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Triggering of Autolytic Cell Wall Degradation in *Escherichia coli* by Beta-Lactam Antibiotics

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A biochemical method was developed to quantitatively compare the effectiveness of beta-lactams in triggering murein degradation (autolysin activity) in *Escherichia coli*. Bacteria prelabeled in their cell walls with radioactive diaminopimelic acid in growth medium were exposed for 10 min to the antibiotics at the appropriate minimal growth inhibitory concentrations and at multiples of these values, and the rate of cell wall degradation was followed during subsequent incubation of the cells in a buffer solution. Beta-lactams with high affinity for the penicillin-binding protein (PBP)-1 were the most effective triggers of autolytic wall degradation; beta-lactams selective for PBP-2 were the poorest; and antibiotics preferentially binding to PBP-3 showed intermediate activities. The relative effectiveness of beta-lactams in autolysin triggering was found to parallel the effectiveness of the same drugs in causing rapid loss of viability, culture lysis, and spheroplast formation. Autolysin triggering was suppressed by inhibitors of protein and ribonucleic acid biosynthesis but not by inhibitors of deoxyribonucleic acid synthesis. The beta-lactam-induced cell wall degradation did not seem to involve a direct stimulation of enzyme activity or synthesis of new enzyme molecules, and murein sacculi isolated from cells that had been preexposed to a triggering dose of beta-lactam treatment exhibited the same sensitivity to crude, homologous autolysins as sacculi prepared from untreated control bacteria. On the basis of these observations, mechanisms are considered for the triggering of *E. coli* autolysins and for the role of autolytic activity in bacterial spheroplast formation, lysis, and death.

Structurally different beta-lactams are known to cause considerably different physiological effects in *Escherichia coli*. For instance, cephaloridine or cephalothin causes rapid lysis of cultures (11, 29). Others, such as cephalexin, cause inhibition of cell division and induce the formation of long filamentous cells (11, 29). Still another group of beta-lactams, namely, mecillinam (10, 21) and, at low concentrations, thienamycin and clavulanic acid (31), cause the formation of ovoid cells. In parallel to their different physiological effects, beta-lactams also seem to differ in their affinities to the penicillin-binding proteins (PBPs) of the *E. coli* plasma membrane (28), and beta-lactams which show strong cell lytic action have high affinity for PBP-1 (PBP-1a and/or -1b) (30, 33). Recent studies on a series of mutants defective in PBPs have indicated that PBP-1 mutants defective in PBP-1b were hypersensitive to penicillin and a double mutant carrying a temperature-sensitive mutation in PBP-1a plus a constitutive defect in PBP-1b would undergo culture lysis when shifted to the restrictive temperature (22, 33).

In several gram-positive bacteria, penicillin-induced cell lysis was shown to involve the activity of murein hydrolases ("autolysins"), since in cells with suppressed autolytic activities penicillin caused only inhibition of growth without cell lysis (1, 7, 36).

Degradation of murein during penicillin treatment of *E. coli* was first reported by Schwarz and Weidel (27). Goodell et al. showed that lytic effects of beta-lactams against *E. coli* may be dissociated from effects on shape, division, and elongation by growth at low pH (9). More recently, direct activation by penicillins of extracted autolysins of *E. coli* and *Klebsiella pneumoniae* was demonstrated (6). Thus, the possibility that autolysins might have an important role in beta-lactam-induced lysis of gram-negative bacteria has already been suggested.

In this communication, a biochemical method is described that has allowed a comparison of beta-lactams with respect to their effectiveness in triggering autolysin activity in *E. coli*. We found that the autolysin-triggering efficiency of various beta-lactams was related to the lytic, bactericidal, and spheroplast-inducing action of these antibiotics. The results are consistent with

a mechanism in which inhibition of a protein in PBP group 1 is presumed to lead to the triggering of the activity of one (or more) of the murein hydrolases of *E. coli* which, in turn, is responsible for lysis, protoplast formation, and loss of viability.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *E. coli* χ 1776 F⁻ *tonA53 dapD8 minA1 supE42 Δ40(gal-uvrB) λ⁻ minB2 rfb-2 gyrA25 oms-2 thyA57* met O65 oms-1 Δ29(bioH-asd) cycB2 cycA1 hsdR2* (3) was used in all experiments. Bacteria were cultured without aeration at 32°C in Penassay broth (Difco antibiotic medium no. 3) supplemented with 2,6-diaminopimelic acid (DAP; 20 μg/ml), biotin (0.2 μg/ml), and thymidine (30 μg/ml) (supplemented Am-3 medium).

Murein was radioactively labeled by adding (DL-*meso*)-2,6-diamino[*U*-^{3}H]-pimelic acid ([^{3}H]DAP, 1.5 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) and L-lysine (1 mg/ml) to the medium ([^{3}H]DAP medium); 1.0 μCi of [^{3}H]DAP was added per milliliter of culture medium, giving a final DAP concentration of 3.126 μg/ml.

Antibiotics and reagents. Benzylpenicillin (Pfizer Inc., New York, N.Y.), cephaloridine and cephalothin (Eli Lilly & Co., Indianapolis, Ind.), ampicillin (Bristol Laboratories, Syracuse, N.Y.), sodium dicloxacillin and 6-aminopenicillanic acid (6-APA) (both from Wyeth Laboratories Inc., Philadelphia, Pa.), mecillinam (Leo Co., Ballerup, Denmark), chloramphenicol (GIBCO Laboratories, Grand Island, N.Y.), and rifampin (Sigma Chemical Co., St. Louis, Mo.) were commercial products. Cephalexin was a gift from Kenneth Price of Bristol Laboratories, Syracuse, N.Y., and mitomycin C was donated by Maria Tomasz of Hunter College, New York, N.Y. Penicillinase and sodium deoxycholate (DOC) were purchased from Riker Laboratories Inc., Northridge, Calif., and Sigma Chemical Co., St. Louis, Mo., respectively. All other chemicals and media components were reagent-grade, commercially available products.

