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# In Vitro Response of *Enterobacter* to Ampicillin

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Three strains of *Enterobacter* were studied for their response to ampicillin. They exhibited a basic level of resistance that depended on the medium used and high-level mutational resistance at a frequency of $10^{-5}$ to $10^{-7}$. Two classes of mutants were selected, one of which showed markedly enhanced antibiotic inactivation as indicated by a biological assay and the other of which resembled the wild type in this regard. Both mutants showed cross-resistance to other β-lactam antibiotics. The results explained discrepancies between traditional broth dilution minimum inhibitory concentration tests and early read automated procedures.

*Enterobacter* species are generally resistant to ampicillin when tested by overnight broth or agar dilution procedures or by the agar diffusion method. Minimum inhibitory concentrations (MICs) of ampicillin are usually 64 μg/ml or higher. In contrast, strains of this genus often appear to be susceptible when tested by automated procedures that read for evidence of growth after only a few hours of incubation in the presence of about 5 to 10 μg of ampicillin per ml. This discrepancy was seen with the Autobac (Pfizer Inc., New York, N.Y.) equipment (8) and, more recently, during evaluative studies of the MS-2 automated susceptibility test system (Abbott Laboratories, Dallas, Tex.) and led to a study of three strains of *Enterobacter* to determine the reason for the differences.

One obvious possibility was that the discrepancies were due primarily to the differences in reading times between the automated and manual methods. The second was that strains of *Enterobacter* were more susceptible to ampicillin in the Isosensitest (IST) medium used with the MS-2 device than in Mueller-Hinton (MH) medium used with the manual methods. In investigating these possibilities, we found that ampicillin resistance of these strains of *Enterobacter* involved a relatively low level of basic resistance that was influenced by medium and a higher level of mutational resistance due to the presence of relatively high-frequency resistant mutants. Mutational resistance was detectable only after several hours of incubation in broth containing ampicillin and when a sufficiently large inoculum was used. This paper documents these phenomena and describes some characteristics of the mutants.

## MATERIALS AND METHODS

**Bacterial strains.** *Enterobacter aerogenes* 2002, *E. aerogenes* 2046, and *Enterobacter cloacae* 2016 were selected for study. They were resistant to ampicillin by diffusion and dilution procedures, but susceptible in the Abbott MS-2 system. They were originally isolated from clinical material by the Clinical Microbiology Laboratory, University of Washington Hospital.

**Antibiotics.** Ampicillin powders were kindly supplied by Bristol Laboratories, Syracuse, N.Y., and the Ames Co., Ames, Iowa. Cefamandole lithium was kindly supplied by Eli Lilly & Co., Indianapolis, Ind.

**Media.** MH broth (Difco Laboratories, Detroit, Mich.; lot 570375) and IST broth (Oxoid Ltd., Basingstoke, Hampshire, England; lot 22026) were used for broth dilution MIC determinations unless otherwise indicated. MH agar medium (Difco lot 640657) and IST solidified with agarose (Calbiochem, La Jolla, Calif.; lot 100430) were used in agar dilution and disk diffusion studies. In one case, MH broth was solidified with agarose. This medium performed similarly to the lot of MH agar.

**Susceptibility test methods.** Disk diffusion tests were performed according to the National Committee for Clinical Laboratory Standards (5) and Food and Drug Administration (2) protocols. All antibiotic disks were obtained from BBL Microbiology Systems, Cockeysville, Md., except for 30-μg cefamandole disks which were kindly supplied by Eli Lilly Co. Agar and broth dilution tests were performed according to the reference methods recommended by an international collaborative study (1). Automated susceptibility tests were made in the MS-2 system. This apparatus allows continuous readings of light transmission in a multichambered plastic cuvette. A single upper chamber is filled with inoculated IST broth, and the cuvette is incubated at 37°C in the machine under shaking conditions. Transmittance is monitored with a light-emitting diode and photocell receptor. When the concen-

tration of organisms reaches approximately $2 \times 10^6$/ml, a 0.85-ml sample of the inoculum is drawn automatically into each of 11 chambers in the bottom part of the cuvette. Ten of these chambers can contain antibiotic elution disks, and the eleventh routinely serves as a growth control. In the case of ampicillin, the chamber(s) contained a final concentration of 10.59 μg/ml. Susceptibility or resistance to this concentration was determined automatically within 3 to 5 h by a comparison of the slopes of growth curves in the antibiotic-containing and control chambers. Details of the procedure have been reported previously by Spencer et al. (7).

