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Induction of Cell Lysis in *Escherichia coli*: Cooperative Effect of Nocardicin A and Mecillinam

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Nocardicin A and mecillinam are two β-lactam antibiotics with poor bacteriolytic activity against *Escherichia coli*. However, the combined use of these drugs resulted in the induction of a fast lytic response in *E. coli* cells. For this cooperative effect to take place, the formation of a complex between penicillin-binding protein 2 and mecillinam is apparently necessary. This suggests that penicillin-binding protein 2 might be actively involved in the response of *E. coli* to bacteriolytic β-lactam antibiotics.

Mecillinam and nocardicin A are two β-lactam antibiotics which are very different chemically. Whereas nocardicin A is a monocyclic β-lactam obtained from the fermentation broth of some strains of *Nocardia uniformis* (1, 4), mecillinam is a synthetic derivative of 6β-amidinopenicillanic acid (6). Both antibiotics are active against *Escherichia coli* and a broad spectrum of gram-negative bacteria (1, 6, 7). This is probably due to their inhibitory effect on peptidoglycan biosynthesis at different levels (1, 10). The bactericidal effect of mecillinam against *E. coli* has been attributed to its interaction with penicillin-binding protein 2 (PBP2), a protein involved in the shaping of the murein sacculus (12–15). The target proteins for nocardicin A are still unknown.

A peculiarity of the bactericidal action of mecillinam and nocardicin A, within the range of the minimum inhibitory concentration (MIC), is that their killing action does not occur until a considerable period of time after the addition of the drugs to the medium. This delay may facilitate the selection of resistant strains.

In this paper we present evidence that nocardicin A and mecillinam act together to induce a fast lytic response in *E. coli* cells. Analysis of several mutant strains with altered PBPs suggests that PBP2 plays an important part in the lytic response of the cells through its interaction with mecillinam. This hypothesis is further supported by the effect of other β-lactams with high affinity to PBP2 on the bacteriolytic activity of nocardicin A.

The possibility of a similar involvement of PBP2 in the synergistic effect of some combinations of mecillinam with other β-lactams (2, 8, 18) seems likely and is now under study.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains employed were *E. coli* W-7 (*dap lys*) (3) and *E. coli* KN126 (K-12 F⁻ *trpE tyr ilv sup-126*) (13) and its derivatives SP 6 (K-12 F⁻ *trpE tyr ilv sup-126 rodA ponA*) and SP61 (K-12 F⁻ *trpE tyr ilv sup-126 ponA*) (16). The strains were kindly supplied by U. Schwarz, Max-Planck-Institut für Biologie, Tübingen, Germany. Cells were grown in batch cultures at 37°C in L broth (LB) (5) supplemented with 4 mg of *meso*-diaminopimelic acid per liter under vigorous aeration.

An increase in cell mass was estimated by measuring the turbidity of the cultures at 660 nm. Viable cell counts (colony-forming units) were determined on LB agar plates after serial dilutions of the samples in 0.05 M phosphate buffer (pH 6.8) and overnight incubation at 37°C.

Determination of MICs. The MICs of mecillinam and nocardicin A were determined by the agar dilution method (19). Freshly prepared stock solutions of the antibiotics were diluted in LB agar to produce test plates (25 mm in diameter, 2 ml of medium per plate) with different concentrations of the drugs. The plates were inoculated with 4×10^4 colony-forming units of the strain to be tested, and MICs were read after incubation for 18 h at 37°C. The MIC was defined as the lowest concentration yielding fewer than five discrete colonies.

Chemicals. *meso*-[3,4,5-^{3}H]diaminopimelic acid (35 Ci/mmol, 2.5 mCi/ml) was purchased from Commissariat à l'Energie Atomique Service des Molécules Marquées, Gif-sur-Yvette, France. The antibiotics nocardicin A, mecillinam, and amoxicillin were generous gifts from T. Nuki (Fujisawa Pharmaceutical Co., Osaka, Japan), W. O. Godfredsen (Leo Laboratories, Ballerup, Denmark), and A. Zugaza (Antibióticos S.A., Madrid, Spain), respectively.