Assay of triggered autolysin. The term "triggering" of autolysin (or autolysin activity) is used throughout this paper to refer to a specific way of provoking cell wall hydrolysis (without any specific mechanistic implications), as defined by the operations to be described next.

After growth in medium supplemented with [^{3}H]DAP for several generations, cells in the exponential growth phase were collected by centrifugation (4,300 × *g*, 5 min), transferred to isotope-free growth medium, and incubated for 50 min (i.e., a period of about one generation) in order to deplete cellular pools of the [^{3}H]DAP. After this period, 1.5-ml portions of the culture were distributed into a number of small tubes containing beta-lactams at various concentrations (representing multiples of the corresponding minimum inhibitory concentration [MIC] values) and incubated for an additional 10 min. The cultures were immediately chilled (ice bath) and then centrifuged at 3,300 × *g* for 5 min at 4°C. Cells were washed with 1.5 ml of ice-cold phosphate buffer (0.1 M, pH 7.0), resuspended in 1.5 ml of the same buffer containing 10 mM $MgSO_4$, and incubated at 32°C. The total time needed to transfer the cells to the buffer was about 5 to 8 min. After 0, 30, 60, 90, and 120 min of incubation, 200-μl portions were removed into prechilled Eppendorf microcentrifuge tubes containing 20 μl of 38% formaldehyde (to stop murein hydrolase activity).

After centrifugation at 12,000 × *g* for 10 min in the cold (4°C), radioactivity in 100 μl of the supernatants was counted. To determine total radioactivity of the reaction mixture, 200-μl portions were mixed with 20 μl of 4% DOC and incubated for 30 min at 32°C; a 100-μl portion of this suspension was used to determine radioactivity. The activity of autolysin triggered by beta-lactams was expressed as the rate of degradation (percentage) of murein during a 2-h incubation of the beta-lactam-treated cells in buffer. The rates were corrected for the spontaneous rate of release of radioactivity from the control (untreated) cells.

Assay of spheroplast-forming ability. Cells in exponential growth phase (10^8 viable units/ml) were collected by centrifugation at 4,300 × *g* for 5 min and suspended in 1/20 volume of supplemented Am-3 medium. Two-milliliter portions of a protoplasting medium (supplemented Am-3 containing 5% sucrose, 0.1% $MgSO_4$, and antibiotics at multiples of their MICs) were distributed into small (11 by 100 mm) test tubes and were inoculated with 0.1-ml portions of the concentrated cell suspension. The suspensions were incubated at 32°C under slow shaking for 1 h, and the percentage of bacteria converted to spheroplasts (defined as spherical cells) was determined by microscopic observation (Carl Zeiss microscope). At least 500 cells (in eight randomly selected fields) were counted for each determination.

Other assay procedures. Culture growth and culture lysis were monitored with a Coleman nephocolorimeter (24). Viable titers of the cultures were assayed by routine plating procedures. Antibiotics were removed before plating by dilution to levels that had no detectable effect on bacterial growth in liquid culture or in agar medium. Growth media were used as diluents. Radioactivity was measured in 6 ml of Biofluor (New England Nuclear Corp., Boston, Mass.) by a Nuclear-Chicago Mark II scintillation spectrometer.

Triggering of murein hydrolase by 5% trichloroacetic acid or 20% sucrose treatment. A suspension of cells (5 × 10^8 viable units/ml) in 0.01 M tris-(hydroxymethyl)aminomethane (Tris)-maleate buffer (pH 6.2) containing 10 mM $MgSO_4$ was mixed with trichloroacetic acid (final concentration, 5%) or sucrose solution (final concentration, 20%), and the sample was kept on ice for 10 min. The cells were washed three times by centrifugation in the buffer and resuspended in the same buffer (12).

Preparation of murein labeled with radioactive DAP from penicillin-treated and nontreated cells. After several generations of growth in [^{3}H]DAP medium, the bacteria were transferred into isotope-free growth medium for one generation. The culture was divided into two portions (8 ml each); one received 500 μg of benzylpenicillin per ml, and the other served as control. After incubation for 10 min, cells were collected by centrifugation (4,300 × *g*, 5 min) and suspended in 1 ml of ice-cold water. The cell suspen-

sion was placed into an equal volume of boiling 1% sodium dodecyl sulfate. After boiling for 10 min, the samples were centrifuged (35,000 × *g*, 60 min, 25°C) to pellet the murein (2). The pellet was washed three times with 5 ml of 0.01 M Tris buffer (pH 7.5) containing 0.02 M NaCl and finally suspended in 1 ml of ice-cold water.

Preparation and assay of murein hydrolase. Exponentially growing cells from 50-ml cultures (cell concentration, 10^8 viable cells/ml) were collected by centrifugation and washed with 10 ml of 0.05 M Tris buffer (pH 7.4) containing 0.08 M KCl, 7 mM $MgCl_2$, and 2 mM ethylenediaminetetraacetic acid (EDTA). Bacterial envelopes were prepared by suspending the cells in 3.5 ml of the same buffer, adding 3.5 g of glass beads (0.7-mm diameter; Minnesota Mining & Manufacturing Co., St. Paul, Minn.), and shaking them for 10 min at 60 Hz in a Mickle cell disruption apparatus (The Mickle Laboratory Engineering Co., Gomshall, England). The envelopes were collected by centrifugation (35,000 × *g*, 60 min) and suspended in 1 ml of 0.01 M Tris buffer (pH 7.8) containing 5 mM EDTA and 2% Triton X-100 at 0°C to solubilize the envelope. Murein hydrolase activity was assayed in 0.01 M Tris-maleate buffer (pH 6.2) containing 0.01 M Mg^{2+} and 1% Triton X-100 at 32°C, using [^{3}H]murein (0.02 mg [dry weight] per ml of incubation mixture) as a substrate (12).

