ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1998, p. 78–82 Vol. 42, No. 1
0066-4804/98/$04.00+0

Postantibiotic Effect in *Escherichia coli* Determined with Real-Time Metabolic Monitoring

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Received 12 March 1997/Returned for modification 22 July 1997/Accepted 18 October 1997

Metabolic activity was used to quantify the delay in recovery of *Escherichia coli* after limited exposures to cefotaxime and piperacillin. This was measured with a microphysiometer, a device which measures changes in pH in the cellular environment, which in turn reflects the metabolic activity of the cells. The antibiotics were rapidly pumped into and flushed from each cellular environment. The length of time that the bacteria were exposed to either antibiotic was determined by programmed valve changes. Metabolic activity was measured during periods in which the instrument's pumps were off. Graphical analysis of the data was used to determine the postantibiotic effect. The lengths of the postantibiotic effects of both drugs (95 to 101 min) determined with the microphysiometer corresponded with the reappearance of short, highly motile cells in significant numbers.

Postantibiotic effect (PAE) is the persistent suppression of bacterial growth after a limited exposure to an antimicrobial agent (9). The inhibitory effect of the antimicrobial agent may last after drug levels are no longer detectable, and thus, knowledge of the duration of PAE may be important in establishing dosing schedules for the treatment of infections since antibiotics may be ineffective during PAE (6, 13). It may thus be prudent to know the duration of PAE for various microorganism and antibiotic combinations. One of the most commonly accepted means for measuring PAE is a viable-count method, although this is a labor-intensive procedure which includes a lengthy incubation step. The viable-count method may yield inaccurate results with some antibiotics, particularly β-lactams (10). Bioluminescence has also been used to measure PAE and is faster than the viable-count method (9, 10), but one recent study has a demonstrated a poor correlation of bioluminescence with the viable-count method (11). PAE has also been measured by morphological studies, the impedance method, or some combination of these methods (14); by measuring hemolysin activity (5); and by a total cell counting technique with a Coulter counter (12).

In any method which measures PAE, antibiotics must be removed from the cells' environment to allow the cells to recover. This is generally accomplished via centrifugation, filtration, or dilution of both cells and antibiotic, with dilution of the latter to a concentration below an inhibitory level. Each of these methods may introduce error into the measurement of PAE since each takes time, may result in the loss of some cells, may leave residual antibiotic, and may affect recovery by damaging cells made fragile by the antibiotic.

In this study, a microphysiometer, a device which measures the metabolism of cells in real time (15), was used to measure the recovery of *Escherichia coli* from antibiotic exposure. This instrument has previously been used to measure the rapid decline of bacterial metabolic activity to a low, stable level and the subsequent recovery of rapidly increasing bacterial metabolism after an antibiotic is introduced and then removed, respectively (3). The instrument has also measured changes in microbial metabolism as responses to extracellular stimuli, including the analysis of ion channels (7) and expression of an antiporter gene (8) in yeasts and the response of *E. coli* to lethal and sublethal levels of an antibiotic (3). The microphysiometer measures metabolism by monitoring the change in the pH or redox potential of the cellular environment. A programmable valve connects each sensor chamber, which holds a cell population in a cell capsule, to two reservoirs containing medium. Coupled with the very small volume of the cell chamber (1 to 2 μl) and a flow rate typically in the range of 40 to 100 μl/min, the fluid around the cells may be rapidly and completely exchanged with the alternative fluid after a change in the valve position directing the alternative fluid through the cells' environment (valve switch). The medium flowing into the cells' environment determines the pH or redox potential around the cells when the pumps are on; metabolic activity is measured by determining the change in pH or redox potential during a brief period in which the pumps are off and the cells modify their environment. A microcomputer is used to program the valve positions at stages in each experiment so that the period of a cell population's exposure to an antibiotic is defined (15).

At sufficiently high concentrations, β-lactam antibiotics cause rod-shaped bacteria to elongate into filamentous forms. After the antibiotics are withdrawn, the return to more normal, short rod shapes is correlated with the end of PAE (1, 5). A microscopic examination of *E. coli* morphology and motility was conducted to compare the PAEs obtained by this established method with the PAEs obtained by the metabolism-based assay.

MATERIALS AND METHODS

Media and antibiotics. Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) was used to culture the bacteria and was also the medium pumped through the fluid paths of the microphysiometer. At the first time point measured in each experiment and between metabolic activity determinations, the pH of the incoming Mueller-Hinton medium was 7.3.

