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Penicillin-Binding Protein 7 and Its Relationship to Lysis of Nongrowing *Escherichia coli*

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The characteristics and possible physiological role(s) of a 30-kilodalton low-molecular-size penicillin-binding protein (PBP), PBP 7, in *Escherichia coli* were investigated. The amount of penicillin required to half saturate PBP 7 was approximately 5 μg/ml, and this PBP bound 15% of the total penicillin bound to all PBPs with a deacylation rate of > 120 min. This PBP was distinguishable from *E. coli* PBPs 1 to 6 by the pattern of [^{3}H]penicillin-labeled peptides generated by partial proteolysis. A unique feature of PBP 7 was its capacity to bind numerous members of penem class antibiotics at relatively low concentrations. A striking correlation existed in drugs between capability of lysing amino acid-starved (nongrowing) *E. coli* and binding to PBP 7. The findings suggest that PBP 7 is a new, physiologically significant target for beta-lactam antibiotics.

Escherichia coli is known to have seven major penicillin-binding proteins (PBPs) ranging in molecular size from 40 to 91 kilodaltons (kDa). The identification of the physiological function(s) of some of these PBPs has been greatly helped by their selective high affinities for certain beta-lactam antibiotics. Additional PBPs with a molecular size range of about 30 to 34 kDa have been reported: PBPs 7 (29 kDa) and 8 (32 kDa) by Spratt (8), PBP 7a and its degradation product 7b (32 to 34 kDa) by Barbas et al. (1), and PBP 8 (34.7 kDa) by Schwarz et al. (7). These proteins have not been studied in detail, and their physiological significance is unknown. Recently, an unusual group of beta-lactam antibiotics has been found which lyses nongrowing *E. coli* (9; E. Tuomanen, K. Gilbert, R. Scartazzini, O. Zak, and A. Tomasz, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 120, 1986). During studies of the mechanisms of action of these antibiotics, it became clear that they shared the ability to bind to a low-molecular-size PBP resembling PBP 7 of Spratt (8). We report here the nature of this PBP and explore its physiological significance.

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MATERIALS AND METHODS

Bacterial strain and growth conditions. *E. coli* W7 (*dap lys*) was grown in M9 minimal salts medium supplemented with 25 μg of L-lysine and 5 μg of diaminopimelic acid per ml (4). Batch cultures were maintained at 37°C with vigorous aeration in a shaking water bath. Under these conditions, the generation time was 0.8 h. To halt growth, bacteria were collected on a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.) and suspended in medium without lysine. All cultures were monitored for turbidity at A_{620} (Sequoia Turner, Mountainview, Calif.) and CFU on supplemented M9 or nutrient agar. Antibiotics were obtained from the following sources: cephaloridine (Eli Lilly & Co., Indianapolis, Ind.); mecillinam (Hoffmann-La Roche Inc., Nutley, N.J.); aztreonam (E. R. Squibb & Sons, Nutley, N.J.); imipenem and cefoxitin (Merck Sharp & Dohme, Rahway, N.J.); MT 141 (Meiji Seika Kaisha Ltd., Tokyo, Japan); cefsulodin, CGP 31608, CGP 36150A, CGP 30779, CGP 17520, and other penems (CIBA-GEIGY, Ltd., Basel, Switzerland).

PBP assay in whole cells. The method of Broome-Smith and Spratt (2) was used to assay PBP in whole cells. Samples (1 ml) of *E. coli* (2×10^8 cells per ml) were pelleted and subjected to three freeze-thaw cycles. More than 100 beta-lactam antibiotics (CIBA-GEIGY) were tested for their ability to bind to the low-molecular-size PBP at 0.1, 1, and 10 times the MIC. Each sample was exposed to a test drug for 15 min at 30°C followed by [^{3}H]penicillin (10 μl of solution, 2.5 μg of drug [140 μCi]; Merck Sharp & Dohme) for 10 min at 30°C. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and PBPs were visualized by fluorography (8).