Incorporation of *meso*-[^{3}H]diaminopimelic acid. An appropriate volume of LB medium, supplemented with 2 μCi of *meso*-[^{3}H]diaminopimelic acid per ml, was inoculated with sufficient overnight culture to give

TABLE 1. MICs for mecillinam and nocardicin A against different strains of *E. coli*

Strain	MIC			
	Mecillinam		Nocardicin A	
	μg/ml	μM	μg/ml	μM
W-7	0.043	0.15	20	40
KN126	0.009	0.03	20	40
SP6	3	10	12	24
SP61	0.009	0.03	21	43

an optical density (OD) of 0.05 at 660 nm. The culture was incubated at 37°C until an OD of 0.2 was reached. The culture was then divided into subcultures which were subjected to different conditions (see below). At regular intervals, 0.2-ml samples were taken from the different cultures and incubated for 15 min at 100°C with an equal volume of 8% (wt/vol) sodium dodecyl sulfate. After cooling to room temperature, the samples were filtered through Millipore HAWP membrane filters which were repeatedly washed with water. The filters were dried, and the radioactivity retained was determined by liquid scintillation counting on an Intertechnique SL-30 counter, with 0.5% (wt/vol) 2-biphenyl-(4)-5-(4-tert-butyphenyl)-1,3,4-oxadiazol in toluene as the scintillation fluid.

RESULTS AND DISCUSSION

Effect of nocardicin A and mecillinam on growth and viability of *E. coli* W-7. The MICs of nocardicin A and mecillinam against *E. coli* W-7 were found to be 20 μg/ml (40 μM) and 0.043 μg/ml (0.15 μM), respectively (Table 1). These concentrations were within the range of those reported for other strains of *E. coli* (1, 2, 13), indicating that W-7 showed a normal response to these β-lactam antibiotics.

Mecillinam and nocardicin A were also tested in exponentially growing liquid cultures to study their effects on the growth and viability of *E. coli* W-7. The results (Fig. 1) showed that nocardicin A at high concentrations (160 μM, or four times the MIC) induced a lytic response in the cells similar to, but somewhat slower than, the response induced by 25 μM (10 μg/ml, or five times the MIC) cephaloridine, a bacteriolytic β-lactam that is very efficient against *E. coli* (12). In contrast, at concentrations near the MIC (40 to 80 μM, equivalent to one to two times the MIC), nocardicin A elicited a rather slow bactericidal and bacteriolytic response in the cells similar to that induced by mecillinam, whose slow, but efficient, bactericidal action is well known (10).