**Inactivation of antibiotic by *Enterobacter*.** Antibiotic inactivation was detected by the "cloverleaf" method of Kjellander and Myrbäck (4). An MH agar plate was seeded evenly with *Micrococcus lutea* ATCC 9341, which was highly susceptible to the β-lactam antibiotics. The *Enterobacter* strains were then streaked heavily onto the seeded plate in the form of a cross. Antibiotic disks were placed in the center of the cross, and the plate was incubated overnight. Inactivation of antibiotic diffusing from the disk by the *Enterobacter* was manifested by a cloverleaf pattern of growth inhibition of the *Micrococcus*. Growth of the *Micrococcus* appeared adjacent to the *Enterobacter* streaks, but there was wide inhibition between them. When there was little or no inactivation of antibiotic, inhibition zones of the *Micrococcus* were approximately circular.

## RESULTS

**Effects of the two media.** The three *Enterobacter* strains were first checked for susceptibility to ampicillin by the different test systems. They were resistant by the diffusion method (zone diameters of <6 mm) and had MICs of >32 μg/ml by the dilution method in MH medium, but were susceptible to approximately 10 μg/ml by the automated procedure in IST medium and when read at 3 to 5 h.

The influence of medium on susceptibility results with ampicillin was examined by testing the three strains by broth and agar dilution procedures (Table 1) and by a diffusion method using both MH and IST media (Table 2). Three different inoculum levels were used in the broth dilution tests. The three strains were more susceptible to ampicillin with all three methods when IST medium was used, but ampicillin MICs were always 16 μg/ml or higher. Results of agar dilution tests on the two media, using very small inocula spread evenly over whole plates, are shown in Table 3 and expressed as the lowest concentration of antibiotic preventing any growth after overnight incubation. These indicated that the individual cells were more susceptible to ampicillin on IST medium. The greater resistance in MH medium was confirmed by testing strain 2002 by the broth dilution

TABLE 1. *Comparison of agar and broth dilution ampicillin MICs for three strains of Enterobacter tested with MH and IST media*

| Strain | Medium | MIC (μg/ml) with following inoculum[a]: | | | |
|---|---|---|---|---|---|
| | | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| 2002 | MH broth | | 256 | 256 | 256 |
| | IST broth | | 32 | 64 | 128 |
| | MH agar | 128 | | | |
| | IST + agarose | 64 | | | |
| 2016 | MH broth | | 256 | >256 | >256 |
| | IST | | 32 (64)[b] | 128 | >256 |
| | MH agar | 64 | | | |
| | IST + agarose | 16 | | | |
| 2046 | MH broth | | 256 | 256 | >256 |
| | IST broth | | 32 (256)[b] | 256 | 256 |
| | MH agar | 128 | | | |
| | IST + agarose | 64 | | | |

[a] Inocula for the agar dilution procedure were organisms per inoculated spot; inocula for the broth dilution procedure were organisms per milliliter.

[b] Numbers within parentheses indicate tubes showing growth in concentrations above those that first inhibited growth (skipped tube phenomenon).

TABLE 2. *Diffusion test results for three Enterobacter strains tested against β-lactam antibiotics on IST and MH media*

| Strain | Agar medium | Zone diam (mm) with following antibiotic: | | | |
|---|---|---|---|---|---|
| | | Ampicillin | Cephalothin | Carbenicillin | Cefamandole |
| 2002 | MH | 6 | 14 | 23 | 24 |
| | IST | 15 | 16 | 24 | 28 |
| 2016 | MH | 6 | 6 | 26 | 28 |
| | IST | 17 | 18 | 27 | 29 |
| 2046 | MH | 6 | 12 | 23 | 24 |
| | IST | 11 | 14 | 25 | 27 |

TABLE 3. *Single-cell[a] ampicillin resistance for three Enterobacter strains tested on MH and IST media*

| Strain | Agar medium | MIC (μg/ml) |
|---|---|---|
| 2002 | MH | 64 |
| | IST | 8 |
| 2016 | MH | 16 |
| | IST | 4 |
| 2046 | MH | 64 |
| | IST | 16 |

[a] The inoculum was approximately $10^2$ cells spread over the surface of the medium.

method in three other batches each of MH and IST with an inoculum of $3 \times 10^3$ bacteria per ml. MICs in the MH batches were 128, 32, and 32 μg/ml, respectively, and those in the IST batches were 8, 16, and 16 μg/ml, respectively. A series of experiments in which components of the two media were supplemented or deleted failed to identify any single factor in the formulations which was responsible for the differences. Thus, the medium used in the automated equipment yielded generally lower MICs for ampicillin than did that used in manual tests; this may account, in part, for the differences encountered between MS-2 and manual test results.

In two of the experiments shown in Table 1, "skipped tubes" were encountered in which growth occurred in a concentration of ampicillin above that which first inhibited the organism. This observation was suggestive of discontinuous mutational resistance.

