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Target for Bacteriostatic and Bactericidal Activities of β-Lactam Antibiotics against *Escherichia coli* Resides in Different Penicillin-Binding Proteins†

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The relationship between cell-killing kinetics and penicillin-binding protein (PBP) saturation has been evaluated in the permeability mutant *Escherichia coli* DC2 in which the antimicrobial activity of β-lactams has been described as being directly related to the extent of saturation of the PBP target(s). Saturation of a single PBP by cefsulodin (PBP 1s), mecillinam (PBP 2), and aztreonam (PBP 3) resulted in a slow rate of killing (2.5-, 1.5-, and 0.8-log-unit decreases in the number of CFU per milliliter, respectively, in 6 h). Saturation of two of the three essential PBPs resulted in a marked increase in the rate of killing, which reached the maximum value when PBPs 1s and 2 were simultaneously saturated by a combination of cefsulodin and mecillinam (4.7-log-unit decrease in the number of CFU per milliliter in 6 h). Inactivation of all three essential PBPs by the combination of cefsulodin, mecillinam, and aztreonam further increased the killing kinetics (5.5-log-unit decrease in the number of CFU per milliliter), and this was not significantly changed upon additional saturation of the nonessential PBPs 5 and 6 by cefoxitin. Similar relationships between PBP saturation and killing kinetics were obtained with imipenem and meropenem at concentrations which inhibited only one PBP (PBP 2), only two PBPs (PBP 1s and 2), or all three essential PBPs. Saturation of one or more PBPs also resulted in a different rate of bacteriolysis, the highest rate being obtained by the cefsulodin-mecillinam combination and by 5 μg of either imipenem or meropenem per ml. All of these conditions caused saturation of PBP 2 and saturation or extensive binding of PBP 1s. However, none of these conditions determined the fastest possible rate of killing, which occurred only when all three essential PBPs were saturated. It was concluded that the actual killing effect of β-lactams is reflected by killing rates that approach the fastest possible rate for the given microorganism and that the targets for the bactericidal activity are precisely those PBPs whose saturation or binding occurs under these conditions.

β-Lactam antibiotics are generally regarded as exerting bactericidal activity. However, this activity is known to vary greatly depending on the β-lactam and the bacterial species or strain considered. Studies on the physiological and biochemical bases of β-lactam activity indicate that growth inhibition depends on inactivation of the enzymes involved in the late stages of peptidoglycan synthesis (22, 29), whereas the bactericidal effect is mainly related to the unregulated activities of autolysins (11, 26). A great deal of evidence, however, has also been provided to show that high killing rates can occur without extensive cell wall degradation or lysis (11, 26, 27).

It was previously found that in *Enterococcus hirae* ATCC 9790 (formerly *Enterococcus faecium*), the essential penicillin-binding proteins (PBPs) 1s, 2, and 3 are each a target for the growth-inhibiting effect of β-lactams, while the nonessential PBP 5 (possibly together with the other PBPs) is the target of the bactericidal activity (15). Studies in *Escherichia coli* have shown that β-lactam antibiotics exert a clear bactericidal activity when they saturate at least two of the high-molecular-weight PBPs (e.g., PBP 1s and 2, PBPs 1s and 3, or PBPs 2 and 3) (2, 3, 5, 10, 20, 30). However, none of these studies tackled the problem of determining the conditions under which β-lactams kill the cells with the fastest kinetics or the role of saturation of nonessential PBPs.

In the work described here we investigated the conditions under which β-lactam antibiotics cause growth inhibition in *E. coli* without a marked killing effect (primary growth-inhibiting activity) and the conditions under which they demonstrate a high (up to the maximum) bactericidal effect (primary killing effect).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strain that we used throughout this work was the *E. coli* mutant DC2 (kindly donated by David Clark), which has been shown to have a breakdown in the outer membrane permeability barrier (6, 17). The strain was kept frozen in brain heart infusion broth with 10% glycerol at −80°C; it was routinely maintained and grown on Mueller-Hinton agar (MHA; Difco Laboratories, Detroit, Mich.), while all experiments were performed with cells exponentially growing in Mueller-Hinton broth (MHB; Difco Laboratories) at 37°C under continuous shaking.

Antibiotics. The activities of all drugs were evaluated before use, and only stocks with activities of at least 98% were used. Working solutions were prepared immediately prior to use, as specified by the manufacturers, and the solutions were added to MHB in adequate concentrations after being sterilized by filtration.

Cefsulodin was provided by CIBA-GEIGY (Basel, Switzerland). Mecillinam was provided by Leo Pharmaceutical Products (Ballerup, Denmark). Meropenem was provided by Zeneca (Milan, Italy). Cilastatin-free imipenem was provided by Merck Sharp & Dohme (Rome, Italy). All other antibiotics were from commercial sources.

