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# *Escherichia coli* Mutants Tolerant to Beta-Lactam Antibiotics

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Two types of *Escherichia coli* mutants tolerant to beta-lactam antibiotics were isolated. One is *E. coli* χ2452, which showed a tolerant response against beta-lactam antibiotics when grown at 42°C, and the others are the mutants C-80 and C-254, selected from mutagenized *E. coli* χ 1776 by cycles of exposure to ampicillin, cephaloridine, and starvation of the nutritionally required diaminopimelic acid. Beta-lactam antibiotics caused rapid loss of viability and lysis in cultures of χ1776 or in χ2452 grown at 32°C. In contrast, the same antibiotics caused only a reversible inhibition of growth in mutants C-80 and C-254 or in cultures of χ2452 grown at 42°C. Beta-lactam antibiotics that show high affinity for penicillin-binding proteins 2 or 3 (mecillinam and cephalexin, respectively) induced similar morphological effects (ovoid cell formation and filament formation) in both parent and mutant strains. In contrast, beta-lactam antibiotics which have a high affinity for penicillin-binding protein 1 (e.g., cephaloridine or cefoxitin), which cause rapid lysis in the parental strains, caused cell elongation in the tolerant bacteria. In contrast to the parental cells, autolytic cell wall degradation was not triggered by beta-lactam treatment of χ2452 cells grown at 42°C or in mutants C-80 and C-254. The total autolytic activity of mutants C-80 and C-254 was less than 30% that of the parent strain. However, virtually identical autolytic activities were found in cells of χ2452 grown either at 42 or 32°C. Possible mechanisms for the penicillin tolerance of *E. coli* are considered on the basis of these findings.

Several types of biochemical observations suggest that lysis of *Escherichia coli* by beta-lactam antibiotics involves the activity of autolytic enzymes (5, 18, 19). Evidence for such an involvement exists in pneumococci since mutants selected for a defective autolytic system were found to be resistant to the lytic (and, to some degree, to the bactericidal) effect of beta-lactam antibiotics, while remaining sensitive to the growth inhibitory effect of these antibiotics (25, 26). Repeated attempts to select for autolysin-defective *E. coli* have failed so far. Therefore, we decided to select for penicillin-tolerant mutants of *E. coli*, i.e., bacteria that could survive treatment with penicillin in a manner that is characteristic of the penicillin-tolerant pneumococci. We describe here three tolerant *E. coli* isolates. Each mutant was found to have a defective autolytic system.

We chose the *E. coli* K-12 strain χ1776 as the parental strain in the mutant isolation because of the presence of several useful properties of which of particular importance were the requirement for diaminopimelic acid (DAP), defective outer membrane, and sensitivity to detergent-induced lysis (4).

Although a complete characterization of the genetic and biochemical basis of tolerance in these bacteria is not yet available, it was felt that the existence of the first penicillin-tolerant, gram-negative mutants was of sufficient interest to warrant a preliminary description.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *E. coli* χ2452 F⁻ *dapD ΔlacZ39 Δ(gal-chl)* λ⁻ *tyrT58 nalA29 ΔthyA57 lysA32 endA1 asd hsdS3* (4), *E. coli* χ1776 F⁻ *tonA53 dapD8 minA1 supE42 Δ(gal-uvrB)40* λ⁻ *minB2 rfb-2 nalA25 oms-2 thyA57 metC65 oms-1 Δ(bioH-asd)29 cycB2 cycA1 hsdR2*, and *E. coli* C-80 and C-254 (beta-lactam tolerant mutants derived form χ1776) were used. Because of the known mechanical fragility of both strains χ1776 and χ2452, bacteria were cultured without aeration. Cultures of 10 ml each were grown in test tubes (18 by 150 mm) at 32 or 42°C in Penassay broth (Difco antibiotic medium no. 3) supplemented with DAP (20 μg/ml), L-lysine (100 μg/ml), biotin (0.2 μg/ml), and thymidine (30 μg/ml) (complete medium, OM). Cells radioactively labeled in the cell wall with DAP were obtained by growth in medium in which the DAP was replaced with 1 μCi of [$^3$H]DAP per ml (giving a final DAP concentration of 3.1 μg/ml).

**Isolation of mutants.** *E. coli* χ1776 was mutagen-

ized by conventional procedures (1). Bacteria, grown exponentially at 32°C in complete medium, were suspended in 0.05 M Tris-maleate buffer (pH 6.0) containing 300 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and were incubated for 30 min at 37°C. Cells were washed once with Tris-maleate buffer to remove the mutagen and suspended in OM. Portions of 2 ml were inoculated into 8 ml of OM and incubated at 32°C for 3.5 h (cell concentration at this point was $5 \times 10^7$ to $7 \times 10^7$ cells per ml). Mutants were selected next by a cycle of treatments that included the following steps.

