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Mechanism of Suppression of Piperacillin Resistance in Enterobacteria by Tazobactam

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Resistance to piperacillin in several isolates of *Citrobacter freundii* and *Enterobacter cloacae* was investigated and confirmed to occur at a frequency of 10^{-7} to 10^{-6}. Development of resistance to piperacillin was significantly suppressed by tazobactam but not by clavulanic acid. To elucidate the mechanism by which resistance suppression occurs, the effect of piperacillin plus tazobactam on the induction of AmpC β-lactamase was analyzed by monitoring the β-galactosidase activity of an inducible *ampC-lacZ* gene fusion in *Escherichia coli*. The combination exerted no inhibitory effect on AmpC β-lactamase induction. Tazobactam also had no effect on the accumulation of a key intermediate in the AmpC β-lactamase induction pathway, 1,6-anhydromurotripeptide, in an *ampD* mutant strain of *E. coli*. However, the addition of tazobactam to liquid cultures of *E. cloacae* 40001 in the presence of piperacillin at four times the MIC caused a delay in the recovery of the culture to piperacillin-induced stress. At 16 times the MIC, a complete suppression of regrowth occurred. Analysis of culture viability on piperacillin plates showed that the culture recovery was due to growth by moderately resistant mutants preexisting in the cell population, which at 16 times the MIC became susceptible to the combination. Evidence from the kinetics of inhibition of the *E. cloacae* 40001 AmpC β-lactamase by clavulanic acid, sulbactam, and tazobactam and from the effects of these drugs on the frequency of resistance to piperacillin suggests that the suppressive effect of tazobactam on the appearance of resistance is primarily mediated by the β-lactamase inhibitory activity.

Bacterial antibiotic resistance continues to be of grave concern to the control of infectious diseases (30). In the gram-negative enteric bacteria *Citrobacter freundii* and *Enterobacter cloacae*, the emergence of resistance to broad-spectrum penicillins and β-lactamase-stable cephalosporins occurs at a frequency of 10^{-7} to 10^{-6} (18, 20). This high frequency of resistance correlates with the presence of a chromosomal, inducible β-lactamase encoded by the *ampC* gene (8). Induction involves a transmembrane signaling pathway composed of the *ampG*, *ampD*, and *ampR* gene products and 1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid (1,6-anhydromurotripeptide) as the putative signal molecule (2, 12, 14, 31). Hyperproduction of AmpC β-lactamase is linked to mutations which destroy the function of AmpD (13, 17, 31).

One strategy in controlling β-lactamase-mediated resistance involves combination therapy with a β-lactam antibiotic and a β-lactamase inhibitor (3). Piperacillin, an expanded-spectrum penicillin, has been used in clinical practice since 1980 and is known to be hydrolyzed by several β-lactamases. Clavulanic acid, sulbactam, and tazobactam are β-lactamase inhibitors currently in clinical use and are important adjuvants to β-lactam chemotherapy. Tazobactam is a penicillanic acid sulfone with strong inhibitory activity against a wide range of commonly encountered plasmid-mediated β-lactamases (4, 25, 28). In vitro studies have shown that tazobactam potentiates the efficacy of piperacillin (1, 9, 25).

A recent study indicated that the combination of piperacillin plus tazobactam suppresses the emergence of resistant mutants in *C. freundii*, *E. cloacae*, and *Proteus vulgaris* in comparison to the suppression of the emergence of resistant mutants with elevated concentrations of piperacillin or ceftazidime alone (11). In this study we examined the mechanism of this potentiation. We reasoned that the suppression of resistance development by the combination of piperacillin plus tazobactam was mediated by interference with the induction of AmpC β-lactamase or by inhibition of the enzyme by tazobactam. Our results suggest that this effect is mediated by the inhibition of AmpC β-lactamase in moderately resistant mutants, present as a small fraction of the cell culture.

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MATERIALS AND METHODS

Strains and plasmids. The strains of *E. cloacae* and *C. freundii* used in this work are listed in Table 1. *Escherichia coli* BNN103 (Δ*lacIOPZYA*)*U169 proH*$^+$ *lon araD135 strA thi hflA150 cbr*::Tn*10* (laboratory collection) harbored plasmids pNU311 (*ampR*) and pNU330 (promoter region of *ampC* fused to a promoterless *lacZ* gene) (21) and was used to analyze the induction of *ampC* by β-lactam compounds. *E. coli* JRG582 is a K-12 derivative strain with an *ampDE* deletion, and *E. coli* JRG58201 is an *ampDG* double mutant constructed from the insertion of the APH cassette into the *ampG* gene of JRG582 (22).

β-Lactam compounds. The following β-lactam antibiotics and β-lactamase inhibitors were used: cefoxitin, from Sigma (St. Louis, Mo.); nitrocefin, from Microbiology Systems (Hunt Valley, Md.); and piperacillin, from Lederle Laboratories (Pearl River, N.Y.). Clavulanic acid, sulbactam, and tazobactam were kindly provided by Beecham Research Laboratories (Brentford, United Kingdom), Pfizer Inc. (Groton, Conn.), and SynPhar Laboratories Inc. (Edmonton, Alberta, Canada), respectively. 1,6-Anhydromurotripeptide was chemically synthesized by the Peptide Institute, (Osaka, Japan). All other chemicals were reagent grade.

Media and growth conditions. Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) (BBL Microbiology Systems, Cockeysville, Md.) were used for all growth experiments. Cultures for the extraction of 1,6-anhydromurotripeptide were grown in Luria-Bertani broth supplemented with vitamin B_1 (0.001%).

In vitro susceptibility testing. The MICs of the β-lactam compounds were determined by the agar dilution method. Plates were spotted with 10^5 CFU and were incubated for 18 h at 37°C.