Susceptibility tests. The MICs were evaluated by broth macrodilution tests, as described by Sahm and Washington (18), by using the standard inoculum of 1×10^5 to 5×10^5 CFU/ml.

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TABLE 1. MICs and MBCs of selected β-lactam antibiotics for *E. coli* DC2

Antibiotic	MIC (μg/ml)	MBC (μg/ml)
Cefsulodin	16	32
Mecillinam	0.06	0.06
Aztreonam	0.12	0.25
Cefoxitin	2	2
Imipenem	0.5	1
Meropenem	0.12	0.12

The MBCs were evaluated by determining the CFU in those tubes used for the MIC determination that showed no visible growth.

Killing rate evaluation. In all experiments in which killing kinetics in the presence of antibiotics (or growth kinetics in the controls) were to be evaluated, samples of 1 ml were taken at intervals, immediately chilled on ice, and centrifuged with a Beckman Microfuge in a cold room for 10 min. The pellet was washed twice with cold saline solution, resuspended in 1 ml of saline, and diluted. A total of 100 μl of each dilution was then spread over the entire surface of MHA plates. The plates were incubated for 24 h at 37°C, and the colonies were counted. The CFU titration at time zero ranged between 5×10^7 and 1×10^8/ml.

Assay of PBPs. PBPs in cell envelopes of *E. coli* DC2 were determined as described by Spratt (23), except that the PBP labeling was done with [^{3}H]benzylpenicillin (20 Ci/mmol; Amersham International, Bucks, United Kingdom). Binding to PBPs was determined for all antibiotics by the standard competition procedure (23). Cell envelopes were incubated for 10 min at 30°C with the desired concentrations of unlabelled β-lactams and, thereafter, for 10 min at 30°C with [^{3}H]benzylpenicillin at a final concentration of 30 μg/ml. PBPs were detected by fluorography after exposure of the gel to an X-ray film (X-OMAT AR; Kodak) for 1 week at −80°C. The intensities of the bands on the fluorograms were determined with a densitometer.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (14). Proteins were detected by Coomassie blue staining.

Other methods. The protein content of the samples was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

TABLE 2. Log_{10} reduction in CFU in presence of various concentrations of β-lactams

β-Lactam (concn [μg/ml])[a]	Log_{10} reduction in CFU at [a]:			PBP(s) bound (% saturation)
	2 h	3 h	6 h	
Cefsulodin				
1	0.09	0.16	0.19	None
5	0.43	0.85	0.92	1s (70)
10	0.57	1.07	2.03	1s (100)
250	0.82	1.57	2.23	1s (100)
500	1.13	1.73	2.50	1s (100)
Mecillinam				
0.01	0.06	−0.05	−0.31	2 (50)
0.02	0.21	0.22	0.34	ND[b]
0.04	0.48	0.63	0.91	2 (100)
0.1	0.61	0.81	1.15	ND
0.2	0.64	0.95	1.20	2 (100)
0.5	0.73	1.03	1.35	ND
5	0.95	1.27	1.54	2 (100)
Aztreonam				
0.05	0.44	0.44	0.59	3 (50)
0.1	0.49	0.49	0.70	3 (70)
0.5	0.54	0.55	0.80	ND
1	0.60	0.62	0.85	1s (20), 3 (100)
5	0.66	0.68	1.00	ND
10	0.77	0.84	1.55	1s (50), 3 (100)
50	0.92	1.10	2.85	1s (70), 3 (100)
100	0.89	1.10	3.04	ND
200	1.19	1.49	3.62	1s (95), 3 (100)

[a] The log_{10} reduction in the initial cell population at the various antibiotic concentrations was determined after 2, 3, and 6 h of incubation at 37°C as described in Materials and Methods. Negative values indicate regrowth. Values are averages of at least three determinations, with a precision of ±10 to 15%.

[b] ND, not determined.

RESULTS

Saturation of only one essential PBP and bacterial death. Cefsulodin, mecillinam, and aztreonam are β-lactam antibiotics with a high affinity for a single PBP (PBP 1s, 2 and 3, respectively) (7, 22) and were used, in the appropriate concentration range, to test the effect of saturation of only one PBP on the viabilities of *E. coli* cultures. The MICs and MBCs of these antibiotics, as evaluated by standard methods, are reported in Table 1.