Electron microscopy. Thin sectioning and electron microscopic observation were carried out according to methods described previously (8).

RESULTS

Culture lysis induced by benzylpenicillin or by DOC. *E. coli* χ 1776 is sensitive to surfactants (3), and addition of DOC to cell suspensions caused lysis as indicated by the rapid decrease in the optical density or light-scattering value (Fig. 1).

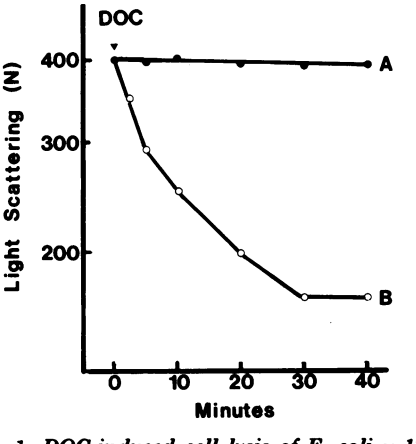

FIG. 1. *DOC-induced cell lysis of E. coli χ 1776. Cells were harvested at the exponential phase of growth, resuspended in 0.1 M potassium phosphate buffer (pH 7.0), and incubated at 32°C. DOC (800 μg/ml) was added to culture B at the times indicated.*

Figure 2 shows the effect of benzylpenicillin (500 μg/ml; corresponding to 8 × MIC) on *E. coli* χ 1776 cultures. Turbidity decrease (cell lysis) started 30 min after the addition of benzylpenicillin, and lysis could not be prevented by the addition of penicillinase as early as 5 to 15 min after the addition of penicillin.

Although both DOC and benzylpenicillin caused cell lysis (as indicated by the optical measurements), the mechanisms of the two processes appeared to be quite different. Whereas penicillin-induced cell lysis was accompanied by an extensive degradation and release of cell wall material into the outside medium, there was virtually no detectable cell wall degradation during DOC-induced culture lysis (Fig. 3). Electron microscopic observation of DOC-lysed and penicillin-lysed bacteria revealed a general fading (decrease in contrast) in the cytoplasm of the DOC-treated cells, whereas penicillin treatment produced cell envelope fragments and empty vesicles (Fig. 4).

Triggering of autolysin activity and cell lysis. A series of experiments was performed to evaluate and compare the effectiveness of various beta-lactams in causing culture lysis (mea-

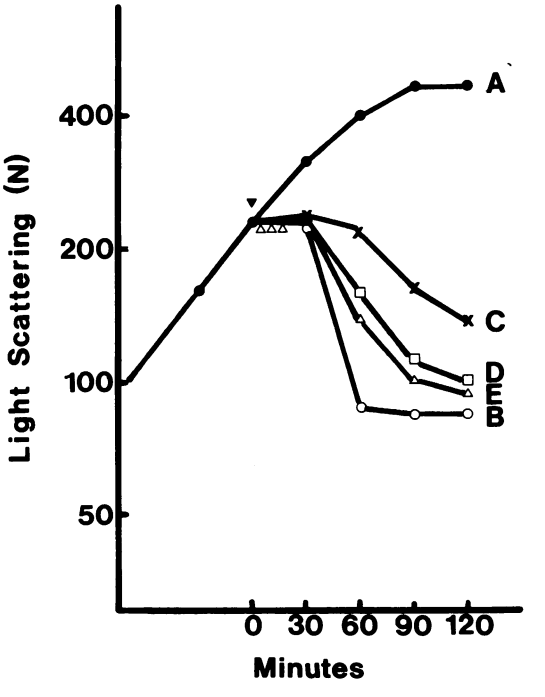

FIG. 2. *Growth of E. coli χ 1776 in the presence of benzylpenicillin. Cultures were distributed to a number of culture tubes at time zero, and cultures B, C, D, and E received 500 μg of benzylpenicillin. Cultures C, D, and E also received 100 U of penicillinase per ml after 5, 10, and 15 min, respectively.*

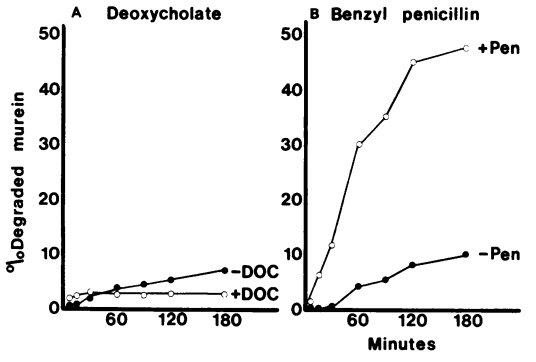

FIG. 3. *Effect of DOC and benzylpenicillin on release of cell wall materials. After several generations of growth in [^{3}H]DAP medium, the bacteria were cultured in medium with nonradioactive DAP for one generation. (A) Five milliliters of the culture was centrifuged at 4,300 × g for 5 min. Cells were suspended in 5 ml of 0.1 M potassium phosphate buffer (pH 7.0), distributed into two tubes (2.5 ml each), and incubated at 32°C. One tube received DOC (800 μg/ml) at time zero. (B) Another 5 ml of the culture was distributed into two tubes (2.5 ml) each and continued to cultivation. One tube received benzylpenicillin (500 μg/ml) at time zero. Portions (200 μl) were taken at the times indicated and put into ice; 20 μl of 4% solution of serum albumin and 20 μl of 50% trichloroacetic acid solution (wt/vol) were added sequentially. After sedimentation of precipitates (5 min at 12,000 × g), radioactivity in 100 μl of supernatant was counted in 6 ml of Biofluor. Degradation rate of murein is expressed as the percentage of radioactive label released.*

sured by the conventional optical procedures) and initiating cell wall degradation (measured by the biochemical technique described in Materials and Methods).

