JOURNAL OF BACTERIOLOGY, Mar. 1988, p. 1129–1136
0021-9193/88/031129-08$02.00/0

Vol. 170, No. 3

Turgor Pressure Responses of a Gram-Negative Bacterium to Antibiotic Treatment, Measured by Collapse of Gas Vesicles

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Received 28 September 1987/Accepted 25 November 1987

The internal hydrostatic pressure of *Ancylobacter aquaticus* was measured by collapsing the gas vesicles with an externally applied pressure. Turgor pressure was measured in conjunction with various antibiotic treatments to elucidate some aspects of the biophysics of gram-negative cell wall function. Differences in the effects of these drugs either alone or in combination with other treatments were related to known biochemical activities of these drugs. Our previous work, demonstrating a heterogeneous cellular response to β-lactam antibodies, was confirmed and extended. Most of the cell wall growth-inhibiting antibiotics resulted in some cells (those in component I) developing a higher pressure, while the remainder (those in component II) lost turgor. Although the fraction of the cells in each component varied a little from subculture to subculture, it did not vary with time or choice of antibiotic treatment. Mecillinam gave a nearly monophasic response. All antibiotics blocking macromolecular synthesis gave monophasic curves. The 50% collapse pressure in some cases, however, was lower higher, or the same as the control.

When a culture of *Ancylobacter aquaticus* is treated with ampicillin, the turgor pressure of about one-quarter of the cells increases (component I), while the turgor pressure of the remaining cells falls to zero (component II) (15, 23). This is the property of recently cloned cells undergoing balanced exponential growth, and here we document that the proportion does not change with drug or time of treatment. Two classes of possible causes of such heterogeneity are: (i) a switch, such as with the *Salmonella* flagellar gene (27); and (ii) an especially sensitive phase of the cell cycle. For the latter possibility, either the antibiotic must prevent the remaining cells from passing through the cell cycle to the sensitive phase, or the cells are able to develop tolerance sufficiently well or rapidly in some, but not all, phases of the cell cycle. In either case, the heterogeneity of response is interesting. Our approach was to test a variety of drugs and combinations of drugs by using the new techniques developed to study the turgor pressure in gas-vacuolated heterotrophic procaryotes (23).

It was found that biphasic collapse curves were produced by certain β-lactam antibiotics. Many inhibitors of macromolecular synthesis affect the turgor pressure either positively or negatively, but all gave a monophasic response. It was found that (i) all penicillins tested, except mecillinam, gave clear evidence of two turgor pressure response classes; (ii) cells in component II lost their turgor before their cellular contents completely leak out through what must be small holes in their cell walls; and (iii) various inhibitors of macromolecular synthesis affected the turgor pressure.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *A. aquaticus* M158 was obtained from A. E. Konopka. The cultures were reestablished from single colonies at monthly intervals. Cultures were grown in CAGV medium and the turbidity of pressurized cells was monitored with a Cary model 16 spectrophotometer as previously described (15). The cell density was kept below 30 μg (dry weight)/ml by repetitive dilution to ensure that cells were in balanced growth for at least 10 generations. The average doubling time was 4.1 h. The growth curve of strain M158 departs from linearity above 50 μg/ml. Growing cultures were maintained in a 30°C temperature-controlled water bath, and air which had been saturated with water containing charcoal was bubbled through the cultures.

Collapse pressure measurements. Collapse pressures of the vesicles in a population of cells were recorded as plots of relative light intensity due to gas vesicles versus externally applied pressure. Increasing the applied pressure gradually collapsed all of the vesicles (15). The pressure was raised to 550 kPa to collapse all vesicles. The light source consisted of a 5.0-mW randomly polarized He-Ne laser, model 105-2 (Spectra-Physics, Mountain View, Calif.), with a ($1/e^2$) beam diameter of 0.83 mm and a wavelength of 632.8 nm. The sample chamber, described previously (15), was a cylindrical glass tube attached to a pressure delivery system. The tube was placed in a holder about 25 cm in front of the laser beam and 8 cm from a silicon photodiode (PIN-1ODP; United Detector Technology, Culver City, Calif.), with a 1-cm^2 active area mounted at a right angle to the beam at that point. At such a large angle, light scattered from the bacteria per se is minimal (14), but the light scattered from the small vesicles is scattered more evenly in all directions. Therefore, a 90° angle of observation is more selective for light scattered by the vesicles. The signal from the photodiode was amplified with a model 101C amplifier (United Detector Technology) and recorded as the ordinate on an *x-y* plotter. The magnitude of the light intensity was adjusted at the start of each series of experiments so that a collapse curve with a height of 15 to 25 cm would result; this allowed reasonably accurate subsequent graphical analyses.