Table 2 shows the results of these experiments. Concentrations of cefsulodin which were clearly bacteriostatic (1 μg/ml) did not bind to the PBP 1 complex (PBP 1 was not resolved into the two components, 1A and 1B, in these experiments), whereas the killing rate increased along with the saturation of PBP 1s. The lowest concentration that saturated PBP 1s, i.e., 10 μg/ml, caused a 2-log-unit decrease in the viable titer of the culture in 6 h. At concentrations greater than 10 μg/ml the overall bactericidal effect of cefsulodin showed no marked increase. Since PBP 1B has a lower affinity for cefsulodin than PBP 1A (7), it was evident that saturation of PBP 1B had to be achieved to yield the highest possible bactericidal effect.

Mecillinam exhibited a very specific affinity for PBP 2, since it bound and saturated only this protein at a wide range of concentrations. The 0.01-μg/ml concentration, which bound approximately 50% of PBP 2, still allowed cell growth, albeit at a reduced rate compared with that of the control. The killing rate increased along with the saturation of PBP 2. The lowest concentration used which saturated PBP 2 (0.04 μg/ml) caused a 1-log-unit decrease in the viable titer of the culture in 6 h. Higher antibiotic concentrations did not cause any marked increase in bactericidal activity.

Aztreonam bound and saturated PBP 3 in the range of 0.05 to 1 μg/ml. Antibiotic concentrations which bound either 50% (0.05 μg/ml) or 100% (1 to 10 μg/ml) of PBP 3 alone showed low bactericidal activity (0.5- to 1.5-log-unit reductions in titers of viable cultures in 6 h). On a further increase in the antibiotic concentrations, a marked increase in the killing rate was observed, but under these conditions aztreonam extensively bound PBP 1s, too.

Saturation of two essential PBPs and bacterial death. Combining the aforementioned β-lactams two at a time, we studied the effects on culture viability of the simultaneous inhibition of either PBP 1s plus PBP 2, PBP 1s plus PBP 3, or PBP 2 plus PBP 3. As shown in Table 3, the combination of 0.2 μg of mecillinam per ml (complete saturation of PBP 2) and cefsulodin at concentrations ranging from 1 to 50 μ/ml yielded a marked increase in bactericidal activity. In the presence of 10 μg of cefsulodin per ml, which was the lowest concentration required to saturate PBP 1s, the overall decrease in the viable titer of the culture was 4.5 log units in 6 h, which was greater than the sum of the decrease caused by the two antibiotics alone (Table 2). No marked increase in the killing rate was observed on further increasing the cefsulodin concentration to 50 μg/ml.

As regards the simultaneous inhibition of PBP 1s and 3 by the specific β-lactams, 0.1 μg of aztreonam per ml, which bound 70% of PBP 3, did not improve the bactericidal activity of cefsulodin (5 μg/ml). Complete saturation of both PBPs by 10 μg of cefsulodin per ml and 5 μg of aztreonam per ml caused a 3-log-unit reduction in the viable titers of the cultures, which roughly corresponded to the sum of the bactericidal

TABLE 3. Log_{10} reduction in CFU in presence of double and triple combinations of β-lactams at various concentrations

β-Lactam (concn [μg/ml])[a]	Log_{10} reduction of CFU[b]			PBP(s) bound (% saturation)
	2 h	3 h	6 h	
CSU (0.5) + MEC (0.2)	0.71	0.75	1.65	2 (100)
CSU (1) + MEC (0.2)	0.95	1.48	2.63	2 (100)
CSU (5) + MEC (0.2)	1.34	1.95	4.21	1s (70), 2 (100)
CSU (10) + MEC (0.2)	1.95	2.51	4.38	1s (100), 2 (100)
CSU (50) + MEC (0.2)	2.21	2.90	4.52	1s (100), 2 (100)
CSU (10) + ATM (0.1)	0.85	0.99	1.92	1s (100), 3 (70)
CSU (10) + ATM (5)	1.02	1.65	2.87	1s (100), 3 (100)
CSU (10) + ATM (10)	0.94	1.43	2.76	1s (100), 3 (100)
MEC (0.2) + ATM (0.1)	0.79	1.19	1.57	2 (100), 3 (70)
MEC (0.2) + ATM (5)	1.39	1.71	2.16	2 (100), 3 (100)
MEC (0.2) + ATM (10)	1.39	1.87	2.33	2 (100), 3 (100)
CSU (5) + MEC (0.01) + ATM (0.05)	1.04	1.22	1.72	1s (70), 2 (50), 3 (50)
CSU (10) + MEC (0.04) + ATM (1)	3.41	4.66	5.29	1s (100), 2 (100), 3 (100)
CSU (50) + MEC (0.2) + ATM (5)	3.87	5.00	5.51	1s (100), 2 (100), 3 (100)

[a] CSU, cefsulodin; MEC, mecillinam; ATM, aztreonam.
[b] The log_{10} reduction in the initial cell population at the various antibiotic concentrations was determined after 2, 3, and 6 h of incubation at 37°C as described in Materials and Methods. Values are averages of at least three determinations, with a precision of ±10 to 15%.

effects of the two antibiotics alone (Table 2). No marked increase in the killing rate was observed on doubling of the aztreonam concentration.