Mutation frequency analysis. The frequency of occurrence of resistant mutants of *E. cloacae* and *C. freundii* was determined on solid medium supplemented with the appropriate concentrations of piperacillin, alone or in combination with a β-lactamase inhibitor (10 μg/ml). Plates were seeded with approximately 10^8 CFU of a fully grown culture and were incubated for 18 h at 37°C. The mutation frequency was expressed as the ratio of the numbers of CFU

TABLE 1. Piperacillin and tazobactam MICs for different isolates of *E. cloacae* and *C. freundii*

Strain[a]	MIC (μg/ml)[b]		Source
	PIPC	TAZ	
C. freundii NI-1	4	128	MS Laboratory Collection[c]
E. cloacae NI-2	2	256	MS Laboratory Collection
E. cloacae S-65	1	64	SynPhar Laboratory
E. cloacae 40001	4	512	SynPhar Laboratory
C. freundii S-40	4	128	SynPhar Laboratory
E. cloacae R538	32	128	Alberta Provincial Laboratory
E. cloacae ATCC 13047	16	128	Alberta Provincial Laboratory
C. freundii U3122	4	256	Alberta Provincial Laboratory
C. freundii R1791	4	256	Alberta Provincial Laboratory
C. freundii R3033	4	256	Alberta Provincial Laboratory

[a] *C. freundii* NI-1 and *E. cloacae* NI-2 were natural isolates, *E. cloacae* ATCC 13047 was from the American Type Culture Collection, and the other strains were clinical isolates.

[b] PIPC, piperacillin; TAZ, tazobactam.

[c] MS Laboratory Collection, University of Alberta Microbiology Student Laboratory Collection.

of resistant colonies formed per the total numbers of CFU plated on non-antibiotic-containing medium.

Killing kinetics. The killing kinetics of the *C. freundii* and *E. cloacae* strains treated with piperacillin alone or in combination with tazobactam were studied in liquid media with *E. cloacae* 40001 as a typical sensitive strain. An overnight culture of *E. cloacae* 40001 was subcultured to 1×10^7 to 5×10^7 CFU/ml into 15 ml of fresh MHB and was grown for 24 h under the following conditions: MHB without piperacillin (0P), MHB supplemented with piperacillin at 16 or 64 μg/ml (16P or 64P, respectively), and MHB supplemented with piperacillin at 16 or 64 μg/ml plus tazobactam at 10 μg/ml (16PT or 64PT, respectively). At regular intervals (typically, every 2 h) the turbidity of the cultures was measured with a Klett-Summerson colorimeter. Simultaneously, 0.5-ml samples of the same cultures were centrifuged at 8,000 rpm in a Costar minicentrifuge (Fisher, Edmonton, Alberta, Canada) for 10 min at 10°C, and the cells were resuspended in an equal volume of saline buffer plus MHB (4:1; vol/vol). This was repeated once, and 50 μl of washed cells, appropriately diluted in the same buffer, was plated onto solid medium under the five equivalent experimental conditions described above. Except for the initial time points, 1.5-ml samples of the cultures were washed as described above and were concentrated five times for the samples collected at the 2-h time point or 15 times for the samples collected at the 3-h time point, and then 50 μl of the cell suspension was plated onto β-lactam-supplemented medium. Under these conditions, the total viable counts of the cultures were determined on 0P plates, and the counts of the resistant mutants were determined on 16P, 16PT, 64P, and 64PT plates. The experiment was repeated twice, and the datum points are the means of quadruplicate values.

1,6-Anhydromurotripeptide analysis. The effect of tazobactam on the accumulation of 1,6-anhydromurotripeptide was analyzed by high-pressure liquid chromatography (HPLC) by the procedure described by Jacobs and coworkers (15), with slight modifications. Hot-water extracts of *E. coli* JRG582 cells (from 0.4 liter of culture) grown to the mid-logarithmic phase in Luria-Bertani broth supplemented with vitamin B_1 (0.001%), in the presence and absence of tazobactam, were prepared in a final volume of 0.5 ml and reduced with $NaBH_4$ as described previously (5). HPLC analysis of reduced hot-water extracts (50 μl) was performed with a Beckman gradient system on a Cosmosil 5C18 column (4.6 by 250 mm) with a linear gradient of from 0 to 30% solvent B (20% acetonitrile in 0.035% trifluoroacetic acid) in solvent A (0.05% trifluoroacetic acid) over a 50-min run, with a flow rate of 1.0 ml/min and at a column temperature of 40°C. Peaks were detected at 206 nm and integrated with Beckman Gold software. Under these conditions the standard, 1,6-anhydromurotripeptide, eluted with a retention time of 32.5 min. Quantitation of the amount of 1,6-anhydromurotripeptide produced by the cultures was derived from the peak area by using the chemically synthesized 1,6-anhydromurotripeptide as a standard.

Enzyme assays. Cell-free enzyme samples were obtained by disrupting the cells in a French press at 16,000 lb/in.^{2}. The kinetic parameters, the K_is of the three β-lactamase inhibitors for the AmpC β-lactamase produced by *E. cloacae* 40001, were determined spectrophotometrically at 490 nm following the hydrolysis of nitrocefin in 50 mM phosphate buffer (pH 7.0) (32). The inhibitor was preincubated with the enzyme at 37°C for 15 min. The 50% inhibitory concentration (IC_{50}) was calculated from the plot of percent inhibition versus concentration of inhibitor. β-Galactosidase activity was measured as described by Miller (26), with *o*-nitrophenyl-β-D-galactopyranoside used as the substrate, and the cells were preincubated with a β-lactam compound for 60 min.

RESULTS

Effect of tazobactam on the resistance of *E. cloacae* and *C. freundii* to piperacillin. To extend the earlier observations of Higashitani and co-workers (11), we examined a number of isolates of *C. freundii* and *E. cloacae* for resistance to piperacillin. The strains were sensitive to piperacillin, with MICs ranging from 1 to 32 μg/ml (Table 1). Incubation of approximately 10^8 CFU on plates containing increasing concentrations of piperacillin (up to 16 times the MIC) resulted in the appearance of resistant mutants at a frequency of 10^{-7} to 10^{-6} for most strains (Fig. 1). The combination of piperacillin plus tazobactam (10 μg/ml) resulted in the complete suppression of resistance at four times the MIC of piperacillin for the strains examined in this study. Sulbactam also showed a suppressive effect on the appearance of resistance, although not to the same extent as tazobactam. Clavulanic acid had little effect.