**Step (i).** A 10-ml mutagenized culture was treated with ampicillin (20 μg/ml, corresponding to about five times the minimal inhibitory concentration [MIC]) for 1.2 h. During this treatment, the turbidity of the bacterial culture first increased by about 30% and then dropped to about 50% of the maximum turbidity value. The cells were collected by centrifugation (5,000 × *g*, 10 min, room temperature), washed once with OM, and resuspended in fresh OM (twice the original volume). After overnight incubation (13 h) at 32°C, the surviving cells grew to a turbid culture that was resuspended in fresh OM and allowed to double in its turbidity ($5 \times 10^7$ to $10 \times 10^7$ per ml), at which time a 10-ml portion of the culture received step (ii) of the enrichment procedure.

**Step (ii).** Step (ii) consisted of treatment with cephaloridine (20 μg/ml; five times the MIC value) at 32°C for 1.5 h. The turbidity changes of this culture were about the same as those observed during the first ampicillin treatment. After the cells were washed with OM, dilution and overnight incubation were the same as after ampicillin treatment. The overnight culture was washed with OM without DAP, resuspended in OM without DAP, and incubated at 32°C for 3 h.

**Step (iii).** During this DAP starvation there was an initial 20% increase followed by a decline (about 30% of the maximum) in turbidity. The culture was washed and resuspended in OM (to half of the culture's turbidity at the end of the DAP starvation) and incubated (32°C) for 3 h, during which time the turbidity doubled. Portions of 10 ml of this culture were then exposed to the final step.

**Step (iv).** Step (iv) consisted of ampicillin treatment (20 μg/ml for 2.5 h). The bacteria were finally washed with OM and resuspended in 10 ml of OM, and frozen stocks were prepared (freezing at −40°C and storage at −80°C).

The final scoring of mutants was done in the following way: frozen stock cultures were melted, diluted, and plated on OM agar plates (about $10^7$ bacteria per plate) containing 10 μg of ampicillin per ml and incubated at 32°C for 16 h. Then the plates were overlayered with 4 ml of soft agar medium (0.6% agar in OM medium) containing 10 U of penicillinase (Rikers Chemicals) per ml and incubated for another 24 h. The colonies that grew up under this condition (about 100 per plate) were picked into liquid medium and checked for a tolerant response against beta-lactam antibiotics. Out of 340 such surviving colonies, 6 were found to show a substantially decreased rate of loss of turbidity (as compared with the parental culture) when challenged with cephaloridine (five times the MIC). These cultures also showed lower autolytic activity (30 to 60% of the parental cells). Two of these tolerant mutants, C-80 and C-254, exhibiting virtually no lysis during cephaloridine treatment, were chosen for more detailed characterization to be described in this paper.

**Assay procedures.** Bacteria were routinely grown at 32°C except when noted otherwise. Culture growth and culture lysis were monitored with a Coleman Nepho-colorimeter (14). Viable titers of the cultures were assayed by routine plating procedures. The assay method of triggered autolysin by beta-lactam antibiotics (13) was as follows.

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 radioactive isotope-free growth medium, and incubated for 50 min (i.e., a period of about one generation) 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-lactam antibiotics at various concentrations (representing multiples of the corresponding MICs) and incubated for an additional 10 to 20 min. The cultures were 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 10 to 15 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 supernatant fluids was counted. To determine total radioactivity of the reaction mixture, 200-μl portions were mixed with 20 μl of 4% deoxycholate 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-lactam antibiotics was expressed as the rate of degradation of murein during a 2-h incubation of the beta-lactam antibiotic-treated cells in buffer. The rates were corrected for the spontaneous rate of release of radioactivity from the control (untreated) cells. The treatment time with beta-lactam antibiotics was 20 min (for *E. coli* χ2452 cells) or 10 min (for the cells of *E. coli* χ1776 C-80 and C-254).

**Triggering of murein hydrolase by trichloroacetic acid.** The method of Schwarz et al. was used (18) (see also legend to Fig. 8) to trigger murein hydrolase by trichloroacetic acid.

**Reagents.** DL-(*meso*)-2,6-Diamino-[*U*-$^3$H]pimelic acid (1.5 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill.). All other materials and chemicals were reagent-grade commercially available products.

## RESULTS

**Tolerant response of *E. coli* χ2452 to beta-lactam antibiotics at 42°C.** Several mutants of *E. coli* K-12 were examined for their response to beta-lactam antibiotics and a mutant, *E. coli* χ2452, was found to show a tolerant response to

beta-lactams at 42°C but not at 32°C (Fig. 1). χ2452 was isolated by Dennis Pereira in the laboratory of Roy Curtiss (D. Pereira, Ph.D. Thesis, University of Alabama Medical Center, Birmingham, Ala., 1979). Although rapid culture lysis occurred after exposure to the MIC of cephaloridine at 32°C, the cells were stable to lysis by the addition of cephaloridine when the bacteria were grown at 42°C. This phenomenon was reproducible by the addition of other beta-lactams such as ampicillin, benzylpenicillin, or cephalothin. The MICs of these beta-lactams were identical at the two temperatures (Table 1).