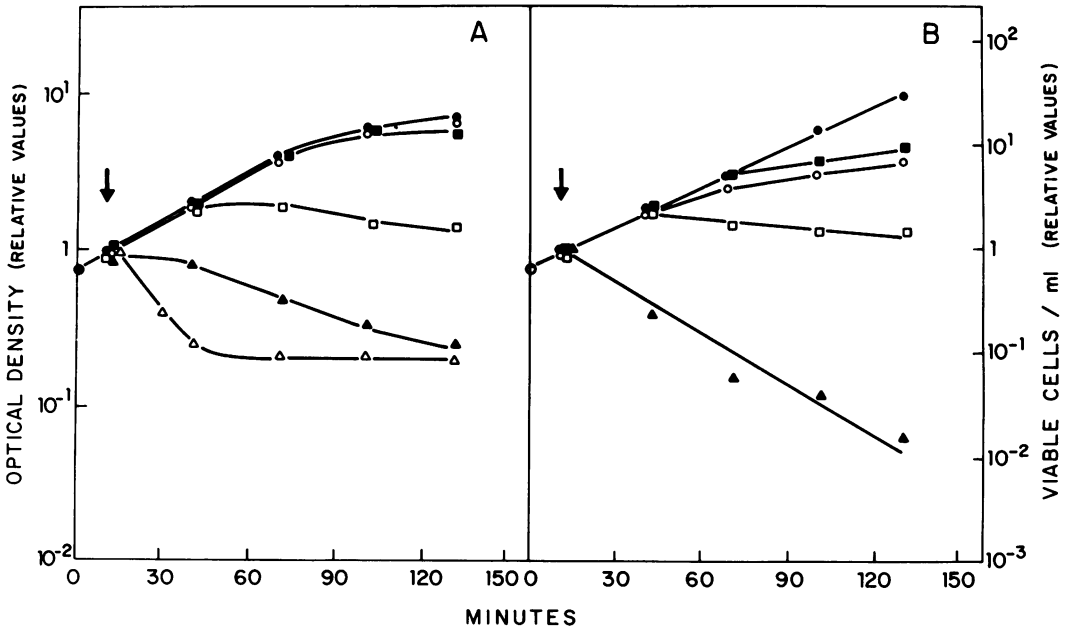

FIG. 1. Action of nocardicin A and mecillinam on the growth and viability of *E. coli* W-7. An exponentially growing culture of W-7 was divided into six equal subcultures at time zero. After 10 min (arrows), each subculture was treated with one antibiotic as indicated below. The effect of the antibiotic on cell mass increase (A) or viability (B) was measured as indicated in the text and plotted against time. To facilitate comparison of the results, the experimental data were converted into relative units. The value of the variables at the time of addition of the antibiotics was taken as the unit of reference. Reference values for this experiment were an OD at 660 nm of 0.270 and 8×10^7 colony-forming units per ml.

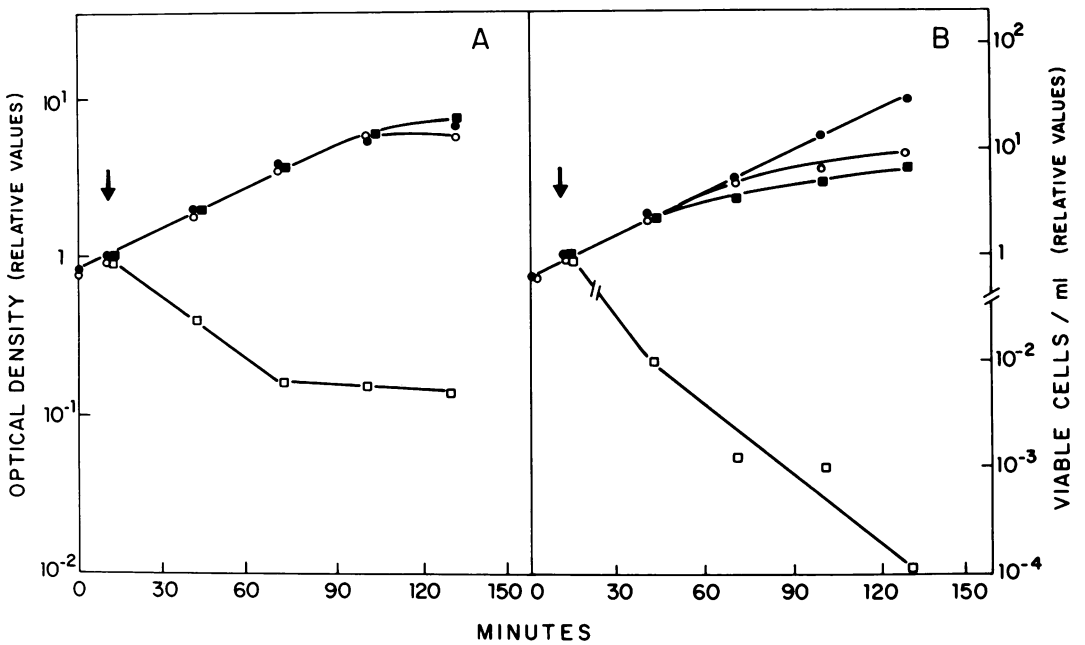

FIG. 2. Effects of nocardicin A plus mecillinam on the growth and viability of *E. coli* W-7. A culture of W-7 growing exponentially in LB medium was divided into four equal subcultures, and the changes in OD (A) or viability (B) in each subculture were determined as indicated, after the following treatments: ●, control culture without antibiotics; ○, nocardicin A added at 10 min (arrow) at a concentration of 40 μM; ■, mecillinam added at time zero at a concentration of 3 μM; □, mecillinam added at time zero at 3 μM plus nocardicin A added at 10 min (arrow) at 40 μM. The data were expressed as indicated in the legend to Fig. 1. The reference values were an OD at 660 nm of 0.245 and 8×10^7 colony-forming units per ml.