The *x* axis of the plotter recorded the rise in applied pressure, where the distance in centimeters was colinear with the millivolt output of a pressure transducer, Omega part PX300 (Omega Engineering, Inc., Stamford, Conn.), which measured the pressure near the sample chamber. The distance from the origin along the *x* axis could then be converted to pressure in kilopascals.

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The 50% collapse pressure of a sample was obtained by determining the midpoint of the sigmoidal curve of light intensity as a function of pressure. The pressure at that point was judged to be the pressure required to collapse 50% of the vesicles in the sample. If the turgor pressure remained and is the same in all cells, the standard deviation (SD) of collapse curve reflects the variability in pressure needed to collapse the individual vesicles. If, on the other hand, the turgor varies from cell to cell either naturally or due to treatment, then the SD will become larger. This SD was estimated by determining the collapse pressures (P) at the points at which 25 and 75% of the vesicles had collapsed (see reference 15): $SD = (P_{75\%} - P_{25\%})/2(0.6745)$.

Antibiotic treatments. Antibiotics were added to growing cultures after being solubilized in a small amount of CAGV. All drugs used were obtained from Sigma Chemical Co., St. Louis, Mo., except mecillinam, which was donated by Hoffmann-La Roche Inc., Nutley, N.J., and nalidixic acid and rifampin, which came from Calbiochem-Behring, La Jolla, Calif. All antibiotics, at the concentrations used, caused a decrease in the growth rate of strain M158 in the times allowed for activity. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was obtained from Sigma Chemical Co.

Estimation of index of refraction of the cytoplasm during leakage of cell components. The individual vesicles are small (100 by 250 nm according to Konopka et al. [17]) compared with the wavelength of light (of 632.8 nm) from the He-Ne laser. Consequently, the scattering of light obeys Rayleigh law (see reference 11 and A. L. Koch, ASM News 50:473–477, 1984). This means that the angular dependence of the intensity of refracted light is independent of the shape and orientation of the vesicles. To scatter light, a colorless object must have an index of refraction different from its environment. In this case, the gases inside the vesicle have an environmental index of refraction of essentially 1, while the environment is essentially that of water, 1.3333. Before liberation, the environmental index of refraction is that of cytoplasm, and this is typically 5 to 6% higher than that of water (14). The formula for the intensity of light scattering depends on many factors which are all held constant during the experiments presented in this paper. Then, by simplification of the Rayleigh formula (see reference 11), the intensity of light scattered by vesicles is proportional to $[n^2/n_0^2 - 1)/(n^2/n_0^2 + 2)]^2$, where n is the refractive index of the inside of a vesicle and n_0 is the refractive index of its immediate environment. This relationship will apply as long as all vesicles remain intact and do not aggregate appreciably. It is a good approximation, even though the index of refraction of the neighborhood of the vesicle (within the distance of the wavelength of light) varies depending on how near the vesicle is to the cell surface. This proportionality was used to estimate the change in the intensity of light scattered by vesicles as the cells gradually leak cytoplasm as a result of antibiotic activity.

