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# Dissociated Resistance Among Cephalosporins

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When disks containing cefuroxime (CXM) or HR 756 (756) were placed next to cefoxitin (FOX) on plates inoculated with *Enterobacter* resistant to FOX, there was marked flattening of the zones of inhibition produced by CXM or 756, but colonies growing near the CXM or 756 disks were found to be fully susceptible to these drugs on retesting. In contrast, when a checkerboard titration with CXM or 756 and FOX was done in broth, organisms growing in the presence of high concentrations of either of the former plus FOX were found to be resistant on retesting. Broth cultures of *Enterobacter* were found to contain small numbers of mutants which were resistant to and inactivated CXM and 756. Evidence is presented suggesting that the addition of FOX induces similar properties in the whole culture, which then becomes resistant to all of the nine cephalosporins tested and to four of six penicillins tested. Such cultures rapidly reverted to susceptible when FOX was removed or inactivated, and an explanation is offered as to why this did not happen in fluid media.

In a recent study at the Whittington Hospital, 900 strains of *Enterobacter* and *Klebsiella* isolated from urine during the past 4 years were tested for susceptibility to cefuroxime (CXM), cefoxitin (FOX), and HR 756 (756) by the disk method. The Stokes method (7) was used. An 85-mm plate was divided horizontally into three parts, the test organism was applied to the center band, and an *Escherichia coli* control was applied to the areas above and below, leaving 2 to 3 mm between adjacent parts. Disks were then placed in this space so that the zones of inhibition were formed half in the unknown and half in the control, making direct comparison easy. By chance CXM and FOX were placed next to each other on one side of the test organism, and it was observed that strains of *Enterobacter* which were resistant to FOX showed marked antagonism between FOX and CXM; the control zones were normal. This effect was readily reproducible and is illustrated in Fig. 1. A similar phenomenon was seen at about the same time at University College Hospital, where a chance observation showed a similar antagonism between 756 and CXM with a strain of *Proteus morganii* which was resistant to the latter. A similar effect between azlocillin and FOX against *Pseudomonas aeruginosa* has been described recently by Kamme and Neuhaus (4). This paper reports an investigation of these effects.

## MATERIALS AND METHODS

Most of the experimental work was done with a single strain of *Enterobacter aerogenes* isolated from urine (Ent 599). The other organisms used were either from the Whittington Hospital collection of urinary isolates or from collections of previous clinical isolates maintained at University College Hospital.

Combined action was originally tested with disks containing 30 μg of the relevant cephalosporin, or, if no disk was available, a punched-out well was filled with a 1,000-μg/ml solution. As the spacing of the disk was obviously very critical, a screening test was devised in which plates were prepared which contained doubling dilutions of CXM or 756. Diluted broth cultures were then streaked across these plates, and blotting paper strips soaked in 1,000-μg/ml solutions of CXM or FOX were placed at right angles across the streaks. Growth only at the side of the strip indicated antagonism, and a measurement of its extent was obtained from the number of dilutions of CXM or 756 above the dilution normally inhibiting the organism on which growth appeared.

Viable counts were performed by preparing serial decimal dilutions in 5-ml volumes and placing 2 drops (0.02 ml each) from each dilution around a well-dried plate.

Assays were done by the plate diffusion method using *Sarcina lutea* as the test organism.

Antibiotic inactivation was also tested for by the cloverleaf method (5). Agar plates were flooded with a suspension of *S. lutea*, the culture to be tested was heavily streaked across the inoculated plate to form a cross, and a disk containing the drug was placed in the center of the cross. If the antibiotic is inactivated, growth of *S. lutea* occurs along the streak inside the inhibition zone, giving this a cloverleaf shape.

Checkerboard titrations were done by preparing a range of doubling dilutions of both drugs singly and in every possible combination either in 2-ml volumes of broth or on agar plates.