Analysis of tazobactam plus piperacillin as a negative effector of *ampC* induction. The possibility that tazobactam mediates its suppressive effect on resistance by interfering with the induction of AmpC β-lactamase was investigated by using an *ampC-lacZ* gene fusion in *E. coli* BNN103/pNU311 and pNU330. This strain contains the *ampR* gene combined with an *ampC-lacZ* transcriptional fusion. This allows the induction of *ampC* to be monitored by measuring the activity of β-galactosidase in β-lactam-treated cells. Figure 2 indicates the level of induction caused by piperacillin, alone or in combination with the β-lactamase inhibitors clavulanic acid, sulbactam, or ta-

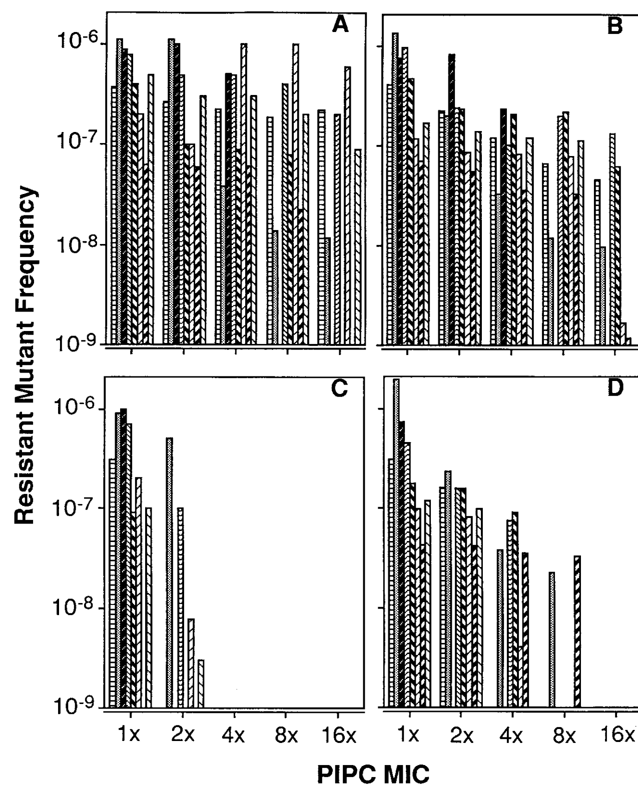

FIG. 1. Mutation frequencies of *E. cloacae* and *C. freundii* for resistance to piperacillin. The mutation frequencies of *E. cloacae* NI-2 (▥), *E. cloacae* S-65 (▨), *E. cloacae* 40001 (▤), *C. freundii* NI-1 (▤), *C. freundii* S-40 (▧), *C. freundii* U3122 (▨), *C. freundii* R1791 (▨), and *C. freundii* R3033 (▧) were determined in the presence of piperacillin (PIPC) alone (A), piperacillin plus clavulanic acid (10 μg/ml) (B), piperacillin plus tazobactam (10 μg/ml) (C), or piperacillin plus sulbactam (10 μg/ml) (D). The piperacillin concentration on the plates was a multiple of the MIC for each strain.

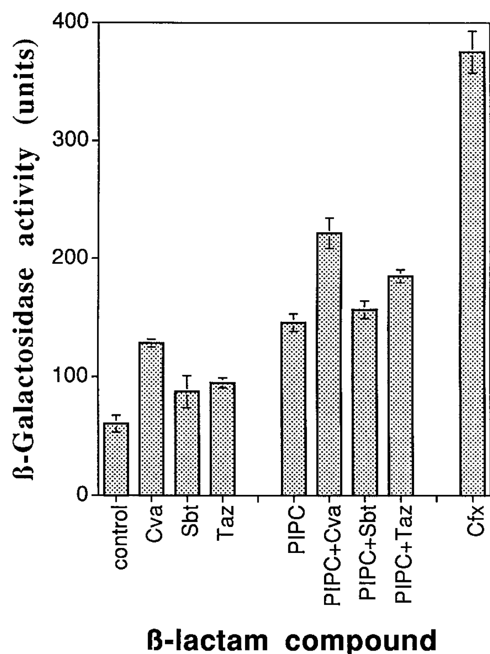

FIG. 2. Effects of piperacillin and different β-lactam inhibitors on the induction of *ampC-lacZ* fusion. Cells of *E. coli* BNN103/pNU311 and pNU330 were treated with different β-lactam compounds separately or in various combinations, and the effects of the compounds on the induction of *ampC* β-lactamase were monitored by measuring the β-galactosidase activity of the AmpC-β-galactosidase fusion protein. Cells were incubated at 30°C with shaking for 60 min in the presence of piperacillin (2 μg/ml) and/or a β-lactamase inhibitor (10 μg/ml) and cefoxitin (2 μg/ml). Control, untreated cells; Cfx, cefoxitin; Cva, clavulanic acid; Sbt, sulbactam; Taz, tazobactam; PIPC, piperacillin.

zobactam. Piperacillin and the β-lactamase inhibitors weakly induced AmpC expression in comparison to the level of induction caused by cefoxitin, a known good inducer, consistent with previous findings (35). The combination of piperacillin with tazobactam did not reduce the level of AmpC β-lactamase induced compared to the level of induction by either one of these compounds alone; rather, the level of induced enzyme was slightly increased. A similar effect was obtained with the combination of piperacillin with clavulanic acid. None of the combinations of piperacillin with the β-lactamase inhibitors tested had a suppressive effect on AmpC β-lactamase induction. We also measured the amount of β-lactamase produced directly by immunoelectrophoresis and found no evidence for the inhibition of β-lactamase expression by tazobactam in strains of *E. cloacae* or *C. freundii* (data not shown).