Prior to the PAE experiments, the MICs of piperacillin (2 μg/ml) and cefotaxime (0.0625 μg/ml) for *E. coli* ATCC 25922 were confirmed by a broth microdilution assay (2). In PAE experiments, the concentrations used in the microphysiometer fluid paths were multiples of these MICs: 16×, 8×, 4×, or 2×. The morphology assay was conducted with both antibiotics at 8× the MIC. Both antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cell capsule preparation. *E. coli* ATCC 25922 was used in all experiments. Inocula were prepared by growing the bacteria for 4 h in 7 ml of Mueller-Hinton broth. The cell density was adjusted to 10^7 cells/ml, and 3 volumes of the cell suspension were mixed with 1 volume of a 0.8% (wt/vol) low-melt agarose (Boehringer Mannheim Corp., Indianapolis, Ind.), the latter having been dis-

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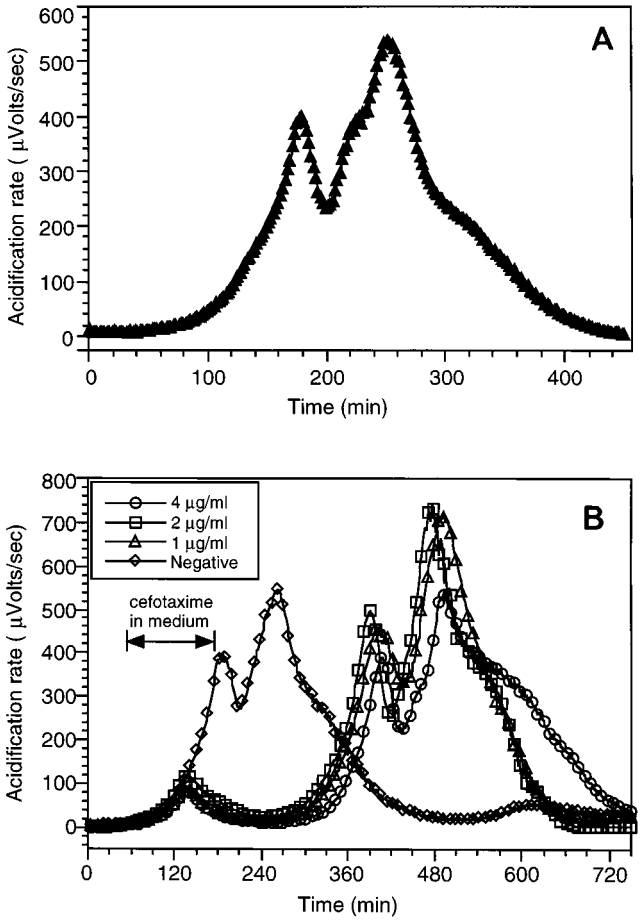


FIG. 1. Metabolic profile resulting from growth of *E. coli* in the microphysiometer (A) and delay of metabolic profiles following exposures to cefotaxime (B).

solved in Hanks' balanced salts solution (Gibco BRL, Grand Island, N.Y.), liquefied, and held at 37°C. Ten microliters of this suspension was pipetted into the centers of eight Transwell cell culture inserts (0.4-μm-pore-size polycarbonate membrane; Costar Corp., Cambridge, Mass.). The suspensions were allowed to solidify at room temperature for 3 min. One milliliter of medium was added below each insert, and 0.25 ml of the medium was added to the interior of the insert. A second polycarbonate membrane insert (0.4-μm pore size; Molecular Devices Corp., Sunnyvale, Calif.) was placed within each larger insert. The cells were thus immobilized in an agarose gel and trapped between two membranes. These completed cell capsules were placed in the sensor chambers of the microphysiometer (15). Except for the negative controls, each cell population within its own sensor chamber was exposed to a single concentration of one antibiotic.

Microphysiometer settings. The instrument used in this study was a Cytosensor System microphysiometer (Molecular Devices Corp.). A microcomputer was used to set the temperature of each sensor chamber to 37°C and the peristaltic pump rate to 100 μl per min. Within each 2-min pump cycle, fluid was pumped for 1.5 min, the pumps were turned off, and metabolic activity was determined in the next 0.5 min. After the completed cell capsules were placed in the instrument, the rate of medium acidification caused by the cells in antibiotic-free medium was measured. A valve switch then introduced Mueller-Hinton broth plus one of the antibiotics at a single concentration into each sensor chamber. Following 2 h or more of antibiotic exposure, a second valve switch was used to remove the antibiotic from the cells' environment by reintroducing the first medium without antibiotic. The initial baselines of the acidification rate measurements for antibiotic-exposed and unexposed cell populations were determined in the Mueller-Hinton broth before significant metabolic activity occurred and are thus identical. These were displayed as having a value of zero at the first time points at which measurements were taken, and all measurements thereafter had values which were offset from zero by the degree of metabolic activity, measured as microvolts per second.