RESULTS

Kinetics of pressure changes. A typical series of collapse curves obtained during ampicillin treatment of strain M158 are shown in Fig. 1. Each curve is the *x-y* plot of the intensity of scattered light versus the applied pressure. The collapse curves changed progressively after addition of 10 μg of ampicillin per ml and could be approximated by a cumulative Gaussian (sigmoidal) curve. Initially, the collapse curve was monophasic; the distinct appearance of the two components

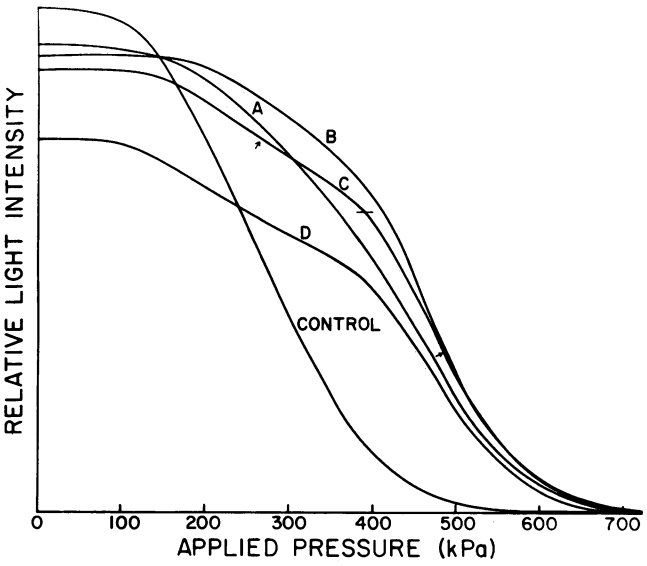

FIG. 1. Collapse pressure curves after different times of treatment with ampicillin. These five curves were obtained at different times by sampling a single culture treated with 10 μg of ampicillin per ml. *x-y* plotter graphs of relative light intensity, set at an arbitrary value before the experiment but not altered during the experiment, are shown. The abscissa is the applied, above atmospheric, pressure. The ordinate is the intensity of scattered light in arbitrary units. The curves were superimposed so that the final values are the same. The total light scattered by vesicles in the control decreased in a sigmoidal curve corresponding to a cumulative Gaussian curve with a mean ± SD of 275 ± 90 kPa. The effect of time of ampicillin treatment in the broadening of the curves is shown. The horizontal line on curve C shows the position chosen to separate component I from component II which provided the best fit to the sum of two cumulative Gaussian distribution. The mean values of these components are indicated by the arrows and are 265 and 489 kPa, respectively. These are indicated by the arrows normal to curve C for components I and II. The times of treatment were as follows: A, 9; B, 16; C, 24; D, 34 min.

was preceded by curves with a much broader slope (Fig. 1A and B) than that of a collapse curve from an untreated growing control culture (Fig. 1, control). The curves at these early stages were almost symmetrical and could be approximated by a cumulative Gaussian curve with a larger SD than the curves obtained before treatment or the components of the curves after longer treatment. As the time of treatment progressed, the slope increased and a break in the curves became evident (Fig. 1C and D), indicating that the collapse pressures of the vesicles were bimodal. In all cases (15, 22), each component was approximately a cumulative Gaussian curve. That is, the two components of the curve appeared to have approximately normally distributed collapse pressures, as indicated by the symmetrical sigmoid shape of each component. Also, the slope of each component was similar to that of the control.

The cells in the first component had a higher turgor pressure than cells growing in normal medium before antibiotic addition. This is indicated because the applied pressure causing collapse of half of the vesicles of component I was lower than the 50% collapse pressure before antibiotic treatment. In our previous studies (15) we found that the pressure causing collapse of 50% of vesicles not subject to turgor was 480 kPa. Component II cells have a 50% collapse pressure of 450 to 550 kPa, and we therefore inferred that the turgor pressure was close to zero in these cells.