Oxoid Isosensitest broth or agar was used throughout. CXM was supplied by Glaxo, FOX was supplied

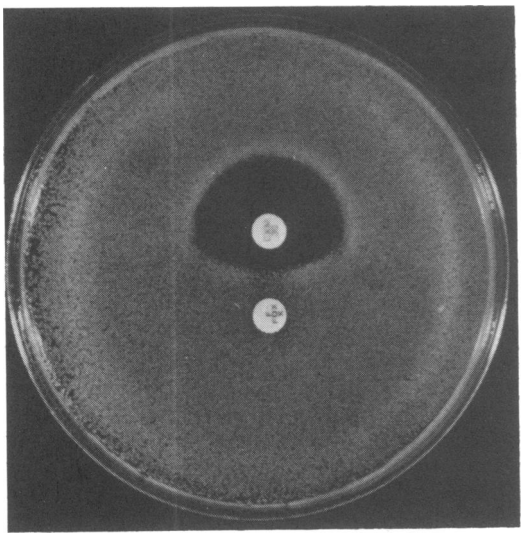

FIG. 1. *Ent 599 on Isosensitest agar. Disks contained 30 μg of FOX (lower disk) and 30 μg of CXM (upper disk).*

by Merck Sharp & Dohme, and 756 was supplied by Roussel.

## RESULTS

All strains of *Enterobacter* which gave little or no zone of inhibition around a disk containing 30 μg of FOX (46 strains in all) showed marked flattening of the inhibition zone produced by a disk containing 30 μg of either CXM or 756 (Fig. 1), but when colonies were taken from within the CXM or 756 zone and retested, they were found to be normally susceptible to these drugs. However, when checkerboard titrations were done in broth with an inoculum of $10^6$ organisms per ml, little or no antagonism was seen, and when tubes showing growth in the presence of both drugs were subcultivated, the subcultures were found to be resistant to CXM (or 756). As the minimal inhibitory concentrations (MIC) of CXM or 756 alone were considerably higher than expected, it seemed likely that the original strain contained a proportion of resistant mutants. A viable count of an overnight broth culture of Ent 599 was then done on plates containing doubling dilutions of CXM. It was found that, although an inoculum of $10^3$ organisms per ml was inhibited by 2 μg/ml, when the inoculum was $10^6$ organisms per ml or more, single colonies appeared in all concentrations up to 64 μg/ml, indicating a mutation frequency of $10^{-6}$ or $10^{-7}$. These organisms were uniformly resistant on subculture. Similar results were obtained with 756, and organisms selected from either CXM or 756 were resistant to both.

An overnight broth culture of the same organism was then counted on an agar checkerboard containing dilutions of CXM and FOX. This showed that the addition of 4, 8, or 16 μg of FOX per ml permitted growth of the entire population on plates containing 32 μg of CXM per ml; even 1 to 2 μg of FOX per ml raised the MIC of CXM to 16 μg/ml and enabled about 40% of the population to grow on 32 μg/ml. A selection of colonies taken from plates containing higher concentrations of CXM plus FOX were all found to be susceptible to CXM.

When the effect of inoculum size was tested in broth, the MIC of CXM was 4 μg/ml when the inoculum was $10^4$ organisms per ml or less but rose to 32 μg/ml when the inoculum was $10^6$ organisms per ml, and skipped tubes were sometimes seen. When 4 μg of FOX per ml was added to CXM dilutions inoculated with $10^4$ organisms per ml, the MIC rose to 32 μg/ml, and organisms from concentrations above the normal MIC were found on subculture to be resistant to CXM.

In view of the similarity of the MIC of CXM in the presence of FOX to that of the naturally occurring resistant mutants, four more strains of *Enterobacter*, together with Ent 599, were tested by the screening method described above; this is illustrated in Fig. 2. The MICs of CXM, 756, and FOX for these strains were also determined by the plate dilution method by using inocula of $10^7$ and $10^3$ organisms per ml. The results of both experiments are given in Table 1; all strains

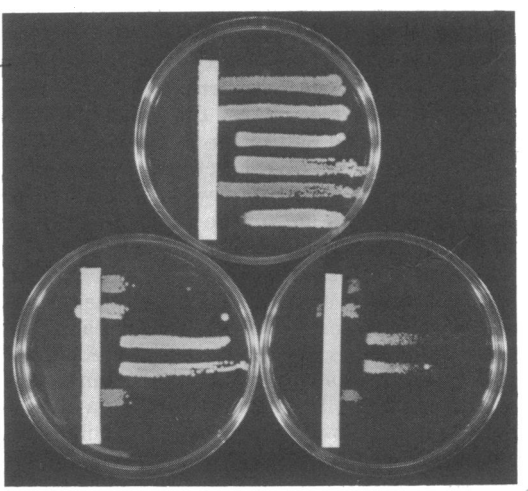

FIG. 2. *Screening test for detecting antagonism between CXM or 756 and FOX. Plates contained no antibiotic (top), 2 μg of CXM per ml (lower left), 32 μg of CXM per ml (lower right). Diluted cultures of five strains of Enterobacter and one Klebsiella strain (bottom streak) were streaked across the plates, and blotting paper strips soaked in a solution of FOX containing 1,000 μg/ml were placed across the streaks.*