Morphology assay. In order to compare the results obtained with the microphysiometer with the results of an established method of PAE measurement, morphological studies of the effects of β-lactam antibiotics on *E. coli* (6) were conducted. The cells were grown to a density of 10^8 cells/ml and were then diluted to 10^6 cells/ml in growth medium containing 8× the MIC of piperacillin (16 μg/ml) or 8× the MIC of cefotaxime (0.5 μg/ml). A negative control lacking antibiotic was similarly inoculated. After 2 h at 37°C, the cells were centrifuged and the medium was removed and replaced with antibiotic-free medium. The resulting dilution of antibiotic was approximately 1,000. The cell suspensions were incubated at 37°C and were periodically sampled by withdrawing a small volume of the suspension. A portion of this aliquot was examined by wet-mount microscopy for the presence of motile cells, and the remainder of the aliquot was placed briefly in an ice bath and then fixed and stained with crystal violet. For each aliquot examined, the proportion of normal to filamentous cells was determined by direct counting of cells with a normal morphology (1.5 to 6.0 μm in length) and filamentous forms (longer than 6.0 μm) in five fields, including any filaments of low contrast which may not have been viable prior to staining (the latter represented a small minority of the filaments present). The lengths of the bacteria were measured by using an ocular reticle, with a stage micrometer used as a reference. The morphology study was repeated several times.

TABLE 1. Effects of cefotaxime and piperacillin on metabolic peak height and time to peak[a]

Antibiotic and concn (μg/ml)	First peak ht (μV/s)	Time to first peak (min)	PAE (min)[b]
Cefotaxime			
0	440 ± 40	186 ± 6	
0.12	508 ± 5	405 ± 9	97 ± 15
0.24	526 ± 31	392 ± 5	84 ± 11
0.48	398 ± 8	408 ± 4	101 ± 10
Piperacillin			
0	482 ± 2	159 ± 1	
4	511 ± 7	358 ± 2	79 ± 3
8	487 ± 31	368 ± 8	89 ± 9
16	427 ± 6	374 ± 4	95 ± 5

[a] Values are means ± standard errors. Data are based on duplicate experiments.

[b] PAE = (time to first peak) − (time to first peak of negative control) − (exposure time).

RESULTS

Metabolic profile of *E. coli* in the microphysiometer. In every microphysiometer experiment conducted ($n = 60$), the rate of acidification of the medium by *E. coli* was observed to rise to a first peak, drop, and rise to a shoulder and a second, higher peak, after which a drop with one shoulder was seen.