Tazobactam does not interfere with the accumulation of 1,6-anhydromurotripeptide. Hyperresistance has been correlated with defects in the *ampD* gene product leading to the accumulation of a peptidoglycan degradation product: 1,6-anhydromurotripeptide (13, 31, 33). This compound may be the signal molecule that interacts with the AmpR transcriptional regulator to activate *ampC* expression. To test if tazobactam interferes with the induction signal, we analyzed its effect on the intracellular accumulation of 1,6-anhydromurotripeptide in cultures of *E. coli* JRG582, a well-characterized *ampD* mutant (5, 15). HPLC analysis of hot-water extracts of cells incubated with or without tazobactam showed the 1,6-anhydromurotripeptide peak in both extracts. Estimation of the cellular content of 1,6-anhydromurotripeptide indicated that 0.6 and 0.5 μmol of this molecule were produced from 1 liter of culture in the absence and presence of tazobactam, respectively. These values were higher than that of 0.4 μmol reported by Jacobs and coworkers (14), presumably due to variations in the procedures for the isolation of the muropeptides. Within experimental error, the results suggested that tazobactam has no effect on the intracellular accumulation of this compound (Fig. 3). As expected, the 1,6-anhydromurotripeptide peak was undetectable from hot-water extracts of *E. coli* JRG58201, an *ampDG* double mutant strain which lacks functional AmpG, the permease for the transport of the ligand (14). The identity of 1,6-anhydromurotripeptide from *E. coli* JRG582 was further confirmed by electrospray mass spectrometry analysis of the isolated peak, which had a mass of 648.6, corresponding to that of a protonated form of 1,6-anhydromurotripeptide. It therefore appears that the suppressive effect of tazobactam on resistance is not mediated by inhibition of or interference with *ampC* induction.

Inhibition of β-lactamase activity correlates with suppression of resistant mutants in *E. cloacae* and *C. freundii*. Since

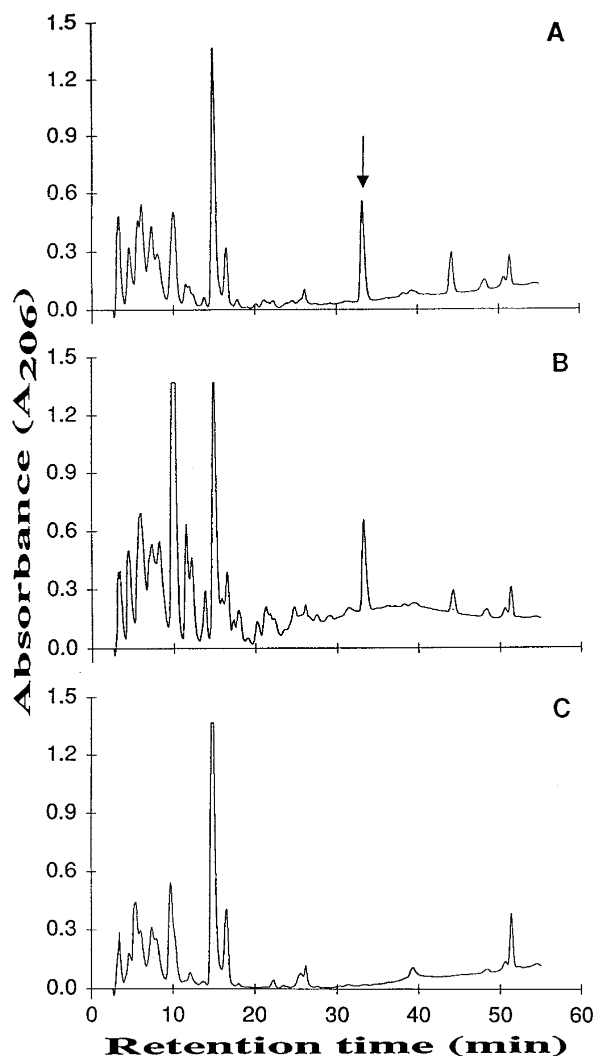

FIG. 3. HPLC analysis of the effect of tazobactam on the accumulation of 1,6-anhydromurotripeptide in *E. coli* JRG582. Hot-water extracts from *E. coli* JRG582 grown in the absence (A) and presence (B) of tazobactam at 10 μg/ml and from *E. coli* JRG58201 (C) were tested. The arrow indicates the 1,6-anhydromurotripeptide peak, with a retention time of 32.5 min. The other major peak in panel B, with a retention time of 10.2 min, represents tazobactam.

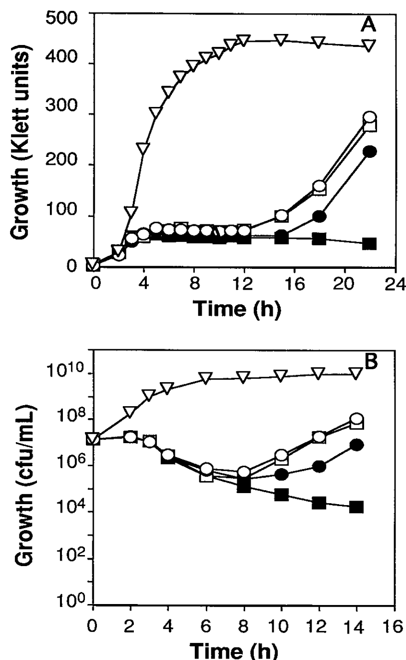

FIG. 4. Effect of tazobactam on the susceptibility kinetics of *E. cloacae* 40001 to piperacillin. Cultures of *E. cloacae* 40001 (1.3×10^7 CFU/ml) were incubated in MHB for several hours at 37°C with shaking under the following conditions: 0P (▽), 16P (○), 64P (□), 16PT (●), and 64PT (■). Growth was monitored by measuring the turbidities of the cultures (A) and the total viable counts on piperacillin-free MHA plates (B).

concentrations of 4 or 16 times the MIC for *E. cloacae* 40001 were essentially identical. The addition of tazobactam delayed by 3 h the recovery of the culture to stress induced by penicillin at four times the MIC but completely suppressed recovery at 16 times the MIC. In fact, the viable counts continued to decrease over the time course of the experiment, with approximately 10^4 CFU/ml remaining after 14 h.

