PENICILLIN-BINDING PROTEINS IN *STREPTOMYCES CACAOI* AND *STREPTOMYCES CLAVULIGERUS*

KINETICS OF [^{14}C]BENZYLPENICILLIN BINDING, TEMPERATURE SENSITIVITY AND RELEASE OF [^{14}C]BENZYLPENICILLIN FROM THE COMPLEX

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On the membrane-bound penicillin-binding proteins (PBP) of *Streptomyces cacaoi* and *S. clavuligerus*, the kinetics of [^{14}C]benzylpenicillin binding, the temperature sensitivity, the release of [^{14}C]benzylpenicillin from the [^{14}C]benzylpenicillin-PBP complexes and the changes of the PBP patterns during the growth cycle were examined. All the PBP in both strains, especially PBP in *S. clavuligerus*, had very low affinity for benzylpenicillin, comparing with other bacteria. As for the temperature sensitivity of the binding ability, all the PBP in *S. cacaoi* were highly sensitive to heat, while PBP-3 in *S. clavuligerus* retained the binding activity after incubation at 60°C for 10 minutes. The release of [^{14}C]benzylpenicillin from the complexes with PBP-1, PBP-2 in *S. cacaoi* and PBP-3 in *S. clavuligerus* was relatively fast initially. However, this soon reached a plateau and the complexes retained [^{14}C]benzylpenicillin even after prolonged incubation. During the growth cycle, the PBP patterns in *S. cacaoi* did not change significantly. However, in *S. clavuligerus*, a band of molecular weight of about 120,000 daltons was observed only in the membrane fraction of early log phase, and PBP-1 (Mr=83,000) and PBP-2 (Mr=79,000) appeared only slightly in this phase.

Most of the *Streptomyces* strains produce β-lactamases with varied properties extracellularly and constitutively[1,2]. Their physiological roles are completely unknown at the present time. However, these enzymes are interesting from the points of the possible evolutional relationships between β-lactamases in *Streptomyces* and pathogenic bacteria[3~6], between β-lactamases and penicillin-binding proteins (PBP) in *Streptomyces*[7~9] and of the possible roles of β-lactamases and PBP in the self-resistance in *Streptomyces* (OGAWARA, Actinomycete Biology, in press). In order to clarify these points further, we examined the PBP of *Streptomyces* which produced β-lactamase or β-lactam compounds[10]. Their properties such as numbers and the affinities for mecillinam, clavulanic acid and methicillin are quite different in *S. cacaoi*, a β-lactamase-producer but β-lactam non-producer, and in *S. olivaceus* and *S. clavuligerus*, β-lactam-producers. These differences may result mainly from the production of β-lactam compounds. Interestingly, however, the affinity patterns of various β-lactams to PBP and the number of PBP in *S. cacaoi* are quite similar to those of *Bacillus subtilis*[11,12], and PBP-2 (Mr=91,000) was suggested to be a killing target of many β-lactam compounds[13].

To elucidate the roles of each PBP in *S. cacaoi* and *S. clavuligerus*, we have studied the kinetics of [^{14}C]benzylpenicillin binding, temperature sensitivity, the release of [^{14}C]benzylpenicillin from the complexes, and the changes of the PBP patterns during the growth cycle.

Materials and Methods

Streptomyces strains

Two *Streptomyces* strains were used: *S. cacaoi* subsp. *cacaoi* KCC S-352 and *S. clavuligerus* IFO-13307. *Streptomyces cacaoi* was a generous gift from Dr. A. SEINO of Kaken Chemicals Co.

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 and benzylpenicillin potassium salt from Sigma Chemicals Co., and [^{14}C]benzylpenicillin (59.5 mCi/mmol) from Radiochemical Centre, Amersham.

Membrane preparation, slab gel electrophoresis and fluorography

Membrane fractions of *S. cacaoi* and *S. clavuligerus* were prepared as previously described[10]. Binding of [^{14}C]benzylpenicillin to the membrane and slab gel electrophoresis-fluorography were performed as described[10,14].