The deacylation rate of the [^{3}H]penicillin-PBP 7 complex was determined in whole-cell preparations with and without lysine supplement in the medium. Both the growing culture and the nongrowing culture (prestarved of lysine for 10 min) were treated with 25 times the MIC of [^{3}H]penicillin (125 μg of drug per ml) for 10 min at 30°C, filtered, and suspended in the same volume of lysine-enriched or lysine-deficient medium containing 1 mg of nonradioactive penicillin per ml. Samples (1 ml) of the cultures were removed at 0, 15, 30, 60, 90, and 120 min and were prepared for SDS-PAGE as described above.

Membrane preparations. Compounds which bound to PBP 7 in vivo were tested for PBP binding affinity in membrane preparations. Inner membranes of *E. coli* were isolated by differential solubility in Sarkosyl (8). Membrane samples containing 200 μg of protein were incubated with 0.1, 1, and 10 times the MIC of the test compounds, followed by 2.5 μg (140 μCi) of [^{3}H]penicillin at 37°C for 10 min. The reaction was stopped with 20% Sarkosyl, and the samples were prepared for SDS-PAGE and visualized by fluorography (8). The amount of penicillin required to half saturate PBP 7 was determined by scanning densitometry (Helena Laboratories, Beaumont, Tex.) by measuring the amount of 10 concentrations (0.1 to 50 times the MIC) of radioactive penicillin bound to the PBPs.

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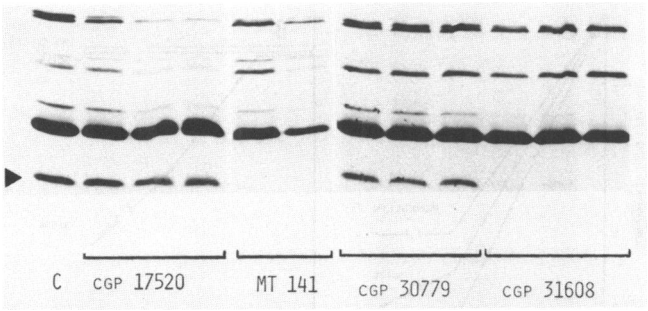

FIG. 1. Binding of beta-lactam antibiotics to PBP 7. Membrane preparations were tested for binding of [^{3}H]penicillin to the PBPs in competition assays. PBP 7 is indicated by the arrowhead. Lane C was treated with [^{3}H]penicillin alone. For all other lanes, the antibiotics indicated were incubated with membrane preparations at 0.1, 1, and 10 times the MIC followed by [^{3}H]penicillin (MT 141 was tested at 1 and 10 times the MIC only). The structures of the antibiotics are shown in Fig. 4.

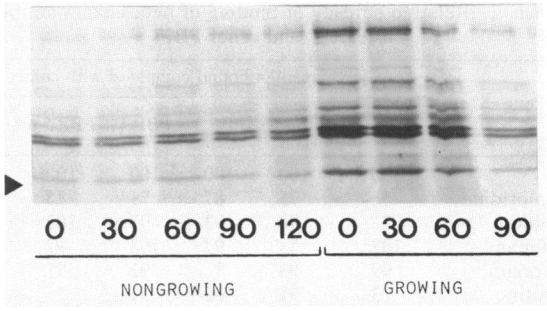

FIG. 2. Deacylation time of the PBP 7-[^{3}H]penicillin complex in the whole-cell PBP assay. [^{3}H]penicillin was allowed to bind to whole cells of growing and nongrowing (deprived of lysine for 2 h) *E. coli*. Upon removal of [^{3}H]penicillin, the loss of [^{3}H]penicillin from PBP 7 (arrowhead) was monitored for 120 min (numbers indicate minutes after removal of [^{3}H]penicillin).

Two-dimensional gel electrophoresis. Limited proteolysis followed by SDS-PAGE was performed as described by Cleveland et al. (3), with membrane preparations containing PBP 7. Samples (200 μg of protein) were treated with 2.5 μg of [^{3}H]penicillin for 10 min at 37°C and prepared for electrophoresis as described above. The first dimension was run in a miniature apparatus (3) with a 5% stacking gel and a 10% separating gel. Gels were stained with Coomassie brilliant blue for 10 min, destained for 45 min, and washed four times with water. After overnight storage at −70°C, a duplicate lane was excised and placed lengthwise on top of the stacking gel (5%) of the second dimension, which contained either *Staphylococcus aureus* V8 protease (2 μg/ml, final concentration; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.), or trypsin (2 μg/ml; Sigma Chemical Co., St. Louis, Mo.). Peptides hydrolyzed during passage through the stacking gel were separated in a 15% separating gel and visualized by fluorography (5). Peptides were assigned to PBPs by comparing horizontal positions with the reserved duplicate lane electrophoresed in only one dimension.