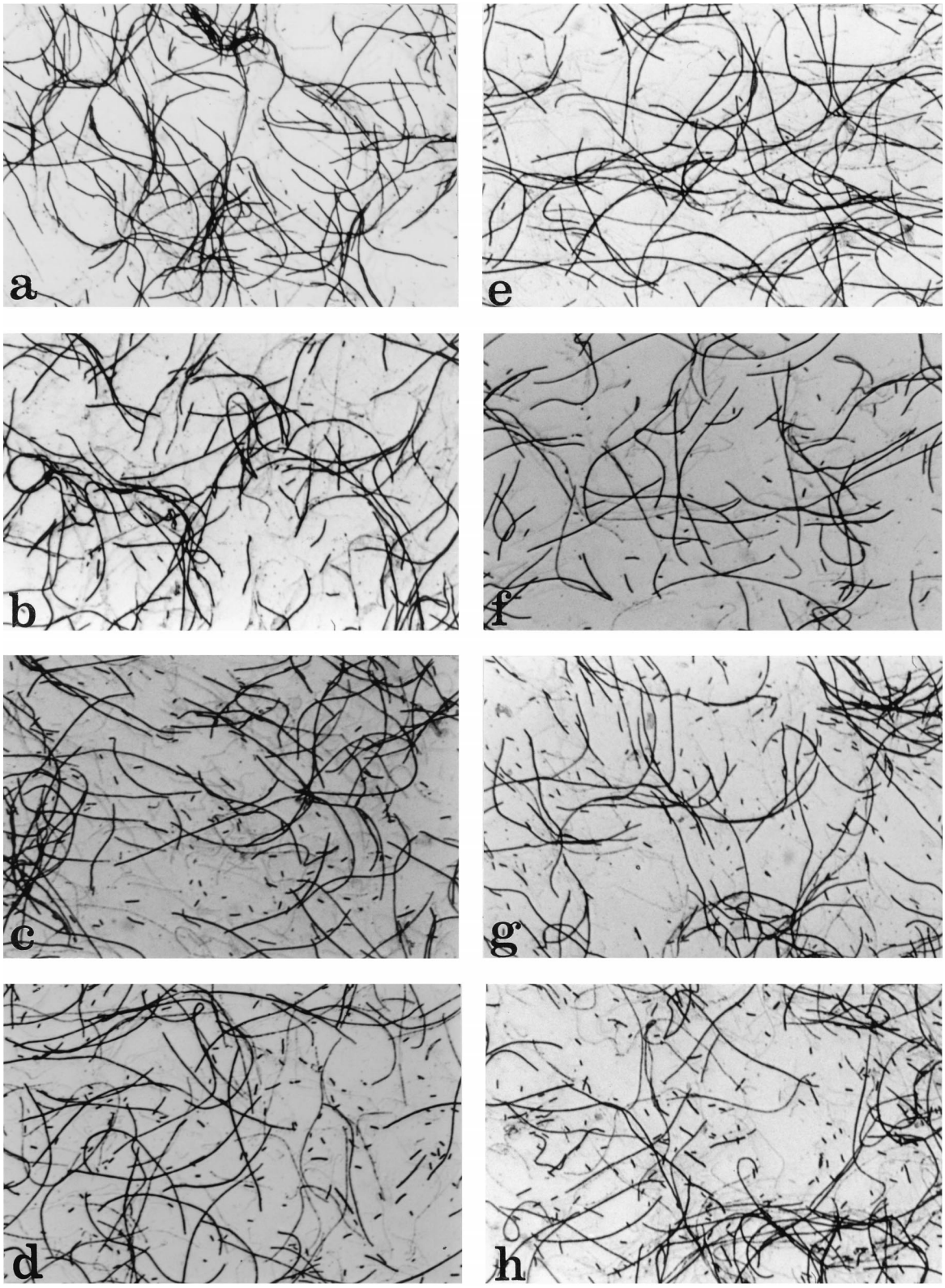
TABLE 2. Effects of different exposures of piperacillin on PAE[a]

Length of exposure (min)	Time (min) to first peak	Time (min) to second peak	Time of PAE (min) from first peak[b]	Time of PAE (min) from second peak[b]	Time of PAE (min) from recovery[c]
0	202 ± 2	280 ± 0			
120	414 ± 8	496 ± 4	92 ± 10	96 ± 4	100 ± 2
240	616 ± 12	710 ± 0	174 ± 14	190 ± 0	190 ± 4
360	760 ± 16	836 ± 6	198 ± 18	196 ± 6	198 ± 4
480	918 ± 16	990 ± 14	236 ± 18	230 ± 14	200 ± 18
720	1,200 ± 18	1,290 ± 10	278 ± 20	290 ± 10	288 ± 12
960	1,530 ± 16	1,584 ± 14	368 ± 18	344 ± 14	374 ± 18
1,440	2,084 ± 40	2,140 ± 40	442 ± 42	420 ± 40	472 ± 28

[a] Values are means ± standard errors. Data are based on duplicate experiments.

[b] PAE was derived from the time to each metabolic peak minus the time to the corresponding peak for the negative control (202 or 280 min) minus the time of each exposure to 32 μg of piperacillin per ml.

[c] Based on graphically displayed data, estimate of time to resumption of increasing metabolism after removal of antibiotic.



This metabolic profile is shown in Fig. 1A. The acidification rate is reported by the microphysiometer in units of microvolts per second, which is approximately equal to milli-pH units per minute (15). After the cells were placed in the microphysiometer, the time required to observe an increasing acidification rate, a result of the fact that cell numbers were increasing to a minimally detectable level, was approximately 30 min after the start of each experiment (it may be difficult to discern an