Kinetics of [^{14}C]benzylpenicillin binding to PBP

The membrane fraction of 50 μl (1 mg of protein) was treated with 10 μl of various concentrations of [^{14}C]benzylpenicillin for 10 minutes at 30°C, and the reaction was stopped by the addition of 20 μl of 0.5 M Tris-HCl, pH 6.8 containing 0.9% benzylpenicillin and 37.5% SDS. After the membrane was solubilized for 10 seconds at 100°C and insoluble fractions were removed by centrifugation at 22,000$\times g$ for 40 minutes at 20°C, the supernatant was used for the electrophoretic analysis.

Temperature sensitivity

This was performed by the method of NOGUCHI *et al.*[15] with some modifications. After preincubation at various temperatures for 10 minutes, a sample of 50 μl (1 mg of protein) of the membrane fraction was incubated with 5 μl of [^{14}C]benzylpenicillin (314 μg/ml, 0.5 μCi) for 10 minutes at 30°C. Then, the reaction was stopped and the membrane was solubilized as described. The supernatant at 22,000$\times g$ for 40 minutes was used for the electrophoresis.

Release of [^{14}C]benzylpenicillin from [^{14}C]benzylpenicillin-PBP complex

The procedure of SPRATT[16] was used with slight modifications. The membrane preparation of 300 μl (6 mg of protein) was incubated at 30°C for 10 minutes with 30 μl of [^{14}C]benzylpenicillin (314 μg/ml, 3 μCi). After the addition of 100 μl of 0.5 M Tris-HCl, pH 6.8, containing 7.5% of benzylpenicillin, the incubation was continued further. At various times, 50 μl aliquots were mixed with 20 μl of 0.5 M Tris-HCl, pH 6.8, containing 37.5% SDS and the supernatants obtained as described above were applied to the gel for electrophoresis.

Effect of growth on membrane-bound PBP

Streptomyces strains were grown at 27°C as described previously[10]. The mycelia were collected at early log phase, log phase, late log phase and lysis phase. Membrane preparation, slab gel electrophoresis and fluorography were performed as described.

Results

The fluorographic patterns of the PBP are shown in Fig. 1. In *S. cacaoi*, six bands were detected, the molecular weights of which were 105,000 (PBP-1), 91,000 (PBP-2), 64,000 (PBP-3), 55,000 (PBP-4), 50,000 (PBP-5), and 47,000 (PBP-6). In *S. clavuligerus*, three bands of PBP-1 (Mr=83,000), PBP-2 (79,000), and PBP-3 (47,000) were reproducibly detected. These results confirmed those of the previous experiments[10]. Even though the PBP were not saturated with [^{14}C]benzylpenicillin as described below, approximate proportions of these PBP determined at a concentration of 50 μg [^{14}C]benzylpenicillin per ml were PBP-1, 3%; PBP-2, 6%; PBP-3, 29%; PBP-4, 20%; PBP-5, 35% and PBP-6, 7% in *S. cacaoi*

Fig. 1. Fluorographic patterns of kinetics of [^{14}C] benzylpenicillin binding to PBP.

[^{14}C]Benzylpenicillin was incubated at final concentrations of 0.001 to 50 μg/ml with membrane preparations of *S. cacaoi* and *S. clavuligerus* for 10 minutes at 30°C. The samples were loaded on a 8.25% SDS-polyacrylamide slab gel (bisacrylamide/monoacrylamide=2.67%), and a fluorogram was prepared by exposure of the dried gel to Fuji X-ray film for 60 days.