RESULTS

Characteristics of PBP 7. PBP 7 fulfilled the requirements for specific binding of penicillin in that binding was abolished by boiling whole cells at 100°C for 20 min before labeling them with [^{3}H]penicillin or by simultaneously adding 1,000 U of penicillinase per ml and 10 times the MIC of [^{3}H]penicillin in the whole-cell assay. The molecular size of PBP 7 was 30 kDa (Fig. 1, control lane). PBP 7 was half saturated at 5 μg of benzylpenicillin per ml. PBP 7 bound 15% of the total penicillin bound to all PBPs in both growing and lysine-starved *E. coli* as determined by densitometric tracings. The whole-cell PBP assay consistently demonstrated PBP 7 in nongrowing and growing *E. coli* (Fig. 2); in contrast, only about one in two inner membrane preparations contained detectable PBP 7. The reason for this inconsistency is not clear at present. The deacylation rate of the penicillin-PBP 7 complex was more than 120 min in both growing and nongrowing *E. coli* (Fig. 2). Peptides carrying the beta-lactam-binding site were generated by partial proteolysis and separated by SDS-PAGE. Both *S. aureus* V8 protease and trypsin cleaved at one site in the vicinity of the penicillin-binding site of PBP 7. The peptide pattern generated from PBP 7 was clearly distinguishable from those of the other PBPs, including the one nearest in molecular size, PBP 5-6 (Fig. 3).

Drug specificity of binding to PBP 7. Of the more than 100 beta-lactam antibiotics tested for affinity to PBP 7, 34 compounds inhibited the binding of [^{3}H]penicillin by >90% at the MIC. This group included MT 141, imipenem, and 32 of 100 penems. The ability to bind to PBP 7 was particularly common among the penem class of antibiotics. No antibiotic tested bound only to PBP 7 at the MIC. At 10 times the MIC, MT 141, imipenem, and the penems showed the greatest affinity for PBP 7, but cefoxitin and, to a lesser extent, cephaloridine also inhibited the binding of [^{3}H]penicillin (Table 1). Mecillinam, aztreonam, cefsulodin, and 68 of 100 penems did not bind at concentrations of up to 10 times the MIC (Table 1).

Comparison of drug structures (Fig. 4) with the ability of the drug to bind to PBP 7 (Fig. 1) demonstrated that subtle alterations in antibiotic structure affected binding to this PBP. The penem CGP 31608 demonstrated high affinity for PBP 7 even at 0.1 MIC, by either the membrane or whole-cell PBP assay. In contrast, a chemical cogener, CGP 30779, with a hydrogen substituted for the methyl group on C-8 and

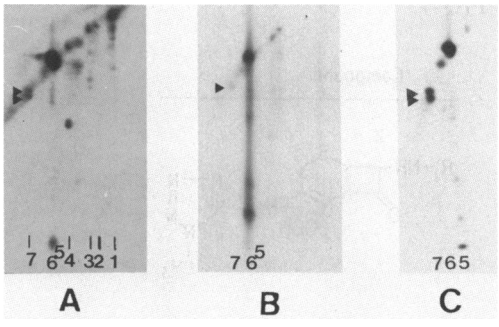

FIG. 3. Electrophoretic profile of peptides generated during partial proteolysis of [^{3}H]penicillin-PBP 1 to 7 complexes of *E. coli*. PBPs are identified by numbers. PBPs 5 and 6 are not generally separable in the first dimension and are thus numbered together in panels A and B. Arrowheads indicate peptide-[^{3}H]penicillin complexes derived from PBP 7. (A) *S. aureus* V8 protease digest of PBPs 1 to 7; (B) control (no enzyme); (C) *S. aureus* V8 protease digest of separated PBPs 5, 6, and 7.