Figure 5 summarizes the effect of various beta-lactams (applied at multiples of their MICs) on cell lysis of *E. coli* χ 1776 and on triggering of autolytic activity. It may be seen that beta-lactams differed widely in their effectiveness both in causing culture lysis and in triggering cell wall degradation. Antibiotics which are known to cause rapid lysis when added to growing *E. coli* cultures, i.e., cephaloridine and cephalothin, were more effective in triggering of autolysin activity than benzylpenicillin. Benzylpenicillin and ampicillin caused the formation of filamentous cells at low concentrations and rapid lysis at high concentrations. Low concentration of these antibiotics did not trigger autolysin, but at high concentrations autolysin was triggered effectively. Cephalexin, which causes the formation of filamentous cells throughout a wide range of drug concentrations, was a poor trigger of autolysin even at high concentrations. Dicloxacillin and 6-APA were also less effective than benzylpenicillin. Mecillinam, which induces the growth of ovoid cells, scarcely triggered autolysin. Thus, cell lysis by beta-lactams in culture conditions was well correlated with the activity of autolysis triggered by short-term treatment of cells with corresponding beta-lactams.

Inhibition of autolysin triggering by inhibitors of protein and RNA biosynthesis.

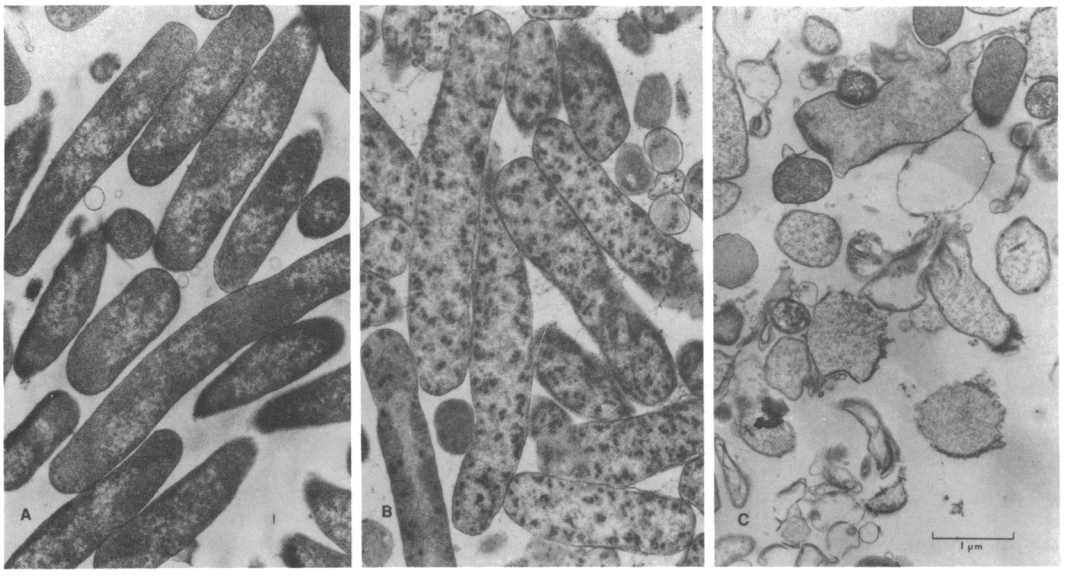

FIG. 4. *Electron micrographs of E. coli χ 1776 cells after DOC and penicillin-induced lysis. Bacteria were fixed in glutaraldehyde after 2 h of treatment with detergent or drug. (A) Control cells; (b) DOC-treated cells; (C) penicillin-treated cells (as described in the legend to Fig. 3).*