Similarly, the 2-log-unit decrease in the viable titers of the culture caused by the combination of 0.2 μg of mecillinam per ml (complete saturation of PBP 2) and 5 μg of aztreonam per ml (complete saturation of PBP 3) amounted to approximately the sum of the bactericidal effects of mecillinam and aztreonam used alone at the same concentrations (Table 2). Once more, no marked increase in the killing rate was observed on doubling of the aztreonam concentration.

Saturation of the three essential PBPs and bacterial death. Since even the most effective of the double combinations still allowed the survival of more than 10^3 CFU/ml and since the aim of our work was to find conditions ensuring the fastest possible killing kinetics, we investigated whether the saturation of all three essential PBPs could improve the killing kinetics.

As shown in Table 3, the combination of cefsulodin, mecillinam, and aztreonam, each at a concentration which saturated 50% of the target PBP, was devoid of efficient killing activity, whereas the same antibiotics at concentrations which fully saturated the target PBP caused greater than 5-log-unit decrease in the number of CFU, which was greater than the sum of the bactericidal effects of each antibiotic used alone (Table 2). This bactericidal effect was not significantly improved by a fivefold increment in the antibiotic concentrations.

It is interesting that the difference between the fastest killing rate observed when two PBPs were saturated (namely, PBPs 1s and 2 with cefsulodin and mecillinam, respectively) and the rate observed when all three essential PBPs were saturated was more striking during the first 3 h of incubation. At this time the killing rate of the triple combination reached its maximum value, with a greater than 2-log-unit decrease in the viable cell count compared with the decrease caused by the cefsulodin-mecillinam combination (Table 3).

Saturation of nonessential PBPs and bacterial death. To ascertain whether the killing rate obtained by saturating the three essential PBPs was indeed the fastest possible, we investigated whether additional saturation of the lower-molecular-weight PBPs, regarded as nonessential for cell growth, could play a role in bacterial killing. Cefoxitin was chosen for its specific affinity for PBPs 5 and 6 (7). This antibiotic also has a very low 50% inhibitory concentration (IC_{50}) for PBP 1A (IC_{50}, 0.1 μg/ml), but the saturation of this protein has no effect on cell growth because the MIC of cefoxitin was 2 μg/ml (Table 1). On the contrary, the IC_{50}s for PBPs 5 and 6 are 0.6 and 0.9 μg/ml, respectively. None of the β-lactams studied to date has a specific affinity for either PBP 4 or the inconstantly detected PBPs 7 and 8, in that no β-lactam binds to these proteins at concentrations lower than those binding the essential PBP 1s, 2, and 3 (7, 12, 28). Thus, only the possible role of PBPs 5 and 6 in bacterial killing could be investigated. Figure 1 shows the bactericidal effects of cefsulodin, mecillinam, and aztreonam at concentrations which saturated PBP 1s, 2, and 3, respectively, combined with 2 μg of cefoxitin per ml, i.e., a

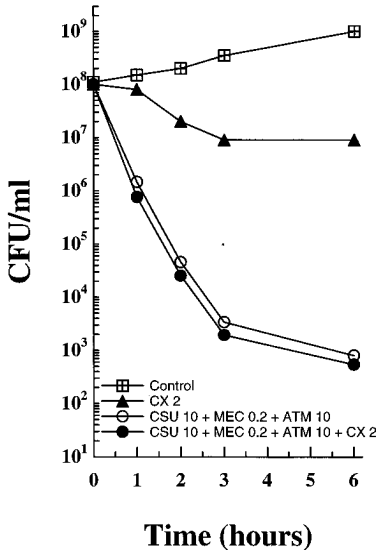

FIG. 1. Saturation of nonessential PBPs and bacterial death. Cells exponentially growing in MHB received, at time zero, triple combinations of cefsulodin (PBP 1s), mecillinam (PBP 2), and aztreonam (PBP 3), each at a concentration saturating the respective PBP target, combined with a concentration of cefoxitin which bound and saturated PBPs 5 and 6. Viable titers of the cultures were determined at appropriate times. Antibiotic concentrations are indicated in micrograms per milliliter. CSU, cefsulodin (10 μg/ml); MEC, mecillinam (0.2 μg/ml); ATM, aztreonam (10 μg/ml); CX, cefoxitin (2 μg/ml); control, cells not exposed to antibiotics.