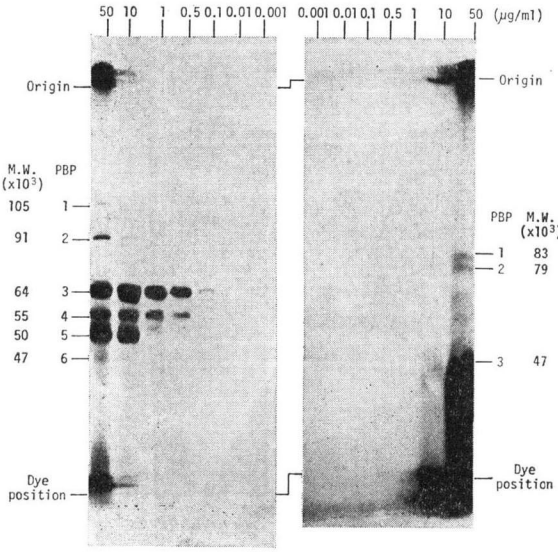


Fig. 2. Kinetics of [^{14}C]benzylpenicillin binding to PBP.

[^{14}C]Benzylpenicillin-PBP complexes detected in the Fig. 1 were estimated by densitometry of the X-ray films. Binding of [^{14}C]benzylpenicillin was plotted as a percentage of that in 50 μg/ml of [^{14}C] benzylpenicillin.

(A) *S. cacaoi*, (B) *S. clavuligerus*.

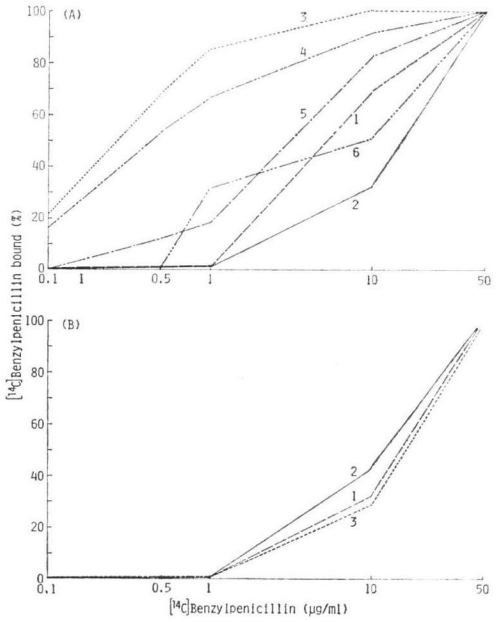
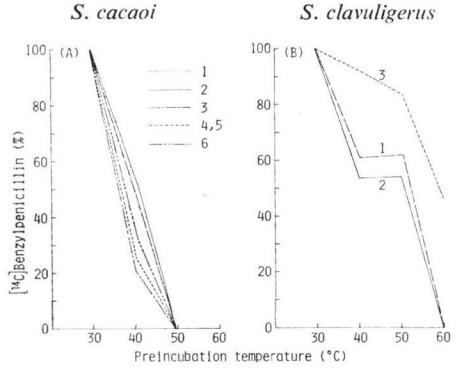


Fig. 3. Temperature sensitivity of penicillin-binding activity of PBP.

Membrane preparations of *S. cacaoi* and *S. clavuligerus* were preincubated at various temperatures as described in the figure. Then, [^{14}C]benzylpenicillin was added and the reaction mixtures were treated as described in Materials and Methods. The resulting [^{14}C]benzylpenicillin-PBP complexes detected on the fluorograms were estimated by densitometry of X-ray films. Binding of [^{14}C]benzylpenicillin was plotted as a percentage of that at 30°C.



and PBP-1, 5%; PBP-2, 6%; and PBP-3, 89% in *S. clavuligerus*, respectively. When the membrane fractions were treated with increasing concentrations of [^{14}C]benzylpenicillin and the fluorographic patterns (Fig. 1) were analysed by densitometer, the intensity of the bands increased differently from each other (Fig. 2). PBP-3 and PBP-4 in *S. cacaoi* were almost saturated with benzylpenicillin at a concentration of 50 μg/ml. However, other PBP, especially PBP-2, showed very low affinity for benzylpenicillin. In *S. clavuligerus*, on the other hand, all the PBP showed very low affinity for benzylpenicillin. Thus, almost no band was detected at 1 μg/ml.

Temperature sensitivity of the PBP was determined in both strains (Fig. 3). In *S. cacaoi*, all the PBP were highly sensitive to heat and lost their binding capacity after incubation at 50°C for 10 minutes. On the contrary, in *S. clavuligerus*,

Fig. 4. Release of [^{14}C]benzylpenicillin from [^{14}C] benzylpenicillin-PBP complexes.