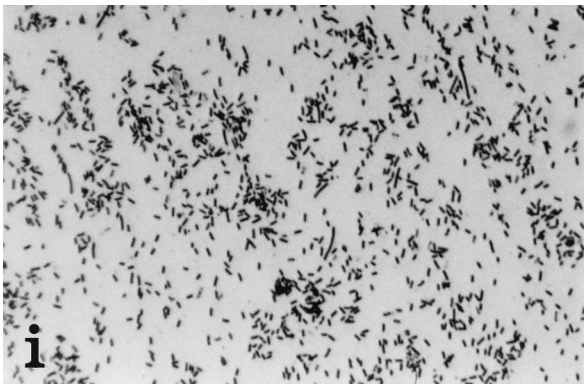


FIG. 2. Short, motile rod forms became more numerous, as illustrated in these photographic progressions, taken 60, 75, 90, and 105 min after removal of piperacillin (a to d, respectively) and cefotaxime (e to h, respectively). Negative control cultures had a similar appearance at all time intervals examined and consisted almost entirely of short, motile rods (i). Magnifications, ×400.

increasing rate at 30 min in Fig. 1A since the scale of the *y* axis has been condensed to include the time for the entire metabolic profile).

PAEs of cefotaxime and piperacillin. A 2-h exposure to cefotaxime in the microphysiometer resulted in a significant delay in growth (Fig. 1B). In these experiments, PAE was determined by one of two methods. By the first method, the time between removal of the antibiotic (the second valve switch) and the resumption of increasing metabolism, determined graphically, was used. This measurement is analogous to the "control-related effective regrowth time" described previously (11). Similar values were obtained by using the time required to reach the first metabolic peak and then subtracting the time required to reach the corresponding peak for the negative control and the time of exposure. In Table 1, which uses data derived by the latter method, no obvious dose dependence for PAE in the range of antibiotic concentrations tested can be detected. The observation that PAE for cefotaxime and *E. coli* is not concentration dependent at less than 32× the MIC has been reported elsewhere as well (10).

A 2-h exposure to piperacillin also resulted in a delay in growth; the data from these experiments were graphically similar to those from the experiment with cefotaxime, the results of which are presented in Fig. 1B. As seen in Table 1, PAE increased with the piperacillin concentration, but the numbers were not sufficiently different to suggest dose dependence.

Table 2 lists the results from a single experiment in which the time of exposure to 32 μg of piperacillin per ml (16× the MIC) ranged from 2 to 24 h. Three graphical features were used to compare possible methods of determining PAE. In the first and second measurements, the time required to reach the first or second peak was determined and then both the time to reach the comparable peak for the negative control and the time of exposure were subtracted. Regardless of whether the first and second peaks were used, the PAEs were similar. The time between removal of the antibiotic and the resumption of increasing metabolism was also used to measure PAE (PAE from recovery in Table 2). The PAEs thus obtained were similar to those determined from the metabolic peaks. All of the methods used to determine PAE for this drug-organism combination demonstrated PAEs that appear to depend on exposure time; i.e., the longer the exposure time, the longer the time required for the survivors to recover.

Comparison of morphological and metabolic assay results. The recovery of *E. coli* from either piperacillin or cefotaxime exposure, determined by microscopic examination, was similar after exposure to either antibiotic (Fig. 2). The effects of both β-lactam antibiotics were apparent at the first time point at which the cells were examined, which was 15 min after the antibiotics were removed. All of the cells in both suspensions appeared as long filaments of 25 to 60 μm in length, whereas in negative control cultures the cells were 1.5 to 6.0 μm long. Very few of the elongated rods were motile, and those that were moved much more slowly than the cells in the negative control cultures. No change in either antibiotic-containing medium was apparent until 75 min after antibiotic removal, when a few short, motile cells began to appear (Table 3). This trend became obvious 15 min later (90 min after antibiotic removal), when more than a third of the cells in each population were of normal length and were highly motile (Fig. 2). This compared with PAEs of 95 and 101 min for the same concentrations of piperacillin and cefotaxime, respectively, determined with the microphysiometer (Table 1).

DISCUSSION

Over the past several years, different PAE studies with the same organisms and antibiotics have come to a fairly wide range of conclusions. For example, a study that used bioluminescence as a method of measuring PAE reported that the PAEs for *E. coli* ATCC 25922 after 2-h exposures to 16× the MIC of piperacillin and cefotaxime are 48 and 60 min, respectively (10). One group using the viable-count method found a PAE of 15 min for a 2-h exposure to 64× the MIC of piperacillin and no PAE after a 2-h exposure to 512× the MIC of cefotaxime (1). The microphysiometer yielded longer PAEs: 95 min for exposure to 16× the MIC of piperacillin and 101 min for exposure to 8× the MIC of cefotaxime. The sizable discrepancies obtained by these different techniques may be due to the different methods of handling damaged cells after centrifugation, filtration, dilution, or fluid flow to remove the antibiotic from the cells. Any method which disturbs potentially damaged cells may prolong recovery time. Initially, it was not known whether the microphysiometer's intermittent flow method would have a dire effect on damaged cells, and the effects of cell immobilization were also not understood.