TABLE 1. Relative efficacy of binding of beta-lactam antibiotics to PBP 7 and other PBPs

Drug	% [^{3}H]penicillin bound compared with control[a]					
	PBPs 1a and 1b	PBP 2	PBP 3	PBP 4	PBP 5-6	PBP 7
None	100	100	100	100	100	100
Cephaloridine	*	75	87	71	75	32
Cefsulodin	*	94	93	97	102	95
Mecillinam	102	*	97	103	92	99
Aztreonam	99	93	*	91	97	87
Cefoxitin	23	91	62	51	*	15
MT 141	27	*	54	37	16	*
Imipenem	22	*	69	*	17	*
CGP 31608	103	*	87	*	32	*
CGP 36150A	93	*	92	*	48	*
CGP 30779	94	*	91	*	79	95

[a] If number is low, then 10 times the MIC of test drug bound well to the PBP, preventing subsequent binding of [^{3}H]penicillin in the competition assay (membrane titrations). Values are the mean of three titrations measured by densitometry, with a lower limit of detection of ~ 10% of the maximum setting. Standard error of the mean was ≤15%; thus values <85% indicate differences from [^{3}H]penicillin binding in controls. *, <10% of control value.

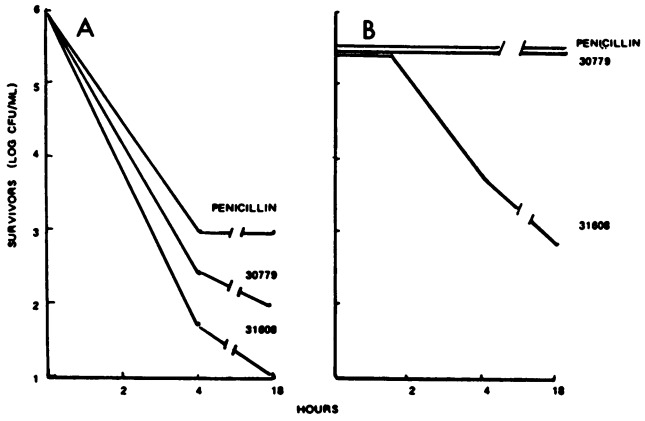

FIG. 5. Bactericidal activity of penem congeners. Growing (A) or lysine-deprived nongrowing (B) *E. coli* were exposed to 10 times the MIC of penicillin, CGP 30779, or CGP 31608, and bacterial survival was assessed over 18 h.

with the same MIC, failed to bind to PBP 7 even at 50 times the MIC. Similarly, MT 141 had a high binding capacity for PBP 7 at the MIC, while the structurally related cephalosporin CGP 17520 did not bind at 50 times the MIC.

Correlation between lytic activity against nongrowing *E. coli* and binding to PBP 7. Cells deprived of the amino acid lysine cannot be lysed by conventional beta-lactam antibiotics such as penicillin, even at concentrations as high as 20 times the MIC (9). However, some antibiotics, most notably from the penem class, are exceptional in that they lyse nongrowing *E. coli* at the MIC (9; Tuomanen et al., 26th ICAAC). Examples from Table 1 include MT 141, imipenem, CGP 31608, and CGP 36150A. When the PBP-binding pattern of these highly lytic compounds was assessed, all 34 agents capable of lysing nongrowing cells also bound to PBP 7 at the MIC. Figure 5 demonstrates that CGP 31608, which binds to PBP 7 (Fig. 1), kills nongrowing *E. coli*, whereas the congener CGP 30779 (with the same MIC) neither binds to PBP 7 (Fig. 1) nor kills nongrowing cells. Only four compounds tested bound to PBP 7 but did not lyse nongrowing cells. Thus, a strong correlation was found between the ability to kill and lyse nongrowing *E. coli* and the ability to bind to PBP 7.

DISCUSSION

The 30-kDa PBP is closest in molecular size to PBP 7 of Spratt (8). It is half saturated at the MIC of penicillin for *E. coli* W7 and has a long deacylation rate of >120 min. It is reproducibly demonstrable in whole-cell preparations of growing and nongrowing cells. Inconsistency in finding PBP 7 in membrane preparations was reported in the original description of PBP 7 (8) and was confirmed in the present experiments. This variability is not yet understood, but may involve a technical artifact reflecting the distribution of this PBP in the inner membrane versus the outer membrane of the cell envelope as has been described for PBP 8 (6). Several properties indicate that the PBP studied here is PBP 7 rather than PBP 8: its smaller molecular size, its lack of ampicillin binding (PBP 8 avidly binds an ^{125}I derivative of ampicillin [7]), and its presence in nongrowing and stationary-phase cells (PBP 8 disappears in stationary phase [6]). The peptide pattern of PBP 7 after partial proteolytic digestion clearly differs from that of PBPs 1 to 6, indicating that PBP 7 is not related to or derived as a degradation product from the other major PBPs.