**Effect of incubation time on growth of *Enterobacter* in the presence of ampicillin.** The effect of incubation time on the growth of *Enterobacter* in the presence of ampicillin was investigated by using the MS-2 device to generate growth curves of the three strains of *Enterobacter* in the presence of 10.6 μg of ampicillin per ml in IST medium. Elution disks containing 9 μg of ampicillin were added to six of the cuvettes (volume, 0.85 ml), and the remaining five served as growth controls. Each strain was tested on four occasions in this manner, and incubation was continued for 20 h. Light transmission readings were automatically recorded on magnetic tape at intervals of 5 min, and computer-generated growth curves from these data were made available to us by H. Spencer of Abbott Laboratories. These plots, one example of which is reproduced in Fig. 1, showed an increase in optical density for approximately 2 h in the presence of ampicillin, followed by a decline to the level of the inoculum. Thereafter, emergent logarithmic growth was detected in most cuvettes after several hours of incubation but varied in the time of initiation. In some cases, no growth occurred in the presence of the antibiotic. These results were again compatible with the presence of a small number of resistant mutants in the inoculum which were randomly distributed to the different cuvettes. Similar experiments with MH medium showed logarithmic growth in the presence of ampicillin parallelling that of the control until approximately 1 h before the control entered the stationary phase.

Manual experiments to generate growth curves in the presence and absence of ampicillin were made using IST and MH media. One experiment is shown in Fig. 2. In this case, 10-ml volumes of IST broth were used in two tubes. One tube contained 8 μg of ampicillin per ml; a second tube without antibiotic served as a growth control. Each was inoculated with $10^5$ *Enterobacter* organisms per ml. Samples of 0.1 ml were removed every hour and serially diluted in sterile broth, and 10-μl amounts of each dilution were spread on blood agar plates for viable counting. In addition, samples were taken for Gram staining and phase-contrast microscopy. The results paralleled those in some MS-2 cuvettes in that there was little, if any, growth in

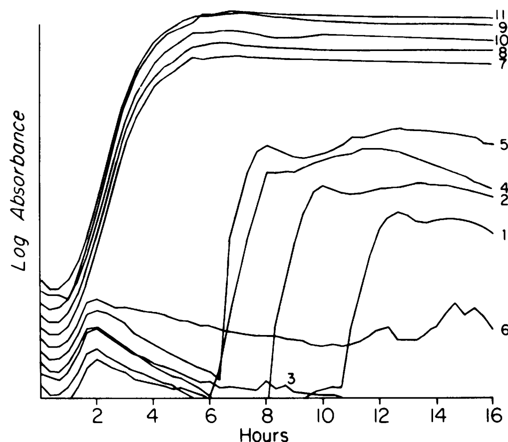


FIG. 1. *MS-2-generated growth curves of one Enterobacter strain in IST broth. Plots 1 through 6 were from samples exposed to 10.6 μg of ampicillin per ml, and plots 7 through 11 were from growth controls in medium containing no antibiotic.*

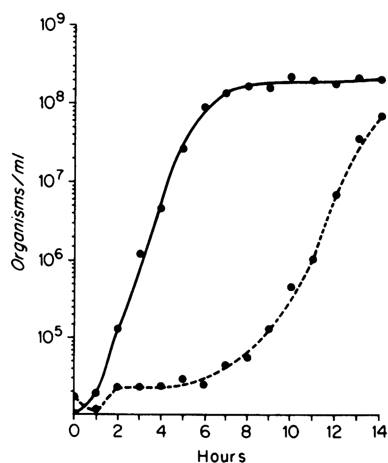


FIG. 2. *Manually generated growth curve of one Enterobacter strain in IST broth. The control (—) contained no antibiotic. The test (— — —) contained 8 μg of ampicillin per ml. Viable counts were recorded on a $\log_{10}$ scale, and time in hours was recorded on an arithmetic scale.*

TABLE 4. *Agar dilution[a] susceptibilities to β-lactam antibiotics of Enterobacter strains and of their ampicillin-resistant mutants*

| Strain | Antibiotic inactivation[b] | MIC (μg/ml) with following antibiotic: | | | |
|---|---|---|---|---|---|
| | | Ampicillin | Carbenicillin | Cephalothin | Cefamandole |
| 2002 | | | | | |
| Mutant 1 | + | 256 | 32 | >2,048 | 64 |
| Mutant 2 | − | 256 | 8 | >2,048 | 16 |
| Wild type | − | 32 | ≤4 | ≤32 | ≤2 |
| 2016 | | | | | |
| Mutant 1 | + | 512 | 128 | >2,048 | 128 |
| Mutant 2 | − | 512 | 64 | >2,048 | 64 |
| Wild type | − | 16 | ≤4 | ≤32 | ≤2 |
| 2046 | | | | | |
| Mutant 1 | + | 1,024 | 16 | >2,048 | 32 |
| Mutant 2 | − | 1,024 | 8 | >2,048 | 16 |
| Wild type | − | 64 | ≤4 | ≤32 | ≤2 |

[a] The medium used was IST solidified with 1.7% agarose.