Changes in cell population dynamics due to piperacillin resistance were monitored by determining the viable counts on plates containing piperacillin at 16 μg/ml. Under these conditions, resistant colonies were detected earlier in the experiment (at 2 h), and the counts of resistant cells paralleled the growth profile thereafter (Fig. 5A). The number of resistant cells increased exponentially from less than 10^2 CFU/ml at 2 h to 10^8 CFU/ml after 14 h of incubation in liquid medium supplemented with piperacillin. In cultures subjected to stress induced by piperacillin (four times the MIC) plus tazobactam, resistant colonies were also detected as early as 2 h, and the growth kinetics were similar to those in the absence of tazobactam. Viable cell counts indicated that at this time, the number of resistant mutants was approximately five times greater in cultures treated with piperacillin alone (16P) than in those treated with the combination (16PT). No recovery was observed at 16 times the MIC in the presence of tazobactam (64PT), although a few resistant mutants were detected at 2 h, but they succumbed to prolonged incubation under these conditions. The numbers of viable cells were also estimated on plates supplemented with both piperacillin and tazobactam, and the results indicated further delays in culture recovery (Fig. 5B). Once viable cells were detected, the growth profile paralleled that seen in the absence of tazobactam. This result the suppression of resistance to piperacillin by tazobactam did not involve a mechanism affecting the induction of β-lactamase, we investigated its role as a β-lactamase inhibitor. Tazobactam and sulbactam suppressed the appearance of resistant mutants, whereas clavulanic acid lacked this effect. The former compounds are penicillanic sulfones with activity profiles different from that of clavulanic acid. We examined the kinetics of inhibition of the β-lactamase from *E. cloacae* 40001 by these three inhibitors. K_i values of 302, 5.65, and 0.86 μM were determined for clavulanic acid, sulbactam, and tazobactam, respectively, indicating that tazobactam is a much better inhibitor of AmpC β-lactamase from *E. cloacae* 40001 than the other two compounds. The IC_{50}s of the three β-lactamase inhibitors (206, 2.72, and 0.49 μM for clavulanic acid, sulbactam and tazobactam, respectively) supported the same conclusion. Similar inhibition profiles were obtained with the enzyme from *C. freundii* NI-1 (data not shown).

Effect of tazobactam on susceptibility of *E. cloacae* 40001 to piperacillin. To examine the hypothesis that tazobactam acts by extending the efficacy of piperacillin, we carried out a study on the growth kinetics of *E. cloacae* 40001 in liquid culture in the presence of piperacillin alone or in combination with tazobactam. The MIC of piperacillin for this organism is 4 μg/ml. An overnight liquid culture of *E. cloacae* 40001 was inoculated into fresh MHB medium containing 0P, 16P, 64P, 16PT, or 64PT. The cultures were incubated at 37°C with shaking, and viable counts and turbidities were monitored over a 24-h period. On the basis of turbidity measurements, all cultures treated with piperacillin except those treated with 64PT recovered from its growth inhibitory effect within the 24-h incubation (Fig. 4A). Figure 4B indicates the total viable counts determined by the standard procedure of plating samples onto antibiotic-free medium. The killing curves of piperacillin at

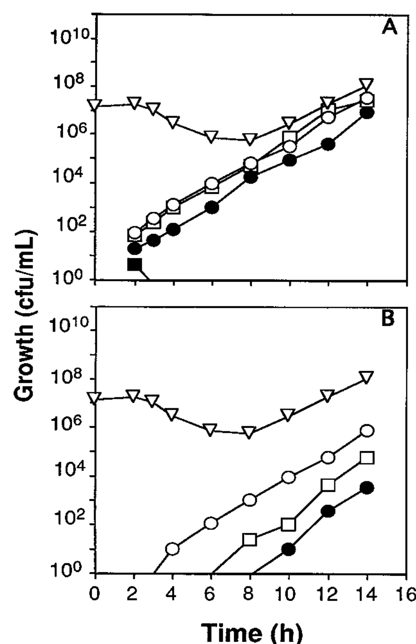

FIG. 5. Effect of tazobactam on the growth kinetics of moderately resistant mutants of *E. cloacae* 40001 in the presence of piperacillin. Cultures of *E. cloacae* 40001 were incubated as described in the legend to Fig. 4. Viable counts of resistant mutants from cultures with 16P (○), 16PT (●), 64P (□), and 64PT (■) were determined on MHA plates containing piperacillin at 16 μg/ml (A) or piperacillin at 16 μg/ml plus tazobactam at 10 μg/ml (B). The total viable counts of the culture with 16P (▽) were determined on piperacillin-free MHA plates as described for Fig. 4B, shown here for comparison purposes.

TABLE 2. Susceptibility analysis of piperacillin-resistant mutants of *E. cloacae* 40001

No. of resistant colonies[a]	Growth medium[b]		MIC (μg/ml)[c]	
	Initial	Final	PIPC	PIPC + TAZ
6 (6)	ON	16P	64	16
2 (2)	0P	16P	64	16
3 (3)	16P	16P	64	16
3 (3)	16PT	16P	64	16
2 (2)	64P	16P	64	16
2 (2)	64PT	16P	64	16
1 (2)	0P	16PT	64	16
1	0P	16PT	512	128
1 (1)	16P	16PT	128	32
4 (5)	16PT	16PT	64	16
1	16PT	16PT	256	64
1 (3)	64P	16PT	64	16
2	64P	16PT	256	64
5 (6)	0P	64P	64	16
1	0P	64P	256	32
3 (3)	64P	64P	64	16

[a] The numbers in parentheses refer to the total number of resistant colonies tested under the described growth conditions.

[b] Initial growth medium refers to the growth conditions of liquid cultures in MHB supplemented with piperacillin with or without tazobactam, from which samples were removed at regular intervals and plated onto MHA plates supplemented with piperacillin with or without tazobactam as indicated in the column describing final growth conditions. ON, overnight culture in MHB. The overnight culture was subcultured at 1.3×10^7 CFU/ml into fresh MHB (0P) or 16P, 16PT, 64P, or 64PT.