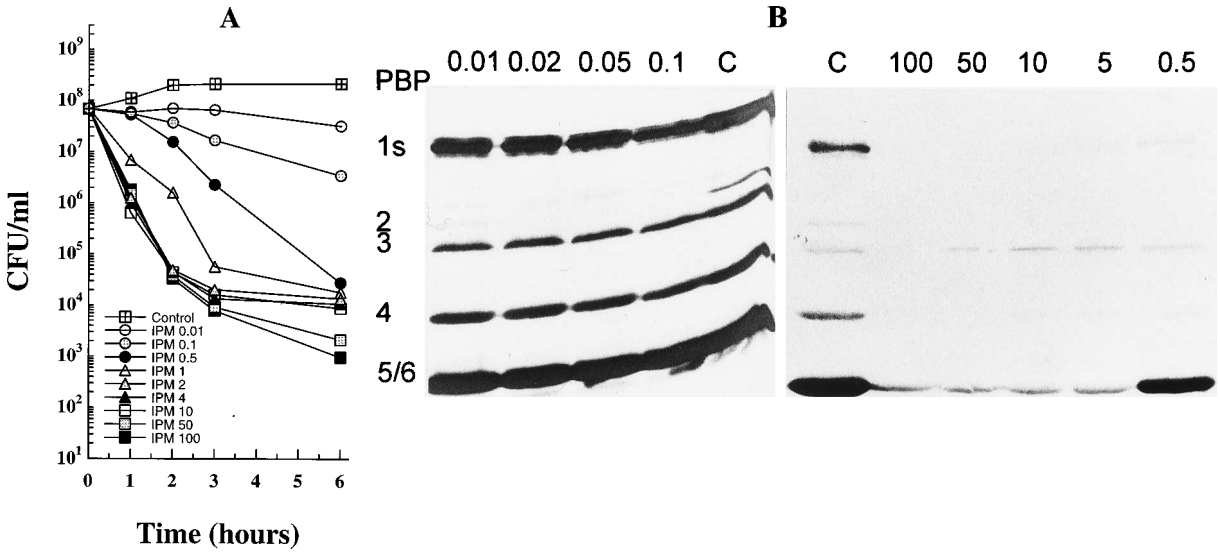

FIG. 2. Bactericidal activity of imipenem at concentrations inhibiting one or several PBPs. Cells exponentially growing in MHB received different imipenem concentrations at time zero, and viable titers of cultures were determined at appropriate times (A). (B) For PBP analysis membrane preparations were exposed to the antibiotic concentrations indicated and then to [^{3}H]benzylpenicillin as described in Materials and Methods. Antibiotic concentrations are indicated in micrograms per milliliter. IPM, imipenem; control (lanes C), cells not exposed to antibiotics.

concentration which extensively bound PBPs 5 and 6 and a relatively small amount of the essential PBPs (7). The combination of the four antibiotics resulted in killing kinetics comparable to those observed when only the three essential PBPs were saturated.

Bactericidal activities of β-lactams which inhibit several PBPs simultaneously. Our results suggested that saturation of individual PBPs was associated with a different killing rate depending on the target involved, and also that saturation of two or all three essential PBPs resulted in bactericidal activity which was the sum of the individual bactericidal effects (and in some cases was even greater than that sum). We therefore analyzed the rate of killing of *E. coli* by β-lactams which at different concentrations could saturate either one, two, or all three essential PBPs. We selected imipenem and meropenem for this study, because these antibiotics exhibited the desired effect at relatively low concentrations (24). Figure 2A shows that imipenem reduced the viable titers of the culture by 1 log unit at a concentration which saturated PBP 2 only (0.1 μg/ml), by 4 log units at concentrations which bound and saturated PBP 1s, 2, and 4 (0.5 to 10 μg/ml), and by 5 log units at concentrations which saturated all three essential PBPs (50 μg/ml). It is worth noting that the killing effect of the drug did not show a marked variation at concentrations ranging from 1 to 10 μg/ml. Above 10 μg/ml, the killing kinetics increased, again in conjunction with increased binding to PBP 3, and reached the maximum value when PBP 3 was saturated. Under these conditions extensive binding of PBPs 5 and 6 also occurred.

Meropenem caused a 1.5-log-unit decrease at a concentration (0.1 μg/ml) which saturated PBP 2 only (Fig. 3A). At higher concentrations (1 to 10 μg/ml) PBPs 1s and 3 were affected in a similar way, so that the killing rates paralleled the simultaneous binding of these two PBPs. Since meropenem had a higher affinity than imipenem for PBP 3, it saturated all three essential PBPs at 10 μg/ml and caused a 5-log-unit decrease in the viable titers of the culture at this concentration. Greater concentrations of meropenem which also interacted with PBPs 4, 5, and 6 did not greatly increase the killing kinetics.