[b] Tested by the cloverleaf technique.

the presence of ampicillin for 6 h, after which a period of logarithmic growth occurred. Microscopic examination of samples before the initiation of logarithmic growth showed bizarre elongated forms. Morphologically normal forms appeared with logarithmic growth. Subcultures from the tube containing ampicillin to non-antibiotic-containing medium yielded colonies that gave marked ampicillin inactivation by the cloverleaf test. Colonies from subcultures of the control tube without antibiotic showed essentially no inactivation by this method. These experiments again suggested that the higher levels of ampicillin resistance in *Enterobacter* were mutational and were not detected after relatively short periods of incubation. They explain, at least in part, the discrepancies between results of the automated and manual procedures.

**Direct selection and characterization of ampicillin-resistant mutants.** Resistant mutants were selected directly from the three strains by incorporating $10^7$ organisms in IST medium solidified with agarose and containing 128 μg of ampicillin per ml. In all cases, between 1 and 10 colonies developed after 18 h of incubation. They were picked and subcultured to agar plates in the absence of antibiotic, and the progeny of single colonies were used for subsequent characterization.

When tested by the cloverleaf procedure, two classes of mutants were apparent. The first showed marked inactivation of ampicillin; the second showed little or no inactivation. Representatives of the two types of mutants from each of the three strains were tested for their susceptibility to ampicillin, carbenicillin, cephalothin, and cefamandole by the agar dilution and diffusion procedures. The results of the dilution procedures, compared with those of the wild types, are shown in Table 4 and demonstrate that the mutants have increased resistance to each of the β-lactam antibiotics. Similar results were seen with the disk diffusion tests. There was no change in susceptibility to antibiotics of other classes as judged by zone diameters in the agar diffusion test.

Cloverleaf tests for antibiotic inactivation were also performed with carbenicillin, cephalothin, and cefamandole. Mutants that inactivated ampicillin also showed marked inactivation of cefamandole, whereas the other mutants and wild types did not. Mutants and wild types all showed inactivation of cephalothin, but it was most marked with the ampicillin-inactivating mutants. There was evidence of a slight inactivation of carbenicillin by the ampicillin-inactivating mutants, but none with the wild types.

## DISCUSSION

Our results showed that the three strains of *Enterobacter* had a relatively low basic level of resistance to ampicillin as detected by agar dilution MICs with small inocula. Superimposed on this was a much higher level of resistance due to resistant mutants that were selected at a frequency in the range of about $10^{-6}$ to $10^{-7}$ on plates containing 128 μg of ampicillin per ml, but from inocula of as low as $10^5$/ml in broth dilution tests. These mutants were stable on subculture in the absence of antibiotics. Basic resistance was to some extent medium dependent in that MICs with MH medium were about fourfold higher than those in IST medium. No single component of either of the media that we used could be identified as accounting for these dif-

ferences. Our results indicate that in automated susceptibility tests, if the concentration of antibiotic is above the MIC of single cells, the organism will be inhibited for several hours until any resistant mutants present in the inoculum reach the level of detection. In macrobroth dilution tests using inocula of $10^6$ cells or more, mutational resistance will almost always be expressed after overnight incubation.

These results are analogous to those previously reported with cefamandole (3). In that case, the majority of the population was susceptible to cefamandole at a level of about 2 $\mu$g/ml, but cefamandole-inactivating and non-inactivating resistant mutants occurred at a frequency of between $10^{-5}$ and $10^{-6}$. The results reported here show that the same mutations were probably involved with high-level resistance to ampicillin (Table 3), because mutants selected with ampicillin were resistant to cefamandole. Similar findings have been reported by Ott et al. (6).

The variability of responses of *Enterobacter* to $\beta$-lactam antibiotics, which is associated with relatively high resistant mutant frequencies, has made it difficult to equate results of overnight with early read automated susceptibility tests. This problem might be lessened by using a medium for automated procedures that enhances the expression of basic resistance. The relatively low level of ampicillin resistance in a majority of the population of some *Enterobacter* strains was unexpected, because traditional overnight broth dilution tests usually reflect the resistance of the mutants. It would be of interest to determine whether a similar situation exists in vivo.


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