[c] The MIC of piperacillin (PIPC) alone or in combination with tazobactam (TAZ) at 10 μg/ml was determined by spotting 10^5 CFU of resistant mutants isolated from the antibiotic-containing plates per 10 μl (see final growth conditions).

indicates either that the combination was more bacteriostatic or that there were fewer viable cells to initiate recovery.

To address this question, the susceptibilities of a number of resistant colonies to piperacillin were analyzed. Attempts were made to select resistant cells detected in the earlier phase of the piperacillin killing curve to minimize the possibility of testing the same resistant cells following multiple cell division cycles. As indicated above, the majority of resistant mutants detected at between 0 and 4 h of incubation in liquid medium were isolated from samples cultured on 16P plates. Other growth conditions, including growth on 16PT, 64P, and 64PT plates, were more inhibitory. MIC analysis revealed that, regardless of the incubation condition in liquid medium, the resistant colonies from 16P plates were identically susceptible to piperacillin (Table 2). For all of the 18 colonies tested the piperacillin MIC was 64 μg/ml, and the colonies were 16 times more resistant than the parent strain, *E. cloacae* 40001. The addition of tazobactam (10 μg/ml) to piperacillin decreased the MIC to 16 μg/ml. No such synergistic effect was observed with the parent strain, *E. cloacae* 40001, as also reported previously (11). Resistant colonies obtained from the plating of liquid culture samples on 16PT plates were also analyzed. Noteworthy is that very few mutant colonies were detected at between 0 and 4 h of incubation in liquid medium under these conditions. As a result, resistant colonies that appeared in the later phase of the piperacillin killing curve were also tested for their resistance levels. The results indicated that this group of resistant colonies was rather heterogeneous in its susceptibility to piperacillin. Of the 11 colonies analyzed, for 5 the piperacillin MIC was greater than 64 μg/ml and went up to 512 μg/ml. Resistant colonies obtained from the plating of liquid culture samples on 64P plates were analyzed as well, and these colonies were found to have a piperacillin susceptibility profile that resembled more those of resistant cells from 16P plates than those from 16PT plates, implying that a higher degree of resistance was required to overcome the stress induced by piperacillin plus tazobactam. On the basis of the killing curves, tazobactam causes a decrease in the number of resistant mutants in the cell population that are able to initiate growth in the presence of piperacillin, and this effect increases with increasing piperacillin concentration.

DISCUSSION

β-Lactamase production is a major determinant of bacterial resistance to β-lactam antibiotics (3, 16, 19). Gram-negative bacilli producing chromosomal AmpC β-lactamases (*C. freundii*, *E. cloacae*, *Pseudomonas aeruginosa*, and *Serratia marcescens*) are responsible for a significant proportion of cases of nosocomial pneumonia and bacteremia (10). In these bacteria, chromosomal AmpC β-lactamase is inducible in wild-type strains, but constitutive hyperproduction of the enzyme occurs in resistant strains that develop at a high frequency. The analysis of clinical strains of *Citrobacter* spp. indicated that the high frequency of resistance to cephalosporins occurred only in strains possessing a β-lactamase that can be induced (8). One of the approaches used to overcome the problem of β-lactamase-mediated resistance has relied on combination therapy of a β-lactam antibiotic and a β-lactamase inhibitor (1, 25). Tazobactam, a recently introduced β-lactamase inhibitor, is used in combination with piperacillin or other antibiotics to which β-lactamase-producing bacteria are susceptible (9).

In this study, several piperacillin-sensitive isolates of *E. cloacae* and *C. freundii* were analyzed for resistance and were found to mutate at a frequency of 10^{-7} to 10^{-6}, consistent with previous findings (8, 18, 20). A combination of tazobactam with piperacillin at concentrations 16 times the MIC proved to be effective in reducing the frequency of resistant mutants. A similar effect was reported by Higashitani and coworkers (11). We investigated whether tazobactam in combination with piperacillin affected the induction of β-lactamase or whether the lower frequency of resistant mutants resulted from increased efficacy of the antibiotic as a result of the presence of the inhibitor.

The AmpC β-lactamase can be induced to various levels by a number of β-lactam compounds (24, 27, 35). Studies investigating the mechanism of induction have shown that the β-lactam compounds do not enter the cytoplasm to induce *ampC* expression; rather, they mediate their effect through muropeptides derived from the degradation of peptidoglycan (12, 14, 31). As a first possibility, we reasoned that tazobactam may exert its effect by interfering with the induction of AmpC β-lactamase. This hypothesis was tested by monitoring the promoter activity of an inducible *ampC* gene in an *ampC-lacZ* gene fusion system in *E. coli* and by analyzing the β-lactamase activity and protein levels in *C. freundii* and *E. cloacae* under inducing and noninducing conditions. Our results indicate that both tazobactam and piperacillin are individually weak inducers of AmpC and that use of the two drugs in combination resulted in additive induction. Recent characterization of the putative signal molecule as 1,6-anhydromurotripeptide has made it possible to analyze the effect of tazobactam on the induction signal transduction pathway. *ampD* mutants of *E. coli* transport the precursor muropeptides from the periplasm via AmpG and accumulate 1,6-anhydromurotripeptide intracellularly (14). Loss of functional AmpG blocks signal transduction, with a consequent lack of accumulation of 1,6-anhydromurotripeptide. Since tazobactam, like other β-lactam

compounds, remains in the periplasm, a possible influence of tazobactam on induction could be monitored by the uptake or the intracellular accumulation of 1,6-anhydromurotripeptide in *ampD* strains. No evidence of interference with the accumulation of this molecule by tazobactam was obtained. These studies suggest that tazobactam does not exhibit an induction inhibitory activity on AmpC β-lactamase in piperacillin-stressed cells.