The tolerant response to beta-lactam antibiotics was temperature dependent, and if temperature was shifted down from 42 to 32°C at 0 to 30 min after the addition of the drug, cells started lysing after a short time lag (Fig. 2).

**Tolerant response of *E. coli* C-80 and C-254 to beta-lactam antibiotics.** Figure 3 demonstrates the effect of cephaloridine on cultures of *E. coli* χ1776, C-80, and C-254. Although growth of the mutants was stopped by the addition of cephaloridine, the cells scarcely lysed even after prolonged (overnight) incubation with the antibiotic. Culture lysis was very slow or negligible in all of the tolerant mutants (including χ2452 grown at 42°C) even during exposure to high concentrations (eight times the MIC) of cephaloridine, ampicillin, and a variety of other beta-lactam antibiotics. Nevertheless, inhibitors of early stages of cell wall synthesis (e.g., the combination of D-cycloserine and fluoro-D-alanine) could still induce lysis of the tolerant bac-

TABLE 1. *MICs of beta-lactam antibiotics against mutants of E. coli*[a]

| Antibiotic | MIC (μg/ml) of strain: | | | | |
|---|---|---|---|---|---|
| | χ2452 | | χ1776 | C-80 | C-254 |
| | 32°C | 42°C | | | |
| Cephaloridine | 3.9 | 3.9 | 2.0 | 3.9 | 3.9 |
| Ampicillin | 3.9 | 3.9 | 3.9 | 3.9 | 3.9 |
| Cephalothin | 15.6 | 15.6 | 31.25 | 62.5 | 62.5 |
| Benzylpenicillin | 31.25 | 31.25 | 31.25 | 62.5 | 62.5 |
| Cefoxitin | | | 7.8 | 7.8 | 7.8 |
| Cephalexin | | | 15.6 | | |
| Mecillinam | | | 7.8 | 7.8 | 7.8 |

[a] The MICs listed in the table were determined by the following procedure: exponentially growing cultures (at cell concentration of about $5 \times 10^7$ cells per ml) received antibiotics at various concentrations, and growth of the cultures was monitored (as described in the Materials and Methods). The minimum antibiotic concentration that caused cessation of growth was taken as the MIC. Inhibition of growth was not instant but became manifest only after a residual increase in the turbidity of cultures.

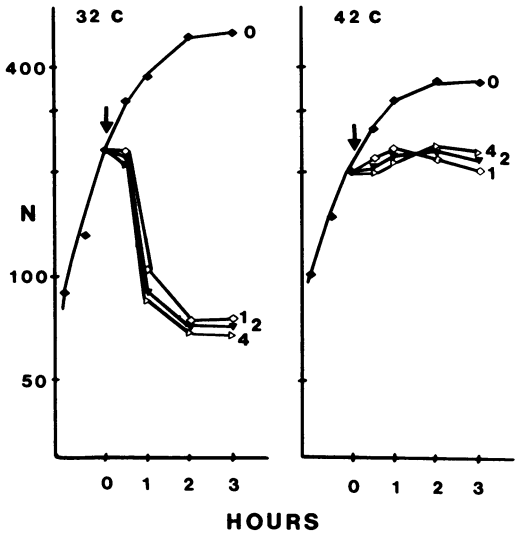


FIG. 1. *Effect of cephaloridine on cultures of E. coli χ2452 growing at 32 and 42°C. Bacterial cultures were grown in OM without aeration. In the late exponential phase of growth (arrow), the cultures received cephaloridine at the concentrations (multiples of MIC; 7.8 μg/ml at both temperatures) indicated by the numbers, and the growth response of the bacterial cultures was followed by nephelometry and plotted in nephelometric (N) units.*

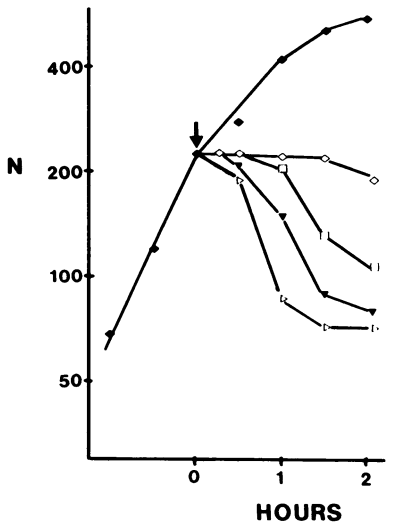


FIG. 2. *Effect of temperature shift during treatment of E. coli χ2452 with cephaloridine. Bacterial cultures were grown in OM at 42°C without aeration. At a cell concentration of about $1 \times 10^8$ viable cells per ml (in the late exponential phase of growth) (arrow), the cultures received 2.5 times the MIC of cephaloridine (19.5 μg/ml), and the temperature of the cultures was shifted to 32°C at 0 (△), 15 (▼), and 30 (□) min after the addition of the drug. Control cultures at 42°C with (◇) or without (◆) cephaloridine are also shown.*