Piperacillin alone is not highly bactericidal for *E. coli* (16).

TABLE 3. Appearance of cells with normal morphologies at various times after antibiotic exposure (2 h) and removal

Time (min) after removal[b]	% normal cells[c]		
	NEG	CEF	PIP
15	>95	<5	<5
30	>95	<5	<5
45	>95	<5	<5
60	>95	<5	<5
75	>95	12 ± 2	8 ± 2
90	>95	38 ± 3	36 ± 4
105	>95	53 ± 3	69 ± 3
120	>95	64 ± 4	75 ± 4
135	>95	78 ± 3	78 ± 2
150	>95	86 ± 3	87 ± 4

[a] Values are means ± standard errors. Data are based on direct counts of five microscope fields for each value determined.

[b] Time after antibiotic is removed and cells are resuspended in antibiotic-free medium.

[c] Percentage of the cells in the total cell population, including filamentous cells of 1.5 to 6 μm in length. Abbreviations: NEG, negative control (no antibiotic in medium); CEF, exposed to 0.5 μg of cefotaxime per ml for 2 h; PIP, exposed to 16 μg of piperacillin per ml for 2 h.

An independent group of investigators found that the MIC of piperacillin for *E. coli* ATCC 25922 was 2 μg/ml, the same value reported here (4). By a viable-count method, a 24-h exposure of this strain to 16× the MIC of piperacillin was shown to result in about a 2.5-log reduction in cell viability through 6 h and little or no change in viability for the succeeding 18 h. Thus, the failure of piperacillin to kill all of the cells in the microphysiometer, given that the same concentration and length of exposure were used, is not surprising. Approximately 7.5×10^4 cells were immobilized in each sensor chamber prior to the introduction of antibiotic. A 2.5-log reduction would yield about 240 surviving cells, whose metabolism would be detected about 450 min after piperacillin was flushed from the cellular environment.

While viable-count methods remain the standard for measuring PAE, morphology is also used to gain an understanding of the relationship between cell form, growth, and metabolism (5, 9). While some filamentous cells may not survive a plating procedure, they may still contribute to the metabolism of the cell population and retain reproductive potential (5). The morphological study described in this report was performed to identify possibly relevant events within each cell population after exposure to one of these β-lactam antibiotics. For more than 2 h after the antibiotics were withdrawn, long filamentous cells, an obvious indication of PAE, dominated each culture. Short, highly motile rods appeared in significant numbers (greater than one-third of the total cell number) at about 90 min after the antibiotics were removed. However, unless one were to arbitrarily designate a particular percentage of normal cells to filamentous cells as the indicator of the end of PAE, it would be difficult to assign a specific time value for PAE from a microscopic examination. This is complicated by the fact that, on a mass basis, filaments continued to dominate both cultures long after 90 min (e.g., normal cells comprising 33% of a culture represent less than 2% of the cell mass if the culture is dominated by filaments with an average length 20 times that of normal cells). The relative contribution of normal cells and filaments to metabolism at this stage is not known. Since motility requires a significant expenditure of energy, the appearance of a high proportion of motile rods in a culture probably correlates with a large surge in metabolic activity. The PAEs derived from microphysiometer experiments may represent a point at which the metabolic contribution of normal-length, motile rods becomes detectable. If true, a more sensitive microphysiometer may yield somewhat lesser PAE values.

Of the two assays performed, the morphological assay was a much more time-consuming assay when the fixing, staining, and counting of normal and filamentous cells in multiple fields were taken into account. The microphysiometer method conferred the ability to program valve changes to remove antibiotics from the cellular environment quickly and easily. This feature made this type of study a walkaway effort, even for the experiment with long exposure times. A greater hands-on effort is required for the morphological, bioluminescence, and other assays which use centrifugation or filtration to remove antibiotics.

The morphological method provided one means of comparing the flow-based method of antibiotic removal with one in which the cells were washed free of antibiotic by centrifugation and resuspension. Since both methods yielded similar PAEs, the flow method of removing antibiotics appears to be a reasonable alternative, and whether the cells are immobilized or remain in suspension may be of little significance. A moving fluid path may more closely mimic some of the environments in which cells may occur in vivo than the static fluids used in many other measuring techniques. Future research efforts may focus on this question.

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