The second possibility is that tazobactam increases the efficacy of piperacillin by inhibiting β-lactamase. The β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam are strong inhibitors of extended-spectrum β-lactamases, with only weak or no activity against cephalosporinases (9, 28). Generally, these compounds inactivate the enzyme irreversibly by forming covalent complexes which resist hydrolysis (23). Of the three inhibitors tested, tazobactam exhibited the highest activity against the cephalosporinase from *E. cloacae* 40001. Similar inhibition profiles were observed for the chromosomal β-lactamase from *Morganella morganii* 985 and *E. cloacae* 772 (28). Although the inhibitory activity of tazobactam may be small in comparison to that shown against penicillinases (4), it is nevertheless significant and is responsible for the delay in the appearance of resistant cells. Unlike tazobactam, clavulanic acid was by far the poorest inhibitor of the *E. cloacae* 40001 enzyme and was also unable to suppress the piperacillin resistance of this strain or the other strains studied. The structural difference between clavulanic acid and the penicillanic sulfones, tazobactam and sulbactam, accounts for the observed effects.

The susceptibility of bacteria producing inducible AmpC β-lactamase is influenced by several factors, including the magnitude of enzyme production, the rate of penetration of the drug to its target, and the stability of the drug to β-lactamase (31). The combination of these factors partly accounts for the different frequencies of resistant mutants observed among the various isolates of *C. freundii* and *E. cloacae* analyzed in this study. The analysis of denser liquid cultures of *E. cloacae* 40001 indicated that the response of this organism to piperacillin was typical of that of gram-negative bacteria, with an initial killing phase followed by regrowth. The cell density used in this study was evidently much higher than that recommended for MIC determinations by conventional tests, but it arguably represents the in vivo situation when the bacteria are exposed to the drug in the course of the infection. In addition, it facilitates the detection of resistant mutants that emerge at a frequency of 10^{-7} to 10^{-6}, thus allowing the resistance suppressive effect of tazobactam to be monitored.

The regrowth by members of the family *Enterobacteriaceae* at large inoculum size has previously been reported to be due to either drug destruction by β-lactamases or filamentous transformation with continuous growth (6, 7). We found that neither of these factors contributed significantly to the recovery of the culture, which results from the spectrum of resistance of the bacteria in the culture. While most cells are sensitive to the antibiotic, a small fraction will have a range of resistance. Plating on antibiotic-containing medium indicated that, indeed, the regrowth was primarily due to resistant cells. Cultures incubated with piperacillin alone (4 or 16 times the MIC) selected cells with moderate levels of resistance. Higashitani and coworkers (11) analyzed the levels of β-lactamase produced by resistant cells of *E. cloacae* 40001 isolated from cultures treated with piperacillin alone or in combination with tazobactam. Resistant cells isolated from a mouse intraperitoneal infection model were β-lactamase-derepressed mutants, and all of them were obtained following treatment with piperacillin alone and not with the combination of piperacillin with tazobactam. The levels of enzyme produced ranged from 4.9 to 8.8 U/mg of protein, which corresponded to an increase of up to 1,000 times that of the parent strain. The few resistant mutants isolated from cultures treated with both piperacillin and tazobactam in vitro produced 2,800 times more β-lactamase than the parent strain, which supports our finding that the combination selects for highly resistant cells. Enhancement of the efficacy of piperacillin by combining it with tazobactam resulted in the killing of the moderately resistant cells at relatively low levels of piperacillin (four times the MIC), while highly resistant cells survived. The highest level of piperacillin tested (16 times the MIC plus 10 μg of tazobactam per ml) was sufficient to kill even the most resistant cells in this culture. Our results suggest that nosocomial infections caused by β-lactam-resistant bacteria can be prevented by using increased concentrations of piperacillin in combination with tazobactam, thus minimizing the problem of the development of resistance associated with the use of β-lactam antibiotics alone (29, 34).

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REFERENCES

1. **Acar, J. F., F. W. Goldstein, and M. D. Kitzis.** 1993. Susceptibility survey of piperacillin alone and in the presence of tazobactam. J. Antimicrob. Chemother. **31**(Suppl. A)**:**23–28.
2. **Bartowsky, E., and S. Normark.** 1993. Interaction of wild-type and mutant AmpR of *Citrobacter freundii* with target DNA. Mol. Microbiol. **10:**555–565.
3. **Bush, K.** 1988. β-Lactamase inhibitors from laboratory to clinic. Clin. Microbiol. Rev. **1:**109–123.
4. **Bush, K., C. Macalintal, B. A. Rasmussen, V. J. Lee, and Y. Yang.** 1993. Kinetic interactions of tazobactam with β-lactamases from all major structural classes. Antimicrob. Agents Chemother. **237:**851–858.
5. **Dietz, H., D. Pfeifle, and B. Wiedemann.** 1996. Location of *N*-acetylmuramyl-L-alanyl-D-glutamylmesodiaminopimelic acid, presumed signal molecule for β-lactamase induction in bacteria cell. Antimicrob. Agents Chemother. **40:** 2173–2177.
6. **Eng, R. H. K., C. E. Cherubin, S. M. Smith, and F. Buccini.** 1985. Inoculum effect of β-lactam antibiotics on *Enterobacteriaceae*. Antimicrob. Agents Chemother. **28:**601–606.
7. **Goldstein, E. J., D. M. Citron, and C. E. Cherubin.** 1991. Comparison of the inoculum effects of members of the family *Enterobacteriaceae* on cefoxitin and other cephalosporins, β-lactamase inhibitor combinations, and the penicillin-derived components of these combinations. Antimicrob. Agents Chemother. **35:**560–566.
8. **Gootz, T. D., D. B. Jackson, and J. C. Sherris.** 1984. Development of resistance to cephalosporins in clinical strains of *Citrobacter* spp. Antimicrob. Agents Chemother. **25:**591–595.
9. **Gutmann, L., M. D. Kitzis, S. Yamabe, and J. F. Acar.** 1986. Comparative evaluation of a new β-lactamase inhibitor, YTR 830, combined with different β-lactam antibiotics against bacteria harboring known β-lactamases. Antimicrob. Agents Chemother. **29:**955–957.
10. **Haley, R. W., D. H. Culver, J. W. White, W. M. Morgan, and T. G. Emori.** 1985. The nationwide nosocomial infection rate: a new need for vital statistics. Am. J. Epidemiol. **121:**159–167.
11. **Higashitani, F., K. Nishida, A. Hyodo, and M. Inoue.** 1995. Effects of tazobactam on the frequency of the emergence of resistant strains from *Enterobacter cloacae*, *Citrobacter freundii*, and *Proteus vulgaris*. J. Antibiot. **48:** 1027–1033.
12. **Höltje, J.-V., U. Kopp, A. Ursinus, and B. Wiedemann.** 1994. The negative regulator of β-lactamase induction AmpD is a *N*-acetyl-anhydromuramyl-L-alanine amidase. FEMS Microbiol. Lett. **122:**159–164.
13. **Honoré, N., M. H. Nicolas, and S. Cole.** 1989. Regulation of enterobacterial cephalosporinase production: the role of a membrane-bound sensory transducer. Mol. Microbiol. **3:**1121–1130.
14. **Jacobs, C., L.-J. Huang, E. Bartowsky, S. Normark, and J. T. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J. **13:**4684–4694.
15. **Jacobs, C., B. Joris, M. Jamin, K. Klarsov, J. van Beeumen, D. Mengin-Lecreulx, J. van Heijenoort, J. T. Park, S. Normark, and J. M. Frère.** 1995. AmpD, essential for both β-lactamase regulation and cell wall recycling, is a novel cytosolic *N*-acetylmuramyl-L-alanine amidase. Mol. Microbiol. **15:**553–559.
16. **Jarlier, V., M. H. Nicolas, G. Fournier, and A. Philippon.** 1988. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in *Enterobacteriaceae*: hospital prevalence and suscepti-