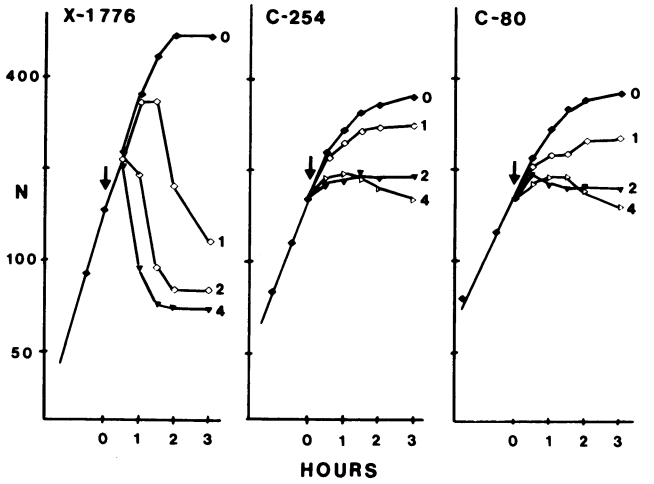


FIG. 3. *Effect of cephaloridine on cultures of E. coli χ1776 and its mutants C-80 and C-254. Bacterial cultures were grown in OM at 32°C without aeration. In the exponential phase of growth (5 × $10^7$ viable cells per ml) (arrow), the cultures received antibiotics at the concentrations (multiples of MIC) indicated by the numbers, and the growth response of the bacterial cultures was followed by nephelometry. The MICs for cephaloridine were as follows: χ1776, 3.9 μg/ml; mutants C254 and C-80, 7.8 μg/ml.*

teria (data not shown).

The MICs of various beta-lactams (for method of MIC determination see footnote *a*, Table 1) were identical to those of the parental cells (Table 1) except for benzylpenicillin and cephalothin; in these cases the MIC for the tolerant cells was higher (maximum, twice those of the parents).

Cultivation of *E. coli* χ2452 at 42°C gave a striking protection against the killing effects of cephaloridine (Fig. 4a) and also against lysis. The killing effects of cephaloridine in the mutants C-80 and C-254 were also very weak as compared with the effects in the parental strain (Fig. 4b).

**Morphological changes of the mutant cells during treatment with beta-lactams.** Figure 5 demonstrates the effect of three beta-lactam antibiotics on the cell shape of *E. coli* χ2452 at 42 and 32°C. Cephaloridine, which has a high affinity for penicillin-binding protein (PBP) 1b (20, 21) caused rapid lysis of cells at 32°C and the formation of empty cells, whereas at the nonpermissive temperature for lysis, cell elongation continued, and bacilli became noticeably longer than the control cells. Mecillinam (which has a high affinity for PBP 2 [20]) caused the formation of ovoid cells at both 32 and 42°C. During the 3 h of treatment with cephalexin, cells grown at 42°C have elongated to lengths varying between 1.5 and 3 times the length of the untreated bacteria grown at this temperature. The same drug caused a more substantial elongation (3 to 6 times the normal length) when

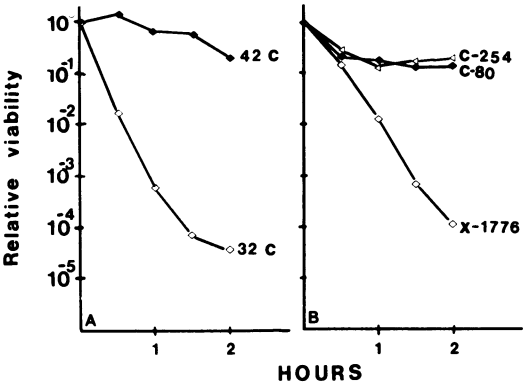


FIG. 4. *Bactericidal action of cephaloridine against beta-lactam-tolerant mutants of E. coli. (A) E. coli χ2452 cells growing in complete medium at 32 and 42°C were treated at 0 min (cell concentration, 1 × $10^8$ bacteria per ml) with 20 μg (about 2.5 times the MIC) of cephaloridine per ml. (B) Cells of E. coli χ1776 C-80 and C-254 growing in complete medium at 32°C were treated at 0 min with twice the MIC of cephaloridine. Samples were periodically removed, diluted with complete medium, and plated to determine the number of viable cells.*

the cells were grown at 32°C. Similar phenomena were also observed with the tolerant mutants C-80 (Fig. 6) and C-254 (data not shown). In contrast, the mecillinam-induced ovoid cell formation was not easy to document in these tolerant strains or in the parental strain χ1776. Mecillinam treatment, at least under the conditions used here (i.e., relatively high cell concen-