PBP 7 fulfills the criteria of a bona fide PBP in that its penicillin-binding capacity is destroyed when the protein is denatured by heat or when the beta-lactam ring is disrupted.

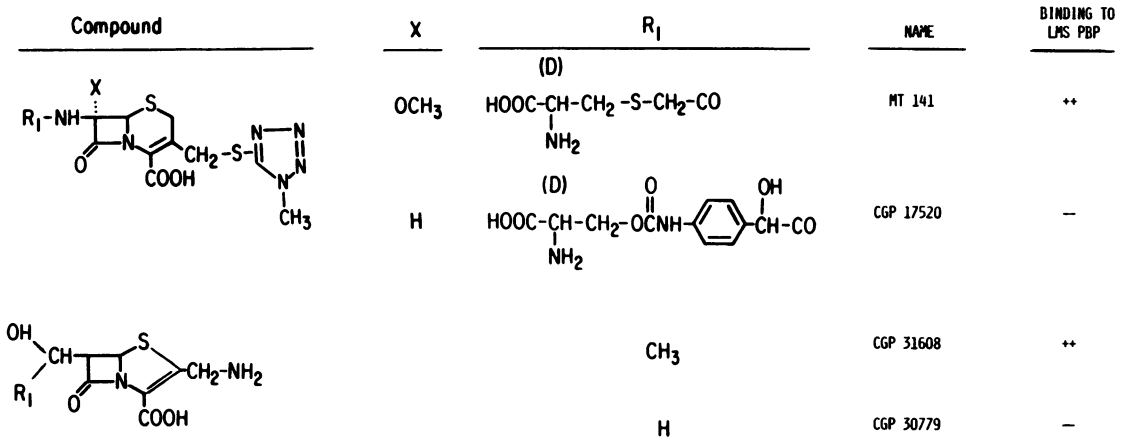

FIG. 4. Effect of variation of drug structure on binding to low-molecular-size (LMS) PBP 7. PBP binding profiles are shown in Fig. 1.

A high degree of specificity to the structural requirement for beta-lactam binding to PBP 7 exists: an alteration as subtle as a change from a methyl group to a hydrogen on C-8 of the penem nucleus abolishes binding. Such drug specificity has been a requirement for the definition of major PBPs of *E. coli*. From the present studies, it is apparent that very few penicillins and cephalosporins bind to PBP 7, while the penem class of antibiotics is particularly effective in binding.

A physiological significance of PBP 7 is suggested by the correlation between the binding of drugs to PBP 7 and the ability of these drugs to kill and lyse nongrowing *E. coli*. All beta-lactams which lyse nongrowing *E. coli* bind to PBP 7, suggesting that binding to PBP 7 is required for lysis of nongrowing cells. Conversely, virtually all beta-lactams which do not lyse nongrowing *E. coli* do not bind well to PBP 7. Four penems bind to PBP 7 but fail to lyse the nongrowing cell, suggesting that PBP 7 inhibition alone is not sufficient for lysis of the nongrowing cell. A reasonable hypothesis may be constructed to explain the role of PBP 7 in protection of the nongrowing cell. We have recently shown that immediately upon growth arrest, *E. coli* begin to produce a peptidoglycan of unusual composition which is more resistant to autolytic degradation and thus may contribute to phenotypic tolerance (10; E. Tuomanen and A. Tomasz, J. Bacteriol., in press; E. Tuomanen, R. Cozens, K. Gilbert, O. Zak, and A. Tomasz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, A-109, p. 19). If PBP 7 is important in the generation of autolysis-resistant, nongrowing peptidoglycan, then inhibition of PBP 7 might allow lysis susceptibility to persist in nongrowing cells. This hypothesis is currently under investigation.

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