Membrane preparations of *S. cacaoi* (A) and *S. clavuligerus* (B) were incubated with [^{14}C]benzylpenicillin for 10 minutes at 30°C. Then, excess of non-radioactive benzylpenicillin was added and incubation was continued further at 30°C. At the times indicated in the figure, SDS was added to aliquots of the mixture and [^{14}C]benzylpenicillin-PBP complexes were detected by fluorography. The amount of [^{14}C]benzylpenicillin still bound to PBP was estimated by densitometry of the fluorograms and was plotted as the density of each [^{14}C] benzylpenicillin-PBP complex at 0 minute was one hundred percent.

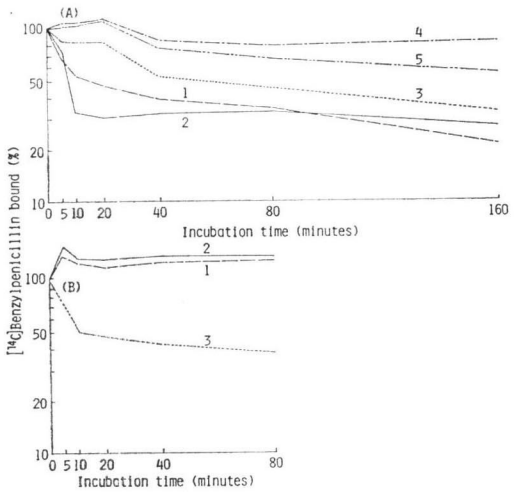
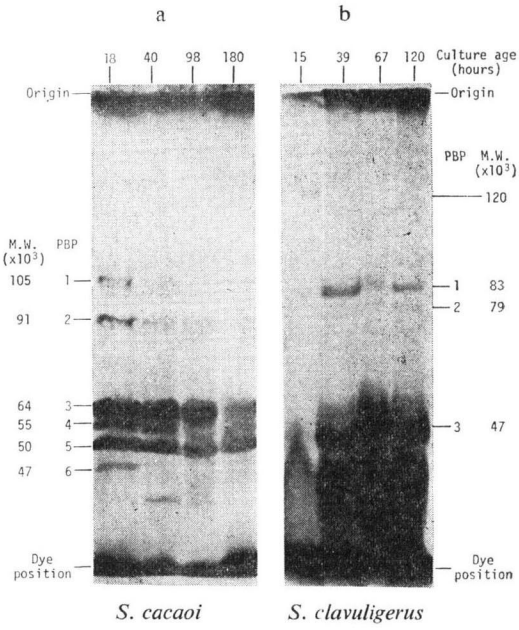


Fig. 5. The fluorographic patterns of PBP in the membranes of different culture age of *S. cacaoi* and *S. clavuligerus*.

Both strains were cultivated at 27°C and the membranes were prepared at the indicated culture age in the figure as described in Materials and Methods.



although PBP-1 and PBP-2 lost their binding capacity for benzylpenicillin at 60°C, PBP-3 retained the binding activity at this temperature.

The release of [^{14}C]benzylpenicillin from the complexes with PBP-1 and PBP-2 in *S. cacaoi* and PBP-3 in *S. clavuligerus* was relatively rapid at the first part of the incubation, the half-life being about 8~15 minutes (Fig. 4). However, this soon reached the plateau and the complexes retained [^{14}C] benzylpenicillin even after prolonged incubation. As for the other complexes, only very slight or no release of [^{14}C]benzylpenicillin was observed.