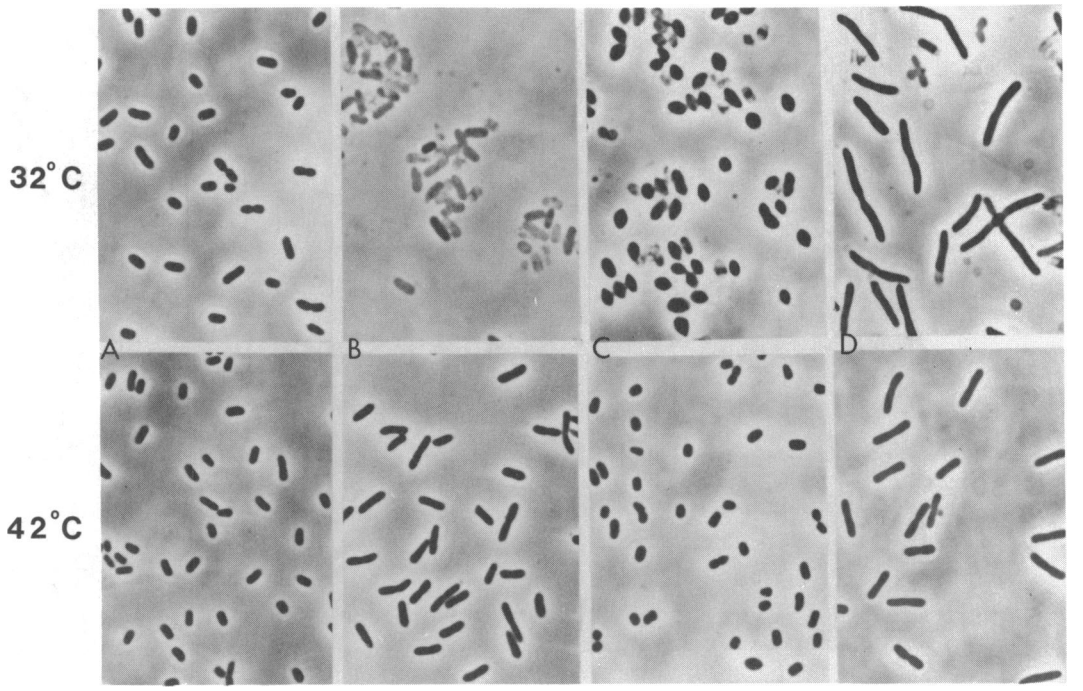


FIG. 5. *Effect of beta-lactam antibiotics on cell shape of E. coli χ2452 grown at 32 and 42°C. (A) Control; (B) cephaloridine; (C) mecillinam; (D) cephalexin. Cells grown at 32 and 42°C were exposed to four times the MICs of various beta-lactam antibiotics for 3 h. Cells were fixed with 2% glutaraldehyde, and phase-contrast micrographs were then made of the cells, using a Zeiss microscope fitted with a Planachromat 100/1.25 phase-contrast oil immersion objective. Kodak Panatomic-X film was used for the photomicrography.*

tration), caused the formation of abnormally shaped bacteria (Fig. 6).

**Triggering of autolytic activity by cephaloridine and by trichloroacetic acid treatment.** The autolytic cell wall degradation of *E. coli* χ2452 was effectively triggered by brief exposure (10 to 20 min) to beta-lactam antibiotics at the lysis-permissive temperature but was not triggered at 42°C (Fig. 7). Autolysis of mutants C-80 and C-254 was not triggered by this treatment either (Fig. 7). Interestingly, exposure to high concentrations of antibiotics (eight times the MIC [Fig. 7]) has occasionally caused a suppression of the rate of cell wall degradation. This type of effect has been consistently observed at very high concentrations (10 to 100 times the MICs) of certain beta-lactam antibiotics (13).

The autolytic activity of *E. coli* χ2452 triggered by 5% trichloroacetic acid treatment (7) was almost the same in cells grown either at 32 or 42°C and was independent of the temperature at which the assays were carried out (Fig. 8a). In contrast, the autolytic activities of C-80 and C-254 triggered by 5% trichloroacetic acid treatment were less than 30% that of the parent strain (Fig. 8b). Identical results were obtained when autolysis was triggered by other methods (exposure to hypertonic sucrose or to freezing and thawing [7]) or in experiments in which the autolytic activities of Triton-ethylenediaminetetraacetic acid extracts (7) (made from tolerant and lysis-prone cells) were compared.