TABLE 1. *Comparison of the concentrations of CXM and 756 in agar inhibiting the growth of an inoculum of $10^3$ organisms per ml, the resistant mutants appearing from an inoculum of $10^7$ organisms per ml, and an inoculum of $10^3$ organisms per ml with FOX added in blotting paper strips soaked in a solution containing 1,000 μg/ml*[a]

| *Enterobacter* strain | MIC of CXM (μg/ml) with an inoculum of: | | | MIC of 756 (μg/ml) with an inoculum of: | | | MIC of FOX (μg/ml) with an inoculum of: | |
|---|---|---|---|---|---|---|---|---|
| | $10^3$ organisms/ml | $10^7$ organisms/ml | $10^3$ organisms/ml + FOX | $10^3$ organisms/ml | $10^7$ organisms/ml | $10^3$ organisms/ml + FOX | $10^3$ organisms/ml |
| 599 | 2 | 64 | >64 | 0.03 | 4 | 2 | >128 |
| 543 | 1 | 32 | 32 | 0.06 | 8 | 4 | 64 |
| 551 | 16 | 64 | 64 | 0.12 | 0.5 | 0.5 | 8 |
| 549 | 16 | >128 | 64 | 0.12 | 0.5 | 0.5 | 8 |
| 635 | 1 | 64 | 64 | 0.12 | 4 | 4 | 128 |

[a] See Fig. 2.

show a marked correlation between the naturally occurring and the induced resistance to both CXM and 756. Although the MIC of FOX for these strains varied, none was fully susceptible, and, unfortunately, no strain identified as an *Enterobacter* has been found which is more sensitive to FOX than those in Table 1.

**Rate of growth in the presence of CXM plus FOX.** The emergence of resistance was then studied by performing serial viable counts of Ent 599 growing in the presence of one or both drugs, all counts being done in duplicate on agar with and without 16 μg of CXM per ml.

The effect of CXM alone is shown in Fig. 3. When the inoculum was $5 \times 10^5$ organisms per ml, the total count fell to $10^3$ cells per ml in 6 h. Resistant colonies first appeared at 4 h and increased logarithmically thereafter, the counts on both plates being similar after 6 h. When the inoculum was $5 \times 10^3$ organisms per ml, the count fell during the first 6 h and then showed little further change, and resistance did not appear.

Figure 4 shows that when the medium contained both 16 μg of CXM per ml and 8 μg of FOX per ml, the count changed little during the first 4 h and then rose parallel to that of the tube containing FOX only (which was almost identical to the drug-free control) until 10 h, after which the total count remained stationary for at least 5 h. Resistant organisms first appeared in the control and in cultures containing FOX only at 6 h but remained at a low level throughout. In the presence of both drugs, resistant organisms did not appear until 10 h, but then the number increased steadily, until it equaled the total count at 24 h.

A second identical tube containing both CXM

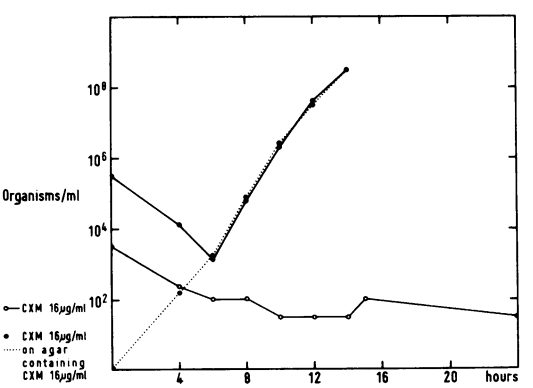


FIG. 3. *Viable counts of Ent 599 growing in broth containing 16 μg of CXM per ml. All counts were done in duplicate, one set on agar containing 16 μg of CXM per ml. Two inocula were used, demonstrating the emergence of resistance from the larger inoculum.*