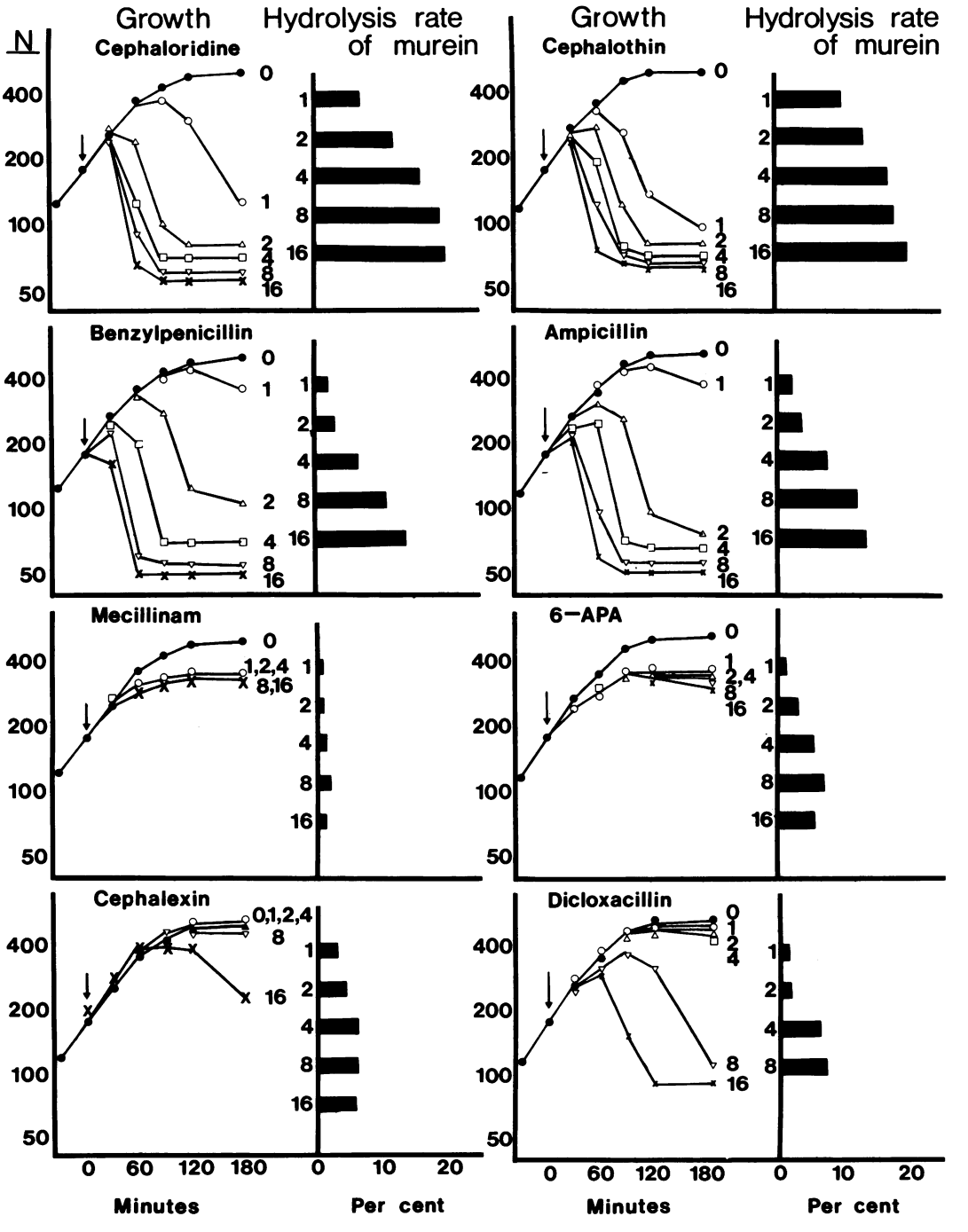

FIG. 5. *Effect of different concentrations of various beta-lactams on cell lysis of E. coli χ 1776 and triggering of autolysin activity. Bacterial cultures were grown in modified antibiotic no. 3 medium without shaking. In the exponential phase of growth (arrow), the cultures received antibiotics at the concentrations indicated by the numbers (multiples of their MICs), and the growth response of the bacterial cultures was followed by nephelometry. Activity of triggered autolysin by various concentrations (multiples of their MICs) of beta-lactams is also illustrated by the histogram at the right side of each figure. The activity is expressed as the degradation rate (percentage) of murein in 2 h (see Materials and Methods).*

It is well known that active cellular growth is required for both the penicillin-induced death and lysis of bacteria (14, 20, 25). We examined the effect of inhibitors of protein and of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) biosynthesis on triggering of autolysin activity. Autolysin triggering was completely inhibited by pretreatment of the bacteria with chloramphenicol or rifampin before exposure to benzylpenicillin. In fact, rifampin treatment was found to suppress even the slow spontaneous degradation of cell walls that is observable during incubation of control cells in the buffer (see curve C in Fig. 6). Pretreatment with an inhibitor of DNA synthesis (mitomycin C) had only minor inhibitory effect.

Triggering of autolysin and cell death. To clarify whether autolysin might cause loss of *E. coli* viability, the viability change during treatment with various beta-lactams was followed by measuring the colony-forming ability. Figure 7 demonstrates the relative viability at 2 h after the addition of beta-lactams at multiples of their MICs. Antibiotics which have strong autolysin-triggering activity were also found to cause the fastest and most extensive loss of viability. In the case of mecillinam under the culturing conditions used, there was no loss of viable titer within 2 h, even after exposure to high concentrations of the drug. Thus, cell death in the early stage of beta-lactam treatment also correlated well with the activity of triggered autolysin.

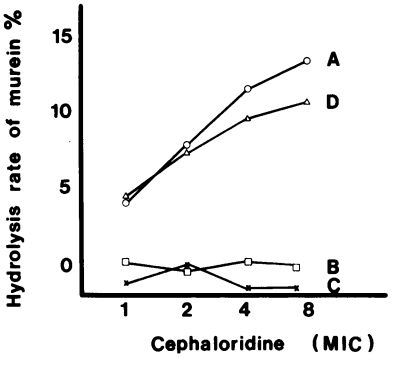

FIG. 6. *Effect of inhibitors of protein, and RNA and DNA biosynthesis on triggering of autolysin by beta-lactams. After several generations of growth in [^{3}H]DAP medium, four tubes of culture (8 ml each) were transferred into isotope-free medium for one generation, resuspended in the same medium, and given (A) no drug, (B) 100 μg of chloramphenicol per ml, (C) 100 μg of rifampin per ml, and (D) 0.2 μg of mitomycin C per ml, respectively. After 20 min, each culture was distributed into five tubes (1.5 ml each) and received cephaloridine at different multiples of its MIC. The activity of triggered autolysin was measured by the method described in the text and expressed as hydrolysis rate (percentage) of murein measured after 2 h of incubation.*

