a new potent cephalosporin with high affinity for penicillin-binding proteins 1 and 3 of *Escherichia coli*. Antimicr. Agents & Chemoth. 15: 20~27, 1979

11) WATANABE. S.; Y. OKA & T. OSONO: Japan Kokai (Patent), 53–15,494, 1978

12) OSONO. T.; S. WATANABE T. SAITOH, T. KAISHIMA & K. MURAKAMI: Japan Kokai (Patent), 52–79,081, 1977

13) KASHIWAGI, T.; I. ISAKA, N. KAWAHARA, K. NAKANO, A. KODA, T. OZASA, I. SOUZU, Y. MURAKAMI & M. MURAKAMI: A new broad spectrum penicillin PC-455: 6-{α-(4-oxo-4H-thiopyran-3-yl-carboxamido) *p*-hydroxyphenylacetamido} penicillanic acid. I. Chemistry and structure-activity relationships. Presented at the 15th Intersci. Conf. Antimicr. Agents & Chemoth., No. 255, Washington, D. C., Sep. 24~26, 1975

14) AOKI, H.; H. SAKAI, M. KOHSAKA, T. KONOMI, J. HOSODA, Y. KUBOCHI, E. IGUCHI & H. IMANAKA: Nocardicin A, a new monocyclic β-lactam antibiotic. I. Discovery, isolation and characterization. J. Antibiotics 29: 492~500, 1976

15) HORIKAWA, S. & H. OGAWARA: Membrane-bound penicillin-binding proteins of *Escherichia coli*. Comparison of a strain carrying an R-factor and the parent strain. J. Antibiotics 31: 1283~1291, 1978

16) LAEMMLI, U. K. & M. FAVRE: Maturation of the head of bacteriophage T4. J. Mol. Biol. 80: 575~579, 1973

17) BONNER, W. M. & R. A. LASKEY: A film detection method for tritium labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46: 83~88, 1974

18) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the FOLIN phenol reagent. J. Biol. Chem. 193: 265~275, 1951

19) OGAWARA, H. & S. HORIKAWA: Penicillin-binding proteins in *Streptomyces cacaoi*. The effects on penicillin-binding proteins and the antibacterial activities of β-lactams. J. Antibiotics 33: 620~624, 1980