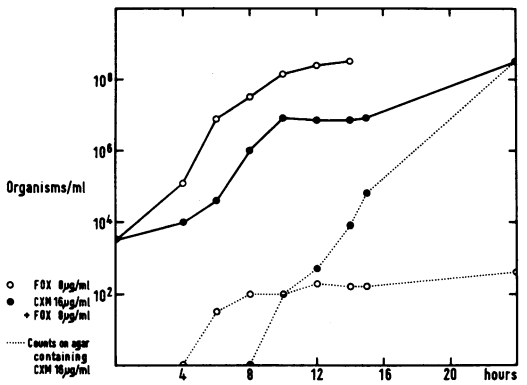


FIG. 4. *Viable counts of Ent 599 growing in broth containing 8 μg of FOX per ml alone or 8 μg of FOX per ml plus 16 μg of CXM per ml, using a light inoculum. All counts were done in duplicate, one set on agar containing 16 μg of CXM per ml. Growth in the drug-free control tube did not differ significantly from that in the tube containing only FOX.*

and FOX was centrifuged after 4 h of incubation, and the pellet was resuspended in broth containing 16 μg of CXM per ml only. Over the next 2 h the count rose to the same level as that in the tube containing both drugs, but then fell sharply over the next 6 h, remaining unchanged thereafter, and resistance did not appear.

**Rate of drug inactivation.** FOX or CXM was assayed in the test mixtures at the same times that viable counts were done. The results are given in Table 2. In the tube containing 8 μg of FOX per ml the level had fallen to 5 μg/ml by 4 h, a trace remained at 6 h, and thereafter none was detected. Inactivation of CXM was slower and even with the heavy inoculum the level had only fallen from 16 to 11 μg/ml by 12 h, by which time resistant organisms numbered nearly $10^8$ cells per ml. In the tube containing CXM plus FOX there was no significant loss of CXM in 15 h, at which time resistant organisms were still present at $<10^5$ cells per ml, but no activity was detected at 24 h.

**Effect of CXM and FOX in agar.** When plates containing 16 μg of CXM per ml with and without 4 μg of FOX per ml were inoculated with Ent 599 to give about 100 colonies on the control plate, no growth occurred in the presence of CXM alone. After overnight incubation colonies on the plate containing both CXM and FOX, although reasonably large, were smaller and more opaque than those on the control plate. However, two of these smaller, opaque colonies had a larger colony of normal appearance growing out of them, and these were found to be resistant to CXM although the others remained sensitive.

TABLE 2. *Rate of inactivation of FOX and CXM in broth inoculated with Ent 599, related to the rate of growth of the organism*

| Time (h) | Test mixture with 8 μg of FOX per ml | | Test mixture with 16 μg of CXM per ml[a] | | Test mixture with FOX + CXM | |
|---|---|---|---|---|---|---|
| | Viable count (organisms/ml) | Concn of FOX (μg/ml) | Viable count (organisms/ml) | Concn of CXM (μg/ml) | Viable count (organisms/ml) | Concn of CXM (μg/ml) |
| 4 | $5 \times 10^3$ | 8 | $5 \times 10^5$ | 16 | $5 \times 10^3$ | 16 |
| 6 | $10^5$ | 5.1 | $2 \times 10^4$ | 16 | $10^4$ | 16 |
| 8 | $10^7$ | Trace | $2 \times 10^3$ | 16 | $6 \times 10^4$ | 16 |
| 10 | $5 \times 10^7$ | ND[b] | $9 \times 10^4$ | 16 | $10^6$ | 16 |
| 12 | $2 \times 10^8$ | | $3 \times 10^6$ | 14 | $10^7$ | 16 |
| 14 | $4 \times 10^8$ | | $6 \times 10^7$ | 11 | $10^7$ | 16 |
| 15 | $5 \times 10^8$ | | $5 \times 10^8$ | 8 | $10^7$ | 16 |
| 24 | | | | 5 | $5 \times 10^8$ | ND |
| | | | | ND | | |

[a] After 24 h of incubation a test mixture containing 16 μg of CXM per ml inoculated with $5 \times 10^3$ organisms showed only slight loss of activity, similar to that of the uninoculated control.
[b] ND, None detected.

To determine whether the drugs were inactivated in agar, a diluted culture of Ent 599 was streaked across a plate containing 16 μg of CXM per ml, and a blotting paper strip soaked in a solution containing 1,000 μg of FOX per ml was placed at right angles to the streak. Growth occurred only beside the strip, and when plugs of agar were punched out from this area with a cork borer and assayed, no drug was detected, although similar plugs taken from other areas of the plate produced large zones of inhibition. A resistant mutant tested in the same way grew in the presence of 16 μg of CXM per ml and was shown to inactivate this drug, both alone and when FOX was present.