## DISCUSSION

The mechanisms by which inhibition of penicillin-sensitive bacterial enzymes (or PBP) brings about interference with the growth of a bacterial cell is not clearly understood presently (24). Chemically different beta-lactam antibiotics can elicit a variety of different morphological effects and distinct growth inhibitory mechanisms in the same bacterium (*E. coli*) (20, 21). There are also examples of the converse situation since different species of bacteria treated with the same beta-lactam antibiotic may show a wide variety of physiological responses, such as inhibition of growth (with only an extremely slow loss of viability), rapid loss of viability, and loss of viability accompanied by cellular lysis (9, 26). Studies on pneumococci and some other gram-positive bacteria have shown that the

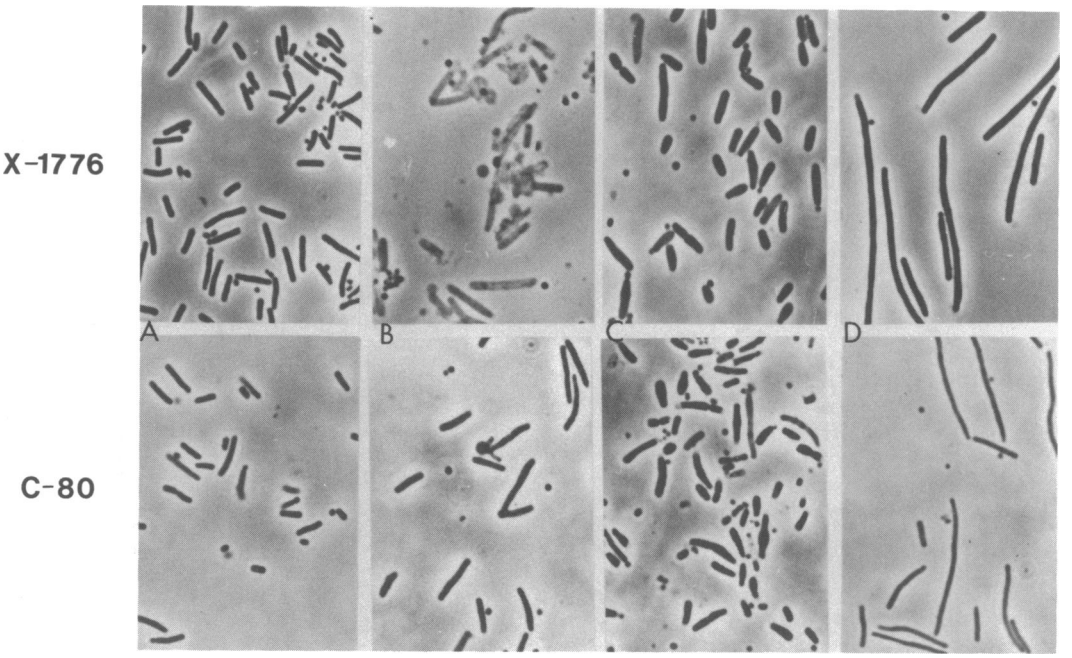


FIG. 6. *Effect of beta-lactam antibiotics on cell shape of E. coli χ1776 and its mutants. (A) Control; (B) cephaloridine; (C) mecillinam; (D) cephalexin. Cultures of the bacteria grown at 32°C were exposed to four times the MICs of the various beta-lactam antibiotics for 3 h. After fixation with 2% glutaraldehyde, phase-contrast micrographs were made of the bacteria, using a Zeiss microscope fitted with a Planachromat 100/1.25 phase-contrast oil immersion objective. Kodak Panatomic-X film was used for the photomicrography.*

beta-lactam antibiotic-induced lysis requires the activity of bacterial autolysins (2, 6, 26). Pneumococci in which the in vivo activity of the *N*-acetyl muramic acid-L-alanine amidase has been suppressed (by mutation or physiological manipulations) show a unique response to penicillin treatment: in such cells the antibiotic causes inhibition of growth (at the normal MIC) and often the rate of loss of viability is also greatly reduced, but no lysis occurs (25, 26).

We proposed the term "antibiotic tolerance" for this phenomenon (25), and recent reports suggest that antibiotic tolerance may not be restricted to laboratory strains of pneumococci but may also occur among clinical isolates of *Staphylococcus aureus* (17) and *Streptococcus sanguis* (10). The involvement of autolytic activity in the penicillin-induced lysis of *E. coli* has been repeatedly suggested in the literature, and penicillin treatment of *E. coli* has been reported to cause enzymatic cell wall degradation (19) and also an increase in the in situ autolytic activity (5). In a recent, more detailed study, we found a striking quantitative correlation between the efficiency of beta-lactam antibiotics to trigger autolytic cell wall degradation and the relative affinity of these antibiotics for the PBP 1 group of *E. coli* (13). In this communication we describe the properties of three *E. coli* isolates that exhibit a tolerant response to treatment with penicillins and cephalosporins. Each one of the isolates has a defective autolytic system.