Surprisingly, when nocardicin A at a low concentration (40 μM) was added to an exponentially growing culture of W-7 shortly after the addition of mecillinam (3 μM), a dramatic bactericidal and bacteriolytic response in the cells was suddenly triggered in the treated cultures (Fig. 2). This remarkable cooperative effect was also seen with peptidoglycan biosynthesis (Fig. 3), as indicated by the inhibition of the incorporation of the specific cell wall precursor *meso*-[^{3}H]diaminopimelic acid in W-7 cells, when treated with nocardicin A (40 μM) plus mecillinam (3 μM).

To analyze the extent to which the onset of cell lysis depended on the concentrations of nocardicin A and mecillinam, we measured the effect on mass growth caused by adding increasing amounts of one antibiotic to cultures growing in the presence of the other and to untreated control cultures. The MICs of nocardicin A and mecillinam able to trigger cell lysis, deduced from these experiments, were about 20 μM for nocardicin A and 0.1 μM for mecillinam, respectively (Fig. 4 and 5). These concentrations were similar to their respective individual MICs. Therefore, the combination of mecillinam and nocardicin A kills the cells faster, but not at significantly lower concentrations of the antibi-

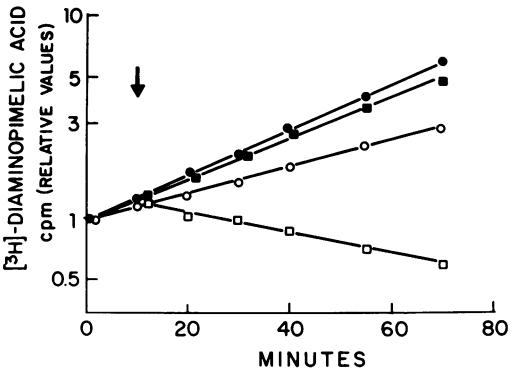

FIG. 3. Effect of nocardicin A plus mecillinam on peptidoglycan biosynthesis in *E. coli* W-7. A culture of W-7 growing exponentially in LB medium supplemented with 2 μCi of *meso*-[3,4,5-^{3}H]diaminopimelic acid (specific activity, 0.1 Ci/mmol) per ml was divided into four equal subcultures. The incorporation of the tritiated precursor in each culture was determined as indicated, after the following treatments: ●, control culture without antibiotics; ○, mecillinam added at time zero at a final concentration of 3 μM; ■, nocardicin A added at 10 min (arrow) at a final concentration of 40 μM; □, mecillinam added at time zero at 3 μM plus nocardicin A added at 10 min (arrow) at 40 μM. The data were expressed as described in the legend to Fig. 1. The reference value was 1.2×10^4 cpm.

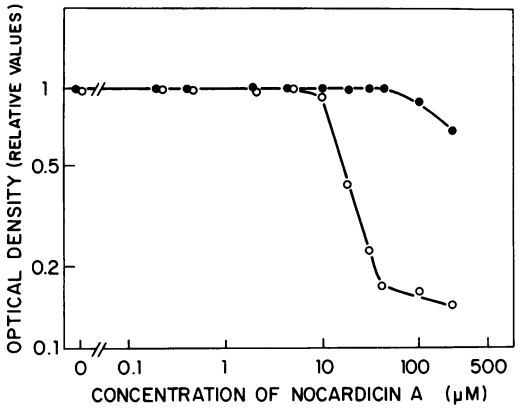

FIG. 4. Determination of the MIC of nocardicin A necessary to induce lysis in W-7 in the presence of mecillinam. An exponentially growing culture of W-7 was divided into two sets of eight similar subcultures. Mecillinam (3 μM) was added to all of the components of one set. After 10 min (time zero), different amounts of nocardicin A were added to separate cultures of each set in parallel and the cultures were incubated at 37°C for 30 min (one generation time). The turbidity of the subcultures was then measured and plotted against the concentration of nocardicin A. The unit of reference for expression of turbidity was the value reached by the control subculture, which was 0.270. Symbols: ●, nocardicin A without mecillinam; ○, nocardicin A plus mecillinam.