**Test for antibiotic inactivation.** When Ent 599 was tested for inactivation of CXM by the cloverleaf method, there was only a very slight indentation of the zone of inhibition produced by a disk containing 30 μg; however, when a resistant mutant taken from a CXM-containing culture was tested there was very marked indentation, indicating considerable inactivation of CXM. A possible explanation of the antagonism between CXM and FOX could be that the latter induced the entire parent culture to inactivate CXM, and Fig. 5 shows this to be the case. *S. lutea* is highly susceptible to FOX, but when the test was done with *E. coli* on plates containing varying amounts of FOX, 2 μg/ml was found to be only slightly inhibitory, and the obvious indentations of the CXM zone and the enhanced growth along the whole length of the streaks are clear evidence of the inactivation of both CXM and FOX.

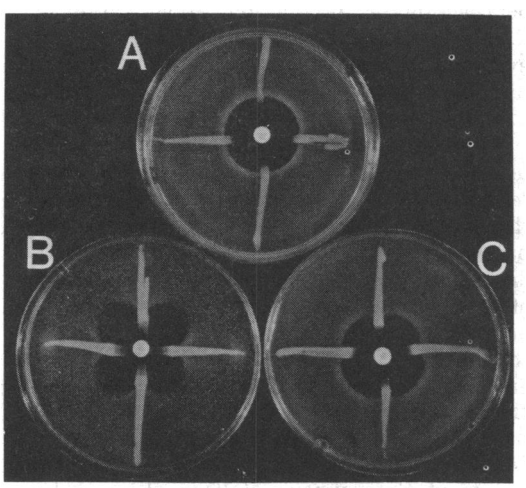


FIG. 5. *Agar plates containing varying amounts of FOX flooded with E. coli. Ent 599 formed the cross, and the disks contained 30 μg of CXM. Plate A, drug-free control; plate B, 2 μg of FOX per ml; plate C, 1 μg of FOX per ml.*

Cloverleaf tests were then done with both the resistant mutant and the parent strain induced with FOX against a variety of cephalosporins and penicillins. There was marked inactivation by both cultures of cephaloridine, cephalothin, cephalexin, cephradine, cefazolin, cefamandole, cefatrizine, 756, penicillin, ampicillin, carbenicillin, and piperacillin, but very little, if any, inactivation of cloxacillin, methicillin, or mecillinam. Similarly, zones of inhibition produced by disks containing any of these cephalosporins were flattened if placed next to a disk containing FOX.

**Antagonism of CXM and 756 by FOX with other species.** A total of 65 strains of various gram-negative bacilli were screened for antagonism of CXM and 756 by FOX by the dilution method described above. The results, related to susceptibility to FOX, are given in Table 3. The most spectacular effect was seen with *Enterobacter,* in which the MIC of both drugs was up to 64-fold higher beside the FOX strip. Antagonism was seen frequently with *Serratia, P. morganii,* and *Proteus rettgeri,* all of which are often resistant to FOX (1), but the increase in MIC was usually only four- to eightfold. It appeared that some degree of resistance to FOX and at least some susceptibility to the other drug was required; almost all strains showing antagonism of CXM behaved similarly to 756, but most strains of *Serratia* and some *P. morganii* strains were resistant to CXM and showed antagonism only with 756. Only 2 of 10 strains of *Providencia* showed any such effect, and in both cases this was very slight. No effect was seen with *Proteus vulgaris* or *Proteus mirabilis,* whether or not the strain produced beta-lactamase, or with *E. coli* or *Klebsiella,* even when these were resistant to FOX.

The MIC of CXM and 756 for a representative selection of these organisms was determined on agar by using four different inocula ranging from $10^7$ to $10^2$ organisms per ml. There appeared to be some correlation between the presence of resistant mutants and of their MIC with the presence and extent of antagonism by FOX.

All of these organisms were also tested for antagonism of 756 by CXM. Surprisingly, in view of the original observation, this was only seen with two strains of *P. morganii* and one of *P. vulgaris,* and the MIC was raised four- to eightfold.