One of the isolates (χ2452) exhibits a tolerant response when grown at 42°C and a lytic bactericidal response when grown at 32°C. The other two mutants (C-80 and C-254) show tolerance at all temperatures of growth. These *E. coli* mutants resemble the autolysis-defective pneumococci in that each one of them exhibits an abnormality in their autolytic system: brief treatment of the mutants (or of χ2452 grown at 42°C) with beta-lactam antibiotics does not trigger cell wall degradation, although the same treatment causes rapid and extensive wall hydrolysis in the parental bacteria and in strain χ2452 grown at the lysis-permissive temperature (32°C). Additional tests have revealed a substantially decreased autolytic activity in the mutant cells (as compared with the parental bacteria). However, cells of strain χ2452 grown at either 32 or 42°C appear to have comparable (normal) levels of autolysin activity. Thus, tolerance of mutants C-80 and C-254 is accompanied by an apparent net decrease in autolysin activity, whereas in χ2452 only the triggering of autolytic activity seems

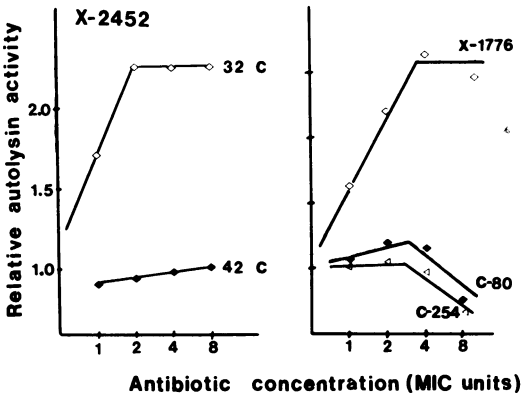


FIG. 7. *Triggering of autolytic activity by cephaloridine. After growth in medium supplemented with [$^3$H]DAP for several generations, cells were grown in radioisotope-free medium for an additional cell generation. After this period, 1.5-ml portions of the culture were treated with increasing concentrations of cephaloridine for 20 min (strain χ2452) or for 10 min (strain χ1776 and its mutant derivatives). The growth temperature was 32°C for all strains except for a portion of χ2452, which was also grown at the lysis-protective temperature of 42°C. For the assay of cell wall degradation, cells of all strains (including the 42°C-grown bacteria) were incubated at 32°C after washing and resuspension in buffer (1.5 ml). Radioactivity released during 2 h of incubation in the buffer was counted, and the autolytic activity was expressed as the rate relative to the spontaneous rate of release of radioactivity from the untreated bacteria. This latter value has amounted to about 8% of the total incorporated radioactivity (13).*

thermosensitive. The titration of the total autolysin content of *E. coli* presents considerable problems since there are several enzyme activities involved (8, 16) and some of these are structure bound. Furthermore, in extracts, some of these hydrolytic enzymes may be restricted in their ability to attack cell walls. For these reasons, it was felt that a determination of in situ autolysin activity (7) as it was done in our experiments may be an appropriate first approximation when comparing autolysin levels of tolerant (mutant) and lysis-prone (parental) bacteria.

The level of autolysin activity was also determined in Triton-EDTA extracts (7) prepared from the parental cells and from the mutants C-80 and C-254. The mutant extracts showed a diminished specific hydrolase activity (about 25 to 30% that of the extract from parent cells). In these assays radioactive DAP-labeled cell walls (murein sacculi [3]) from the parental bacteria were used as substrate (data not shown). It is not yet known whether the lowered autolysin activity in the mutants involves one or more of the multiple types of murein hydrolases of *E. coli* (8, 16).

The mechanism by which interference with cell wall synthesis provokes autolytic cell wall degradation in *E. coli* is not yet known. In the case of pneumococci, we proposed that the suicidal activity of the autolytic amidase may be caused by a defect that rapidly upsets the in vivo control of this enzyme after the addition of penicillin to the bacteria (26). This suggestion was based on the demonstrated release of an autolysin inhibitory agent (Forssman antigen) from pneumococci during penicillin treatment (26). There is no evidence at this time for the existence of analogous autolysin inhibitors in *E. coli*. The triggering of in situ autolytic wall degradation of *E. coli* after exposure of the cells to various chemical treatments (e.g., cold trichloroacetic acid, ethylenediaminetetraacetic acid, high concentration of sucrose [7]) or mechanical disruption has been explained as the disruption of a "barrier" (plasma membrane?) that was suggested to separate the autolysins of *E. coli* from their substrate in the normal cells (7). Rapid release of substantial quantities of lipids and other membrane components have been demonstrated during penicillin treatment of several autolysin-defective gram-positive bacteria (9, 11, 12). Preliminary experiments indicate that penicillin-induced release of lipid material also occurs in some strains of *E. coli* (A. Tomasz, unpublished data), and it is conceivable that such a process may damage the hypothetical