otics. Similar results were obtained with *E. coli* KN126 (Fig. 6A) and other unrelated bacterial strains, indicating that the cooperative action of nocardicin A and mecillinam is not due to any peculiarity of W-7.

Action of nocardicin A and mecillinam on mutants with altered PBPs. As mecillinam apparently works against *E. coli* by its specific interaction with PBP2, analysis of the response of PBP2 mutants to the action of nocardicin A and mecillinam could give additional information about the nature of the cooperation between these antibiotics. The bacterial strains SP6 (defective for PBP1a and PBP2), its parent KN126, and SP61 (a spontaneous revertant of SP6 with a normal PBP2) were used for this purpose. We found that the MICs for nocardicin A and mecillinam in these strains indicated a susceptibility comparable with that of W-7 except for SP6, which is resistant to mecillinam because it possesses the *rodA* (PBP2$^-$) mutation (Table 1) (15). When the parental strain KN126 and SP61, the revertant of SP6 which possesses PBP2, were subjected to experiments similar to those described in the legend to Fig. 2, they behaved like W-7 (Fig. 6A and C) in that lysis was easily induced by the sequential addition of mecillinam and nocardicin A. However, in similar experiments, SP6 was not lysed by this combination of antibiotics (Fig. 6B).

The behavior of KN126, SP6, and SP61 suggests that a functional PBP2 may be needed by the cells for them to be susceptible to the induction of lysis by mecillinam plus nocardicin A. This suggests that the action of mecillinam is due not only to the inhibition of PBP2 but also to some modification of its activity, leading to a state in which the cells become hypersusceptible to nocardicin A.

Induction of cell lysis in *E. coli* W-7 by nocardicin A plus amoxicillin. We determined whether the induction of cell lysis by the joint action of nocardicin A and mecillinam specifically requires mecillinam as a modifier of PBP2 by replacing mecillinam with amoxicillin, another β-lactam with high affinity for PBP2. In this experiment, W-7 cells were treated with increasing amounts of nocardicin A either in the presence or in the absence of 4 μM (1.5 μg/ml) amoxicillin, a concentration which allows preferential binding to PBP2 as determined in competition experiments with [^{14}C]benzylpenicillin (9, 13).

The effect of amoxicillin on W-7 cells in the presence of nocardicin A was very similar to that of mecillinam (Fig. 7), indicating that there is no absolute requirement for mecillinam as a modifier of PBP2. Similar results were obtained with clavulanic acid, another β-lactam with pref-

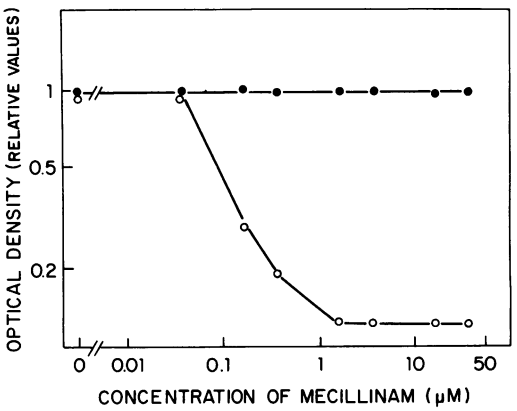

FIG. 5. Determination of the MIC of mecillinam necessary to induce lysis in *E. coli* W-7 in the presence of nocardicin A. The conditions were the same as described in the legend to Fig. 3, except that different amounts of mecillinam were added to parallel cultures of each set. Nocardicin A (40 μM) was added after 10 min to all of the subcultures of one set. After incubation at 37°C for 30 min, the turbidity of the samples was measured and plotted as in Fig. 4. The turbidity reference value was 0.290. Symbols: ●, mecillinam control without nocardicin A; ○, mecillinam plus 40 μM nocardicin A.