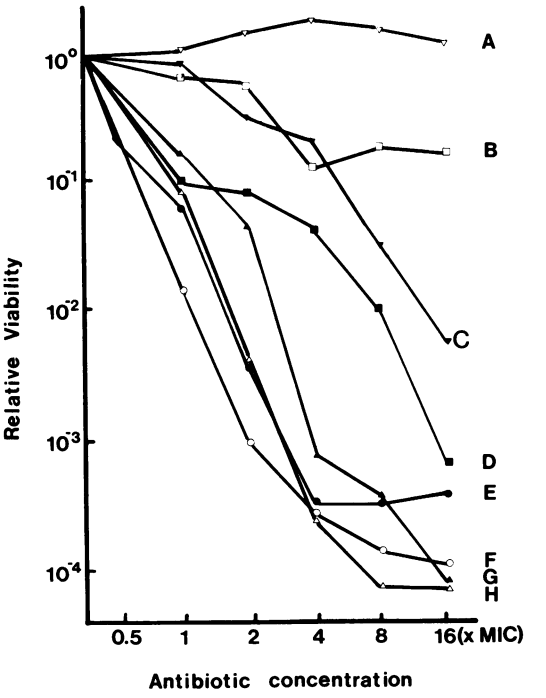

FIG. 7. *Bactericidal action of various beta-lactams. The cultures in the exponential growth phase (2×10^7 cells/ml) received antibiotics at the concentrations (multiples of their MICs) indicated by the numbers. After 2 h, 200 μl portions of the cultures were immediately diluted with modified Am-3 medium, and the number of viable cells was determined. Antibiotics: (A) mecillinam; (B) cephalexin; (C) 6-APA; (D) dicloxacillin; (E) cephaloridine; (F) cephalothin; (G) benzylpenicillin; (H) ampicillin.*

Triggering of autolysin and spheroplast formation. Induction of spheroplast formation by penicillin has been known for some time (19), and it seems likely that this phenomenon also depends on the action of autolysins. The spheroplast-forming ratio (at 1 h after the addition of beta-lactams in sucrose-containing medium) was measured by microscopic observation and plotted against the drug concentration. Spheroplast formation was most effectively induced by beta-lactams which had strong autolysin-triggering activity (Fig. 8).

Inhibition of murein hydrolysis by beta-lactams. As described above, treatment of cells with beta-lactams enhanced the degradation rate of murein in situ; i.e., beta-lactams triggered autolysin. To learn about the mechanism of this phenomenon, a number of exploratory experiments were carried out.

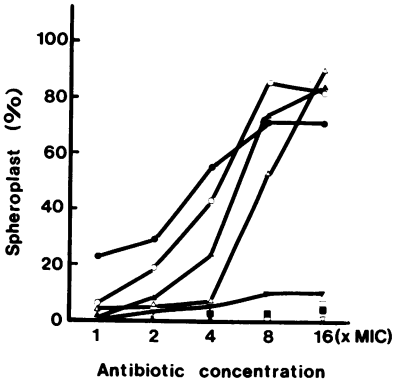

FIG. 8. *Spheroplast-forming ability of various beta-lactams. The cells in exponential growth phase (10^8 cells/ml) were collected by centrifugation, suspended in modified Am-3 medium supplemented with 5% sucrose, 0.1% $MgSO_4$, and beta-lactams at multiples of their MICs, and incubated for 1 h at 32°C. The proportion (percentage) of spheroplasts in the cultures was determined by microscopic observation. Antibiotics: (●) cephaloridine; (○) cephalothin; (▲) benzylpenicillin; (△) ampicillin; (▼) cephalexin; (□) 6-APA; (▽) mecillinam; (■) control cells.*

The effect of various beta-lactams on an already triggered cell wall degradation was first examined by using cells exposed to a triggering dose of benzylpenicillin. After the usual 10-min exposure (to trigger autolysis), the bacteria were suspended in the lysis buffer, and it was in this medium that some of the suspended cells received additional beta-lactam treatment. None of the beta-lactams tested stimulated the rate of cell wall degradation (that had already been triggered by the first exposure to benzylpenicillin) (Table 1). In fact, and surprisingly, it was found that high concentrations of certain beta-lactams suppressed the rate of cell wall degradation. Interestingly, the more effective autolysin-triggering beta-lactams (such as cephaloridine and cephalothin) were also found to be the more effective inhibitors of wall degradation; poor triggering agents (e.g., mecillinam) had no inhibitory activity. A similar inhibitory action by beta-lactams was also observed in cells triggered (to degrade their cell walls) by other agents such as exposure to 5% trichloroacetic acid or 20% sucrose (not documented). The significance of these unexpected observations is not clear at the present time. On the other hand, the data show clearly that beta-lactams did not stimulate the in vivo activity of autolysins as measured by our assay procedures.

Autolysin content of penicillin-treated and control bacteria. The rapid degradation of cell walls after treatment of bacteria with certain beta-lactams might be caused by the induced synthesis of new autolysin (hydrolase) molecules in the penicillin-treated cells; this possibility has already been considered in the earlier literature (25). We compared the total autolysin activities of control cells and of cells pretreated with a triggering dose of penicillin by two methods. In the first method, control and penicillin-treated bacteria were treated with detergent solution to extract autolysins, and the specific activities of such extracts were compared by determining the rates of degradation of added radioactive cell walls. No differences were detectable between the control and penicillin-treated extracts by this assay (not documented). In a second type of assay, bacteria were labeled with radioactive cell wall precursors, and a portion of the cells were subsequently treated with a triggering dose of penicillin. Next, both control and penicillin-treated cells were briefly exposed to cold trichloroacetic acid by a procedure that is known to induce cell wall degradation in *E. coli* (12). The rate of cell wall degradation was again found to be very similar in the control and drug-treated cells (Fig. 9). These experiments do not absolutely rule out the possibility of differences in autolysin concentration; nevertheless, no differences were apparent within the limitations of the method.