Saturation of PBPs and bacteriolysis. To establish a relation between bactericidal activity and bacteriolysis associated with saturation of one or more PBPs, we measured the turbidity of an *E. coli* culture treated with β-lactams with a high affinity for a single PBP, either alone or in combination, at the minimum concentrations saturating the specific target (Tables 2 and 3). It is evident from Fig. 4 that among the β-lactams used alone, only cefsulodin was able to trigger autolytic activity. This agrees with the finding that this antibiotic showed the best bactericidal activity at the minimum concentration saturating PBP 1s (Table 2). A marked effect on the onset of autolysis was shown by the simultaneous saturation of two PBPs obtained by the combination of mecillinam and aztreonam (PBP 2 and 3) and also by the combination of cefsulodin and mecillinam (PBPs 1s and 2). The combination of cefsulodin (PBP 1s) and aztreonam (PBP 3) did not greatly improve the bacteriolytic effect achieved with cefsulodin alone. Saturation of all three essential PBPs had a bacteriolytic effect after 6 h similar to that exerted by simultaneous inhibition of PBP 1s and 2 (Fig. 4), even though the former condition caused a decrease in the titer of the viable culture which was roughly 1 log unit greater than that obtained with the latter condition (Table 3).

Similar results were obtained when bacteriolysis caused by imipenem and meropenem was studied at concentrations saturating one or more PBPs. Saturation of PBP 2 alone by the two antibiotics did not cause any significant decrease in turbidity. Binding of PBP 1s in addition to PBP 2, which was obtained with imipenem at 0.5 μg/ml and meropenem at 5 μg/ml, resulted in significant triggering of autolysis comparable to that observed when mecillinam and cefsulodin were combined at concentrations saturating PBP 1s and 2. Additional saturation of PBP 3 by meropenem at 10 μg/ml and imipenem at 100 μg/ml did not significantly increase the autolysis rate, whereas bacterial death was increased by 1 log unit or more (Fig. 2 and 3).

DISCUSSION

The work described here shows that β-lactams, at concentrations that inhibit the growth of *E. coli*, cause bactericidal

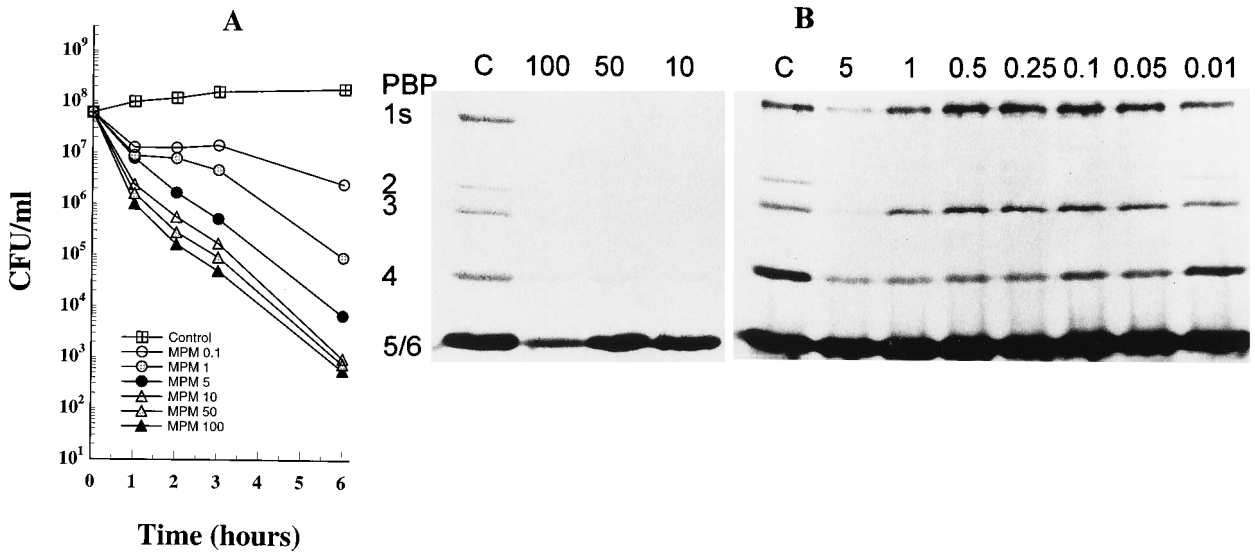

FIG. 3. Bactericidal activity of meropenem at concentrations inhibiting one or several PBPs. Cells exponentially growing in MHB received different meropenem concentrations at time zero, and viable titers of cultures were determined at appropriate times (A). (B) For PBP analysis membrane preparations were exposed to the antibiotic concentrations indicated and then to [^{3}H]benzylpenicillin as described in Materials and Methods. Antibiotic concentrations are indicated in micrograms per milliliter. MPM, meropenem; control (lanes C), cells not exposed to antibiotics.