bility patterns. Rev. Infect. Dis. **10:**867–878.

17. **Korfmann, G., C. C. Sanders, and E. S. Moland.** 1991. Altered phenotypes associated with *ampD* mutations in *Enterobacter cloacae*. Antimicrob. Agents Chemother. **35:**358–364.
18. **Lampe, M. F., B. J. Allan, B. H. Minshew, and J. C. Sherris.** 1982. Mutational enzymatic resistance of enterobacter species to beta-lactam antibiotics. Antimicrob. Agents Chemother. **21:**655–660.
19. **Lindberg, F., and S. Normark.** 1986. Contribution of chromosomal β-lactamases to β-lactam resistance in enterobacteria. Rev. Infect. Dis. **8**(Suppl. 3)**:**S292–S304.
20. **Lindberg, F., L. Westman, and S. Normark.** 1985. Regulatory components in *Citrobacter freundii ampC* β-lactamase induction. Proc. Natl. Acad. Sci. USA **82:**4620–4624.
21. **Lindquist, S., F. Lindberg, and S. Normark.** 1989. Binding of the *Citrobacter freundii* AmpR regulator to a single DNA site provides both autoregulation and activation of the inducible *ampC* β-lactamase gene. J. Bacteriol. **171:** 3746–3753.
22. **Lindquist, S., K. Weston-Hafer, H. Schmidt, C. Piel, G. Korfmann, J. Erickson, C. Sanders, H. H. Martin, and S. Normark.** 1993. AmpG, a signal transducer in chromosomal β-lactamase induction. Mol. Microbiol. **9:**703–715.
23. **Livermore, D. M.** 1993. Determinants of the activity of β-lactamase inhibitor combinations. J. Antimicrob. Chemother. **31**(Suppl. A)**:**9–21.
24. **Livermore, D. M., M. Akova, P. Wu, and Y. Yang.** 1989. Clavulanate and β-lactamase induction. J. Antimicrob. Chemother. **24**(Suppl. B)**:**23–33.
25. **Livermore, D. M., and P. Seetulisingh.** 1991. Susceptibility of *Escherichia coli* isolates with TEM-1 β-lactamase to combinations of BRL42712, tazobactam or clavulanate with piperacillin or amoxycillin. J. Antimicrob. Chemother. **27:**761–767.
26. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Minami, S., A. Yotsuji, M. Inoue, and S. Mitsuhashi.** 1980. Induction of beta-lactamase by various beta-lactam antibiotics in *Enterobacter cloacae*. Antimicrob. Agents Chemother. **18:**382–385.
28. **Moosdeen, F., J. D. Williams, and S. Yamabe.** 1988. Antimicrobial characteristics of YTR 830, a sulfone β-lactamase inhibitor, compared with those of clavulanic acid and sulbactam. Antimicrob. Agents Chemother. **32:**925–927.
29. **Mouton, Y., C. Beuscart, and C. Soussy.** 1986. Efficacité et tolérance de la pipéracilline chez 333 malades. Presse Med. **15:**2347–2350.
30. **Neu, H. C.** 1992. The crisis in antibiotic resistance. Science **257:**1064–1073.
31. **Normark, S., E. Bartowsky, J. Erickson, C. Jacobs, F. Lindberg, S. Lindquist, K. Weston-Hafer, and M. Wikström.** 1994. Mechanism of chromosomal β-lactamase induction in gram-negative bacteria, p. 485–503. *In* G. J.-M. and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Science B.V., Amsterdam, The Netherlands.
32. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for the detection of β-lactamase by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1:**283–288.
33. **Park, J. T.** 1995. Why does *Escherichia coli* coli recycle its cell wall peptides? Mol. Microbiol. **17:**421–426.
34. **Sanders, C. C., and W. E. Sanders.** 1983. Emergence of resistance during therapy with the newer β-lactam antibiotics: role of inducible β-lactamases and implications for the future. Rev. Infect. Dis. **5:**639–648.
35. **Weber, D. A., and C. C. Sanders.** 1990. Diverse potential of β-lactamase inhibitors to induce class I enzymes. Antimicrob. Agents Chemother. **34:** 156–158.