Hydrolysis of murein sacculi isolated from penicillin-treated and nontreated cells. Beta-lactams are known to inhibit the cross-linking reaction of newly formed peptidoglycan, and such poorly cross-linked murein sacculi might be hypersensitive to the action of murein hydrolases (24, 32). To test this point,

TABLE 1. *Effect of beta-lactams on hydrolysis of murein by penicillin-triggered autolysin*[a]

Antibiotic	Relative activity		
	1[b]	10	100
Benzylpenicillin	87.0	74.5	56.5
Ampicillin	98.2	90.2	84.5
Cephaloridine	98.0	55.4	28.8
Cephalothin	74.5	41.3	28.8
Cephalexin	95.5	98.2	100.0
Dicloxacillin	85.0	60.0	
Mecillinam	98.0	94.5	93.0
6-APA	99.0	98.5	

[a] [^{3}H]DAP-labeled cells were treated with 500 μg of benzylpenicillin per ml for 10 min, washed, and suspended in 0.1 M phosphate buffer (pH 7.0) containing 10 mM $MgSO_4$. A 1.4-ml portion of cell suspension was mixed with 0.1 ml of antibiotics to give final concentration of 0 (100% relative activity), 1, 10, or 100 times MIC and incubated at 32°C for 120 min. The hydrolysis rate of murein was expressed relative to that in mixture without beta-lactam of 100.

[b] Antibiotic concentration (× MIC).

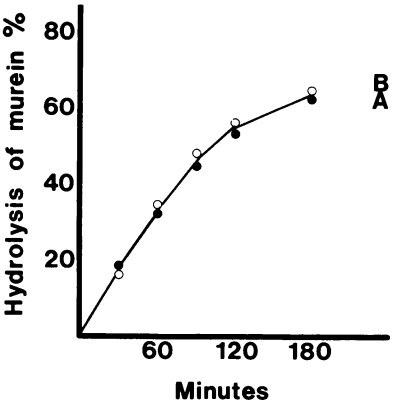

FIG. 9. *Hydrolysis rate of penicillin-treated and nontreated cells after triggering with trichloroacetic acid. After labeling with [^{3}H]DAP for several generations, cells were collected and transferred to isotope-free medium for 50 min. Two milliliters of this culture was treated with 500 μg of benzylpenicillin per ml for 10 min (A), and another 2-ml culture received no drugs (B). Cells of both tubes were collected, treated with cold trichloroacetic acid (see Materials and Methods), washed, and incubated in buffer at 32°C; the degradation rate of murein (percentage) was plotted against incubation time.*

murein sacculi were isolated from penicillin-treated and nontreated cells by the hot sodium dodecyl sulfate extraction method, and the rates of hydrolysis of these murein sacculi by a crude murein hydrolase extract were compared. No differences were apparent (Table 2).

DISCUSSION

The essential role of murein hydrolases in penicillin-induced lysis has been established in several gram-positive bacteria (1, 7, 35, 36). The relationship of murein hydrolases to cell lysis is less clear in the case of *E. coli*. Lysis of *E. coli* cultures after treatment with beta-lactams has been described repeatedly, and a role of murein hydrolase activity has also been suggested (6, 26, 27, 38). A variety of cell wall degradation products have been isolated from penicillin-induced spheroplasts (27). More recently, it has also been recognized that structurally different beta-lactams may differ greatly in their ability to induce cell or culture lysis at or above their corresponding MICs (29). However, we are not aware of definitive evidence that penicillin-induced lysis of *E. coli* is caused by the triggered bacterial autolysin activity. The experimental results described in this communication should establish a more definitive and quantitative relationship between the triggering of autolysin activity and beta-lactam-induced cell lysis in *E. coli*.

The importance of establishing by a direct biochemical assay the involvement of murein hydrolase activity in bacterial lysis (deduced from optical measurements) is illustrated by the results in Fig. 3. Both DOC and penicillin caused lysis (turbidity drop), but only in the latter process was there evidence for the involvement of murein hydrolase activity. The observations described clearly show that the rate of cell wall degradation was a function of the antibiotic concentration, and with many beta-lactams it could be initiated by a brief exposure to the antibiotics at or near the MIC. Even more striking is the correlation between the autolysin-triggering efficiency (i.e., specific activity expressed per MIC unit) and beta-lactam structure; cephaloridine, cephalothin, benzylpenicillin, and ampicillin had the highest and mecillinam and 6-APA had the lowest efficiencies. In *E. coli* strain χ 1776, the PBPs and their relative affinities for the various beta-lactams appear to be similar to those of other *E. coli* K-12 strains (29; unpublished data). From the beta-lactams examined, it seems that those with high affinity for PBP-1 have the highest and those with special affinities for PBP-2 (mecillinam) have the lowest triggering efficiencies, whereas beta-lactams with selective affinities for PBP-3 (cephalexin or dicloxacillin) occupy intermediate positions. These experiments strongly suggest that the rapid disintegration (lysis) of *E. coli* after exposure to benzylpenicillin and many cephalosporins is caused by the triggering of murein hydrolase activity.

During penicillin treatment of most bacteria, including *E. coli*, the rate of loss of viability is generally much faster than culture lysis (35). In several bacteria cultured under lysis nonpermissive conditions, or in autolysin-defective mutants of pneumococci, however, it was noted that loss of viability was much slower than in the

TABLE 2. *Hydrolysis of murein sacculi isolated from penicillin-treated and control cells*[a]

Amt of enzyme (μl)	Degradation rate of murein (%)	
	Nontreated sacculi	Penicillin-treated sacculi
20	44.0	46.0
50	67.5	63.0

[a] Reaction mixture containing 20 μl of murein sacculi, 20 or 50 μl of enzyme (see Materials and Methods), and 130 μl of 0.01 M Tris-maleate buffer (pH 6.2) containing 0.01 M Mg^{2+} and 1% Triton X-100 in a final volume of 200 μl was incubated at 32°C for 60 min. The reaction mixture was chilled on ice, and 20 μl of 4% serum albumin and 20 μl of 50% trichloroacetic acid were added. After centrifugation at 12,000 × *g* for 5 min, radioactivity in 100 μl of supernatant was counted.