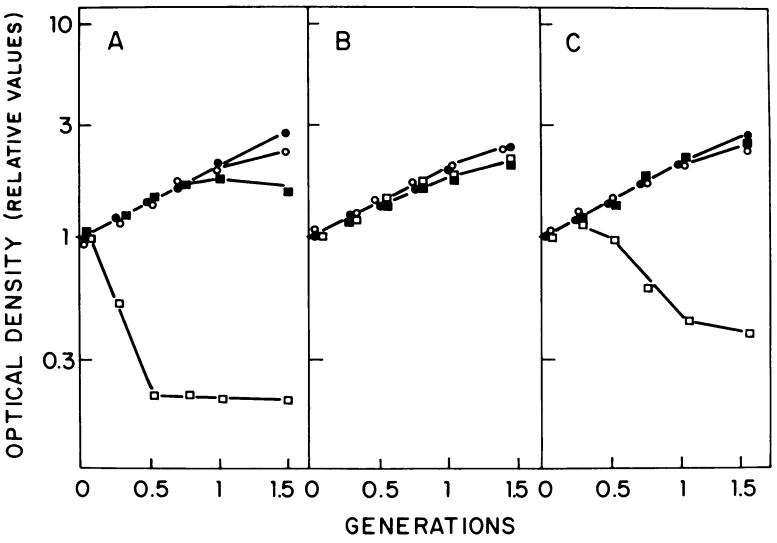

FIG. 6. Induction of cell lysis by mecillinam plus nocardicin A in *E. coli* KN126 and derived strains. Exponentially growing cultures of KN126 (A), SP6 (B), and SP61 (C) were treated with mecillinam (3 μM) and nocardicin A (40 μM) as described in the legend to Fig. 2. To facilitate comparison among strains, incubation time in the presence of the drugs was expressed as the number of doubling times after the addition of nocardicin A to the cultures. The doubling times were: KN126, 40 min; SP6, 55 min; SP61, 65 min. The data were expressed as described in the legend to Fig. 1, and the reference values were: KN126, 0.240; SP6, 0.18; and SP61, 0.230. Symbols: ●, control, no antibiotics; ○, 3 μM mecillinam added; ■, 40 μM nocardicin A added; □, 3 μM mecillinam and 40 μM nocardicin A added.

erential binding to PBP2 (17) (data not shown). The modification of PBP2 by the formation of a PBP2–β-lactam complex is probably enough to trigger cell lysis when nocardicin A is present in the growth medium.

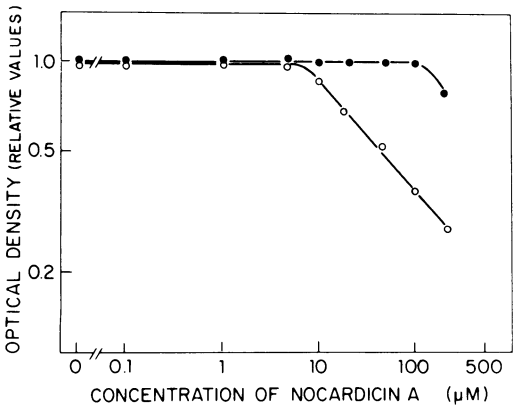

FIG. 7. Induction of cell lysis in *E. coli* W-7 by combinations of amoxicillin and nocardicin A. The experimental procedure was identical to that described in the legend to Fig. 4, except that mecillinam was replaced by 4 μM amoxicillin. The data were expressed as in Fig. 4, and the reference value was 0.305. Symbols: ●, control without amoxicillin; ○, 4 μM amoxicillin added.

The recently published results of Schmidt et al. (11), who postulated the active participation of PBP2 in the lysis of *E. coli* cells induced by the β-lactam furazlocillin, give additional support to our working hypothesis and suggest that the involvement of PBP2 in the response of *E. coli* to β-lactams may be a general phenomenon.

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