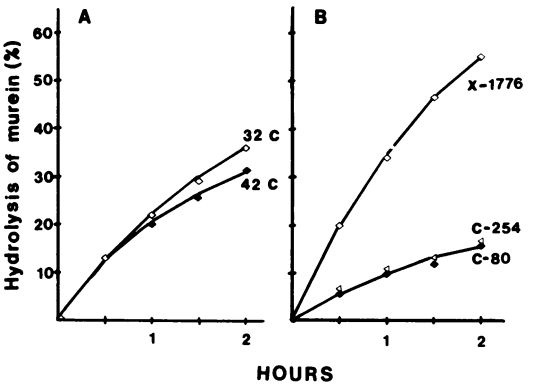


FIG. 8. *Autolytic activity triggered by trichloroacetic acid treatment. [$^3$H]DAP-labeled cells were suspended in 0.01 M Tris-maleate buffer (pH 6.0) containing 10 mM $MgSO_4$ and mixed with an equal volume of 10% trichloroacetic acid and allowed to react for 10 min in ice (7). The cells were washed three times by centrifugation in the buffer, resuspended in the same buffer, and incubated at 32°C. The radioactivity released into the supernatant solution was assayed at different times by the procedure described in Materials and Methods. (A) Cells of χ2452 grown at 32°C (◇) or 42°C (◆). (B) Cells of χ1776 (◇) and its mutants C-80 (◆) and C-254 (△). Bacteria were grown at 32°C.*

barrier separating autolysins and the murein in a manner somewhat analogous to the damage that may be responsible for triggering by chemical treatments. If this were true, then the lack of autolysin triggering in strain χ2452 grown at 42°C may be caused by the presence of a more stable barrier (different membrane composition). Alternatively, cell wall synthesis at the higher temperature may produce a murein of low autolysin sensitivity at a critical area of the mutant cell wall.

It should be emphasized that the nature of autolysin triggering is obscure at the present time. There is strong evidence implicating the functional involvement of both the binding proteins 1 (PBP 1a and 1b) (21–23) as well as the autolytic enzyme(s) of *E. coli* in the penicillin-induced lysis of this bacterium. However, it is not clear how and why inhibition of PBP leads to initiation of autolytic cell wall degradation, and the term triggering refers to this poorly understood connection between the functions of PBP 1 and the control of autolysin activity. Besides the hypothetical barrier function, triggering may involve accumulation of cell wall precursors or even the local modification of the murein substrate, e.g., by introduction of poorly cross-linked material into areas of the cell wall during penicillin treatment (15).

In the thermosensitive tolerant mutant χ2452 the heat-sensitive element in the penicillin response seems to be this hypothetical triggering process since both autolytic activity and the PBP patterns are normal at each temperature (data not shown). The substantially lowered autolytic activity and antibiotic tolerance of mutants C-80 and C-254 are reminiscent of the properties of autolysis-defective pneumococci. On the other hand, lysis of the *E. coli* mutants can still be induced by treatment with inhibitors of early steps in cell wall biosynthesis, whereas the autolysis defective pneumococci are resistant to the lytic effect of all cell wall inhibitors (25). It is conceivable that the tolerant phenotype has a more complex biochemical and genetic basis in *E. coli* than in pneumococci because of the more complex nature of autolysin regulation and the differences in the mode of cell wall assembly in *E. coli* and in pneumococci.

The morphological changes induced in the tolerant *E. coli* during treatment with different beta-lactam antibiotics require comment. It seems that inhibitors of PBP 2 (mecillinam) and PBP 3 (cephalexin) can elicit their typical morphological effects in these cells (i.e., ovoid cell formation and filament formation), whereas the response of the tolerant *E. coli* to inhibitors of PBP 1 is changed: instead of the appearance of "rabbit ear" forms and lysis, such cells would elongate (Fig. 5). These findings support the notion that in *E. coli* triggering of autolysin activity by beta-lactam antibiotics is a specific consequence of the inhibition of PBP 1. The tolerant cells appear to be defective in the sequence of events leading from PBP 1 to autolysin triggering; they respond to inhibitors of PBP 1 by reversible inhibition of growth (instead of loss of viability and lysis). These observations suggest that triggered autolysin activity may be the cause of viability loss (as well as cell lysis) in *E. coli*.

It is hoped that these mutants will be helpful in the analysis of the mechanism of the antimicrobial effect of beta-lactam antibiotics. A mutant of *E. coli* strain K-12 that is tolerant to lysis by cephalexin has been isolated independently by T. Nikaido, S. Tomioka, and M. Matsuhashi (personal communication). No defect in a peptidoglycan-lytic enzyme activity has so far been demonstrated.

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