## DISCUSSION

Dissociated resistance is a term which was first used by Garrod (3) to describe strains of staphylococci which were resistant to erythro-

TABLE 3. *Effect of FOX, applied in blotting paper strips, on the MICs of CXM and 756 on agar plates*[a]

| Organism | No. of strains | No. of strains with the following susceptibility to FOX:[b] | | | Increase (fold) in MIC of: | |
|---|---|---|---|---|---|---|
| | | S | M | R | CXM | 756 |
| *Enterobacter* | 5 | | 2 | 3 | 4–64 | 4–128 |
| *Klebsiella* | 10 | 7 | 3 | | 0 | 0 |
| *E. coli* | 6 | 3 | 3 | | 0 | 0 |
| *Providencia* | 10 | 9 | 1 | | 0–4[c] | 0 |
| *Serratia* | 10 | | 10 | | 0–2 | 0–8 |
| *P. mirabilis* | 4 | 4 | | | 0 | 0 |
| *P. morganii* | 8 | 2 | 6 | | 0–2 | 0–8 |
| *P. vulgaris* | 5 | 4 | 1 | | 0 | 0–4[c] |
| *P. rettgeri* | 7 | 3 | 4 | | 0–8 | 0–8 |

[a] Tested as described in the legend to Fig. 2.

[b] Sensitivity based on the size of the inhibition zone on the drug-free control plates. S, Sensitive, zone not >4 mm smaller than that of the control *E. coli*; M, moderate, zone of at least 3 mm but >4 mm smaller than that of the control; R, resistant, zone <3 mm.

[c] Single strains.

mycin but susceptible to other related antibiotics, such as oleandomycin. These strains become resistant to oleandomycin, etc., in the presence of erythromycin, and Weaver and Pattee (8) showed that erythromycin is the specific inducer of this type of resistance. They also showed that if the erythromycin is removed, reversion to susceptibility will take place within 90 min.

The marked antagonism among the cephalosporins against *Enterobacter* has some features in common with this. Resistance is induced by only one drug, FOX, but all other cephalosporins are then affected. Similarly, if FOX is removed, the culture rapidly reverts to being susceptible. The main difference, apart from its nature, is that this resistance to cephalosporins arises spontaneously in a very small proportion of cells. of cells.

The existence of these mutants can explain why an organism is found to be resistant to CXM after overnight exposure to CXM plus FOX in broth, although colonies taken from agar containing both drugs remain susceptible. In the experiment illustrated in Fig. 3 and 4, FOX had almost disappeared from the tube containing this drug alone at 6 h, by which time the total count was almost $10^7$ organisms per ml. It was not possible to assay FOX in the presence of CXM, but if it is assumed that its inactivation was the result of bacterial growth, it might also be assumed that the FOX in the tube containing both drugs was inactivated by 10 h, by which time the count had reached $10^7$ organisms per ml. The organism would then revert to susceptible and would be killed by CXM, which showed no loss of activity at this time. However, resistant mutants, in the same proportion as in the control tube, had already appeared, and the suppression of the rest of the culture by CXM would permit these organisms to grow out, as happens when the heavy inoculum is added to CXM alone (Fig. 2) to give a resistant culture after 24 h. Presumably the rate of death of the susceptible organisms was similar to the rate of growth of the resistant mutant, with the result that the total count remained stationary for at least 5 h and rose only when resistant organisms outnumbered the susceptible organisms.

It seems that organisms which are resistant to CXM only in the presence of FOX acquire the same characteristics as the resistant drug-inactivating mutants, but it is not clear whether this resistance (in either culture) is due solely to beta-lactamase production. Inactivation of CXM in the medium is relatively slow and is evidently the result of bacterial growth and not its cause; nevertheless, intracellular enzyme could presumably account for the resistance.

Similar mutants capable of inactivating cefamandole were found in all strains of *Enterobacter* tested by Findell and Sherris (2), although they found no evidence of inactivation by the parent strains, which were susceptible to the drug. The enzyme produced by these mutants is of some interest, as the susceptibility of *Enterobacter* to cefamandole, CXM, and 756 is thought to be due to the resistance of these antibiotics to the cephalosporinase produced by this species. FOX is generally believed to be insensitive to beta-lactamase, but according to Onishi et al. (6) it is inactivated by occasional strains of *Enterobacter cloacae,* although to a lesser extent than other cephalosporins. Findell and Sherris (2) suggested that mutation of the structural gene may have extended its range of activity. Alternatively, do *Enterobacter* perhaps produce two beta-lactamases, one of which is

frequently lost, and if so, how readily do they reacquire this property?

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