effects of very different intensities, depending on the PBPs that are partially or fully saturated. The intensity of the bactericidal effect was lowest when the β-lactams bound and saturated only one of the essential PBPs, higher when two of them were bound, and highest when all three essential PBPs were saturated. It was also interesting to find that concentrations exceeding those necessary for saturating the latter PBPs did not increase the kinetics of bacterial killing and that binding and saturation of nonessential PBPs 5 and 6 had no effect on the kinetics of cell death.

Binding of only one of each of the three essential PBPs caused a relatively slow bacterial death so that at 6 h after the beginning of incubation with the antibiotic a large number of cells (10^6 to 10^7) were still alive. In contrast, saturation of all essential PBPs caused not only very rapid and almost complete extinction of bacterial populations as large as 10^8 but also cell death with the fastest possible kinetics. It thus emerged that inactivation of one of the essential PBPs by β-lactams primarily caused inhibition of growth and then the relatively slow death of bacterial cells, while inactivation of the three essential PBPs at the same time caused, as its primary effect, cell death, which is responsible for the apparent growth inhibition. Consequently, two different targets for the inhibitory activities of β-lactams against *E. coli* can be discerned: one represented by each of the essential PBPs and primarily responsible for a primary growth-inhibiting effect and the other consisting of the

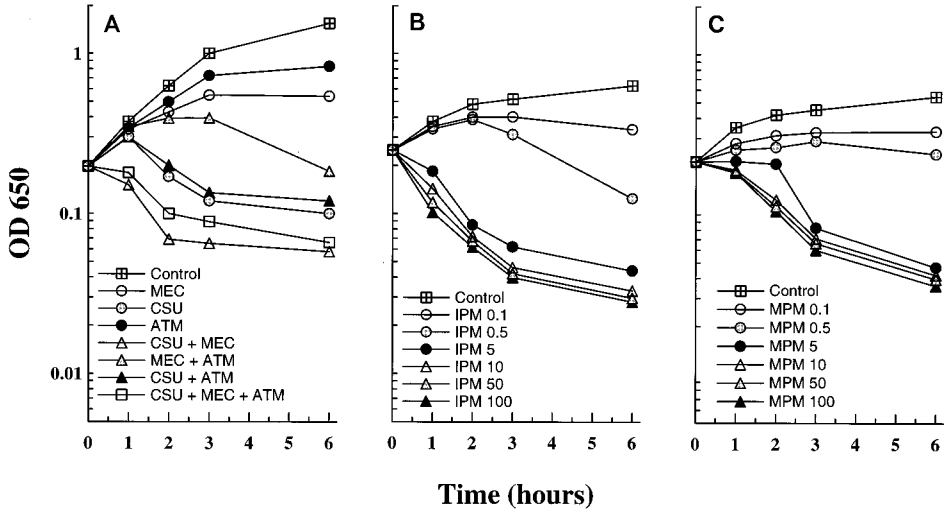

FIG. 4. Saturation of PBPs and bacteriolysis. Cells exponentially growing in MHB received, at time zero, antibiotics with a high affinity for a single PBP (cefsulodin, PBPs 1; mecillinam, PBP 2; aztreonam, PBP 3) alone and in double and triple combinations, each at a concentration saturating the respective PBP target (A), imipenem at different concentrations (B), and meropenem at different concentrations (C). Turbidity was measured at the appropriate times. Antibiotic concentrations are indicated in micrograms per milliliter. CSU, cefsulodin; MEC, mecillinam; ATM, aztreonam; IPM, imipenem; MPM, meropenem; control, culture not treated with antibiotics; OD 650, optical density at 650 nm.

three essential PBPs together and primarily responsible for cell killing. That inactivation of only one of the essential PBPs is not per se a primary lethal event is also indicated by the fact that conditional mutants of *E. coli* which lack one of these PBPs do not grow, but neither are they killed under the nonpermissive condition (25, 31).