lysis-prone bacteria (36), suggesting that autolysin activity may be involved in the loss of viability as well. Our experiments indicate that the rate of loss of viability of beta-lactam-treated *E. coli* parallels closely the specific autolysin-triggering activity of the corresponding beta-lactams. In the case of cephalexin, although the activity of triggered autolysin is not so strong, viability loss might still be caused by the enzyme. In the case of mecillinam, however, quite different mechanisms might work to kill the cells, because autolysin was scarcely triggered and no viability loss occurred within 2 h. These findings suggest that autolysin activity may be responsible for the rapid loss of viability observable in *E. coli* cultures treated with beta-lactams that can efficiently trigger these enzymes. Additional evidence for this comes from the observation that conditions known to antagonize the irreversible antibacterial effects of penicillins, such as treatment with inhibitors of protein or RNA synthesis, also inhibit triggering of autolysin activity (see Fig. 6). Further supportive evidence for this notion is provided by the penicillin response of penicillin-tolerant *E. coli* mutants recently isolated in our laboratory (K. Kitano and A. Tomasz, manuscript in preparation). These mutants respond to treatment with benzylpenicillin (and other beta-lactams with high affinity for PBP-1) by inhibition of growth; both lysis and loss of viability are suppressed, and the mutants exhibit a lowered autolytic activity (Kitano and Tomasz, submitted for publication).

Penicillin is known to induce spheroplast formation in *E. coli* (19), and the spheroplast-forming ability of various beta-lactams is also quite parallel to the autolysin-triggering efficiency of these antibiotics (Fig. 8). Thus, cell lysis, cell death in early stages, and spheroplast formation in *E. coli*, which occur when cells are treated with beta-lactams, may all be phenomena in which the rate-limiting step is the activity of triggered autolysin.

Table 3 summarizes the relationships between beta-lactam structure and the various known physiological effects of these antibiotics, including autolysin-triggering efficiency, induction of cell lysis, cell death and spheroplast formation, affinity for PBP-1, and effect on the morphology of the cells. All the data indicate strongly that PBP-1 is the autolysin-triggering target. On the other hand, it is not at all clear how and why the inhibition of PBP-1 activity (presumably the major murein transpeptidase of *E. coli* [34]) should cause the triggering of autolytic enzyme activity. The data presented here indicate that autolytic cell wall degradation rapidly follows inhibition of the activity of PBP-1. It is conceivable that poorly cross-linked areas within the murein act as activators or attachment sites of one (or more) of the *E. coli* murein hydrolases. It is also possible that autolysin triggering is a response to the accumulation of poorly cross-linked murein oligomers or other cell wall precursors. Studies with pneumococci suggest that in at least some gram-positive bacteria autolysin-inhibitory substances (lipoteichoic acids, lipids) are released from the penicillin-treated cells (34, 35), and such a loss of as yet unidentified endogenous inhibitors may also occur in *E. coli*. It has been suggested that the autolytic cell wall degradation after treatment of *E. coli* cells with a variety of agents (such as EDTA, cold trichloroacetic acid, or mechanical disruption) may

TABLE 3. *Properties of beta-lactams giving various physiological effects*

Beta-lactam	MIC (μg/ml)	Affinity to PBP-1[a] (μg/ml)	Morphological effects		Efficiency of:		
			Low concn[b]	High concn[c]	Autolysin triggering[d]	Killing[e]	Protoplast formation[f]
Cephaloridine	3.9	1.9	Lysis	Lysis	1.5	1.57	3.5
Cephalothin	15.6	0.4	Lysis	Lysis	1.0	1.15	4.4
Ampicillin	3.9	3.6	Filaments	Lysis	6.0	1.63	6.7
Benzylpenicillin	31.25	1.7	Filaments	Lysis	7.5	2.60	7.6
Cephalexin	15.6	59.0	Filaments	Filaments (slow lysis)	>100	≫16	≫16
Dicloxacillin	121.0		Filaments	Filaments (slow lysis)	>8	8.0	≫16
6-APA	62.5		Ovoid	Ovoid (slow lysis)	>16	12.8	≫16
Mecillinam	7.8	>500	Ovoid	Ovoid	≫100	≫16	≫16

[a] Concentration of beta-lactam required to give 50% competition (30).
[b] At approximately MIC concentration.
[c] At approximately 10 × MIC concentration.
[d] Concentration (× MIC) of antibiotic required to trigger autolysin to give 10% of hydrolysis rate of murein in 2 h.
[e] Concentration (× MIC) of antibiotic required to give 99% death in 2 h.
[f] Concentration (× MIC) of antibiotic required to give 50% spheroplast-forming ratio by 1 h of incubation.

be due to the disruption of an anatomical barrier (plasma membrane) that separates the cell wall and the hydrolytic enzymes in normally growing cells (12). Leakiness of the plasma membrane (16, 17, 37) and loss of lipid material (15, 18) have been observed during penicillin treatment of several species of bacteria, and it is possible that a similar damage to the *E. coli* plasma membrane might also be involved in the beta-lactam-induced autolysin triggering. The exploratory experiments described in this paper concerning the mechanism of autolysin triggering have yielded only negative results. Using rather crude assays, we found no evidence in the penicillin-treated cells for an increase in total autolysin activity or any increase in the autolysin susceptibility of cell wall material, nor did beta-lactams stimulate an already triggered autolytic wall degradation. In fact, unexpectedly, some beta-lactams at high concentrations were found to suppress the rate of cell wall degradation. A mechanism of this type may be the basis of the zonal effect (Eagle effect), i.e., the phenomenon that the irreversible effects of penicillins require a concentration optimum in some bacteria (5, 6). An endopeptidase sensitive to high concentrations of penicillin has been described in *E. coli* (13), and it is conceivable that the activity of this enzyme takes part in the penicillin-triggered cell wall degradation.

The method described for the comparison of autolysin-triggering efficiency of beta-lactams may be used as a simple, fast, and quantitative test to evaluate one specific effect of beta-lactams, their ability to trigger bacterial autolysins.

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