The effect of growth on the PBP patterns was examined. Through the whole cycle of the growth, the PBP patterns in *S. cacaoi* did not change significantly. Fig. 5a shows the patterns of the PBP in the membrane fractions prepared from *S. cacaoi* of the indicated culture age in the figure. On the contrary, the PBP patterns in early log phase (15-hour culture) of *S. clavuligerus* were definitely different from those in other phases (Fig. 5b). In this membrane fraction, PBP of the molecular weight of about 120,000 dalton was clearly observed but PBP-1 and PBP-2 appeared only slightly. In this medium, cephamycin C detected by the antibacterial activity against *Proteus* spp. was produced from 40-hour culture and the maximum activity was observed at 96-hour cultivation. Clavulanic acid, on the other hand, was detected from 24-hour culture and the maximum potency was observed in 67-hour culture.

Discussion

The membrane-bound PBP in *S. cacaoi* and *S. clavuligerus* were compared in the binding affinity, temperature sensitivity of [^{14}C]benzylpenicillin binding and the release of [^{14}C]benzylpenicillin from the complexes. As reported previously[10,13], the PBP in these strains are quite different from each other in numbers and the affinity for various β-lactam compounds. In addition, the following differences were observed in the present experiments: First, PBP-3 and PBP-4 in *S. cacaoi* were almost saturated with [^{14}C]benzylpenicillin at a concentration of 50 μg/ml, but all the PBP in *S. clavuligerus* had very low affinities for benzylpenicillin as in the case of PBP-2 in *S. cacaoi*. Second, although the molecular weights of PBP-6 in *S. cacaoi* and PBP-3 in *S. clavuligerus* were similar (47,000), the former was a minor component (7%), but the latter was a major band (89%). Furthermore, these PBP were quite different in their sensitivity to heat (Fig. 3). Third, the release of [^{14}C]benzylpenicillin from PBP-1 and PBP-2, the higher molecular weight components, was relatively rapid in *S. cacaoi*, while that from PBP-1 and PBP-2 in *S. clavuligerus* could not be observed even after a prolonged incubation (Fig. 4). Rather, the release from PBP-3, the smallest component, was faster in *S. clavuligerus*.

These differences are quite distinct because *S. cacaoi* and *S. clavuligerus* belong to the same genus. Although *Escherichia coli* and *Pseudomonas aeruginosa* belong to different genera, the properties of the PBP in these bacteria are similar[15]. This is also the case with *Bacillus subtilis*[11], *B. licheniformis*[17] and *B. megaterium*[17,18]. The major reason for these differences may result from the production of β-lactam compounds in *S. clavuligerus*. However, the idea that the PBP in *S. clavuligerus* is already saturated with the β-lactams produced by itself such as cephamycin C and clavulanic acid was negated by the experiment with hydroxylamine[10]. Thus, the property of producing β-lactam compounds in itself may affect the apparent PBP patterns in *Streptomyces*. In accord with this, the numbers of PBP are generally smaller in the β-lactam-producers than in the β-lactam-non-producers (Ref. 10, and unpublished data).

Another interesting property of PBP in *Streptomyces* is their low affinity for [^{14}C]benzylpenicillin in general. PBP-2 in *S. cacaoi* and all the PBP in *S. clavuligerus* could not be saturated with benzylpenicillin at a concentration of 50 μg/ml. Even this value (50 μg/ml) is extremely high compared with that of PBP in *E. coli* (less than 8 μg/ml)[16], and that of PBP-2, a supposed killing target[11,12] in *B. subtilis* (less than 0.15 μg/ml)[19]. The low affinity of PBP-2 in *S. cacaoi* is in good accord with the proposal that PBP-2 may be a killing target of many β-lactam compounds in this strain[13], and low affinity of all the PBP in *S. clavuligerus* may be due and necessary for its production of β-lactam compounds. The low affinity for [^{14}C]benzylpenicillin in *Streptomyces* reflects on the high minimum inhibitory concentration (MIC) values for benzylpenicillin in *Streptomyces* in general[1]. The MIC value for benzylpenicillin in *S. cacaoi* and *S. clavuligerus* is more than 30 times higher than that in *B. subtilis*[2,13]. Thus, *Streptomyces* strains protect themselves from their own metabolites, β-lactam compounds, by at least two mechanisms: low affinity penicillin-binding proteins as described above and a hydrolyzing enzyme, β-lactamase[20].

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