In previous works we have demonstrated that PBP 1s, 2, and 3 of *E. hirae* are essential and that, depending on the growth conditions, inhibition of only one (PBP 3) or two of these causes growth inhibition (4, 8, 9). We have also suggested the concept that two different targets for the inhibitory activities of β-lactams exist in this microorganism, one of which is responsible for bacteriostatic activity and the other of which is responsible for bactericidal activity (15). The findings of the present work indicate that different targets for these different effects also exist in *E. coli*, thus making the concept more general and reinforcing its value. We also demonstrated in *E. hirae* that when the target for the bactericidal activities of the drugs is fully saturated no increase in the kinetics of the bactericidal activities is observed (15), a situation similar in all respects to the one described here for *E. coli*. Since these two species are not related to one another, we believe that other species, too, may carry two targets for the two inhibitory effects of β-lactams.

Various β-lactams have been shown here to vary in their abilities to bind to the two targets. Some, such as cefsulodin, aztreonam, and mecillinam, appeared to be capable of specifically binding only the target for the bacteriostatic effect, while others (such as imipenem and meropenem) were also partially or completely capable of binding the target for bactericidal activity, but only at concentrations greater than the MIC. Because the target for the bactericidal effect consists of all three essential PBPs, its inactivation was partial when only two of these PBPs were bound and was complete when all three of them were saturated. We cannot rule out the possibility that, in microorganisms other than *E. coli* and *E. hirae*, some β-lactams saturate the target for bactericidal activity at concentrations no greater than the MIC. In this instance (so far only a hypothesis), the antibiotics would only be capable of inhibiting bacterial growth by killing the cells. None of the β-lactams so far described have such a property, but most of them could exert a similar effect at concentrations exceeding the MIC which saturate at least two of the essential PBPs. Such concentrations would differ from antibiotic to antibiotic and would depend on the relative affinity of the drug for the three PBPs.

Although inhibition of growth by β-lactams was always accompanied by a certain degree of bactericidal activity, there can be no doubt that the killing effect observed when the specific target was inhibited was qualitatively different. In fact, when the target for bactericidal activity was inhibited, large bacterial populations were almost completely killed within a period of time which was a small multiple of the generation time of the microorganism and the killing effect was not increased by concentrations that significantly exceeded the concentration required to inhibit the target.

In order to provide a clear and more complete description of the two different types of bactericidal effects that β-lactams can exert, we suggest defining as directly bactericidal those antibiotics (not only β-lactams) which, at the MIC, are able to kill at least 99.9% of a large bacterial population (10^8) with kinetics that are the fastest possible for that antibiotic and microorganism. This bactericidal effect should occur within a time that is a relatively small multiple (four- to eightfold) of the generation time. For those antibiotics proving to have a bactericidal effect in conventional assays, the minimum concentration at which they are directly bactericidal might be defined as the minimum directly bactericidal concentration (MDBC).

In previous works we and other groups have demonstrated that bacterial killing by β-lactam antibiotics both in *E. hirae* and in *E. coli* is more rapid when larger numbers of PBPs are saturated. The novelty of the present study resides in the demonstration that (i) in *E. coli* the fastest killing is observed only when all three PBPs are saturated, (ii) the concentrations of β-lactams exceeding those necessary for saturating these proteins do not significantly increase the killing rate, and (iii) in *E. coli*, as opposed to *E. hirae*, the so-called nonessential PBPs have no role in β-lactam killing kinetics.

The bactericidal effect of β-lactams has often been described as being due to the lysis triggered by the drugs. In the aforementioned studies of other investigators (1–3, 5, 10, 30), the bactericidal effect of saturation of two PBPs was directly related to the actual extent of lysis. However, other studies carried out both in *E. coli* and in other microorganisms have actually suggested that lysis is not the only mechanism whereby β-lactams kill bacteria (11, 13, 26, 27). The data presented here lend further support to this latter conclusion. We have found that after two high-molecular-weight PBPs are saturated, saturation of the third, while causing a further decrease in the viable counts, had virtually no effect on the decrease in culture turbidity.

At present, it is not possible to provide a conclusive explanation of the mechanism whereby saturation of the three high-molecular-weight PBPs causes such a rapid death of *E. coli* populations. However, as suggested previously (16, 19, 21), in *E. coli* two mechanisms exist for the growth of the bacterial envelope, one of which is responsible for lateral wall formation and the other of which is responsible for septum formation. It has been speculated that when only one of these mechanisms is inhibited, the cell can survive for awhile since the other mechanism allows surface expansion, which creates additional intracellular room for the newly synthesized molecules. On the contrary, the inhibition of both mechanisms would have a more rapid bactericidal effect since it deprives the cell of any chance of protecting the increasing cytoplasmic content (21). Since PBPs 1s, 2, and 3 are involved in both mechanisms of envelope expansion, the strong bactericidal effect associated with their simultaneous inhibition by β-lactams may be the consequence of complete inhibition of cell surface expansion.

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