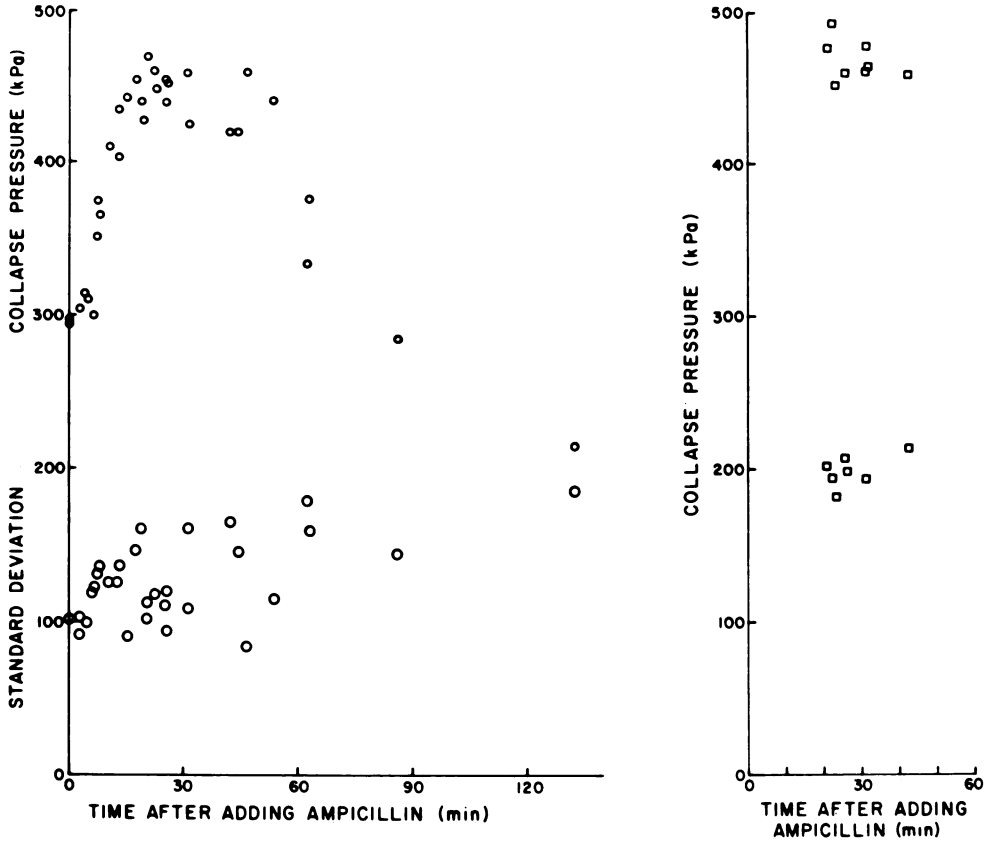

FIG. 2. The 50% collapse pressures, SD of the collapse curve, and 50% collapse pressures of components I and II. The collapse curves of 6 to 10 runs obtained from four separate experiments were analyzed. Each culture had been given 500 μg of ampicillin per ml as described in Materials and Methods. The applied pressure at the midpoint of the light intensity versus applied pressure and SD of the collapse curves was measured at different times after ampicillin addition and is plotted in the left-hand panel by open small and large circles, respectively. The SD of these collapse curves was calculated as described in Materials and Methods. The SD is a crude, but objective measure of breadth. When possible, this crude measure was supplemented by deconvolution of the collapse curves into two cumulative normal curves. The 50% collapse pressure of the separate components I (below) and II (above) estimated are shown in right-hand panel as open squares.

The curves in Fig. 1 were obtained at different times during a single experiment, using 10 μg of ampicillin per ml. The same type of experiment done with 500 μg of ampicillin per ml gave very similar results despite the 50-fold difference in concentration. Both concentrations are well above the inhibitory concentration. The lowest concentration of ampicillin causing a decrease in the growth rate of strain M158 was 2 μg/ml. A set of four experiments at 500 μg/ml, each consisting of several runs as time of treatment progressed, was used to construct the left-hand panel of Fig. 2 in which the collapse pressure at the midpoint of each curve and the SD (calculated as in Materials and Methods) is plotted against treatment time. For those curves in which the two components were graphically distinct, the midpoints of the two components were estimated separately and are shown in the right-hand panel of Fig. 2.

The magnitude of the light intensity measured in a particular component is indicative of the proportion of cells in that turgor pressure class (assuming vesicle concentration per unit volume of cytoplasm remains constant). The definitive proportion of cells in component I was evident in about 10 to 15 min. The percentage rose to a particular value for each particular experiment and remained constant over time (Fig. 3). In experiments not shown the proportion remained constant for at least 2 h. There appears to be some slight upward trend for the experiments at the lower ampicillin concentration. This is not unexpected since a higher concentration of drug will probably penetrate the cells, bind to penicillin-binding proteins (PBPs), and act more quickly.

Estimation of index of refraction in the environment of

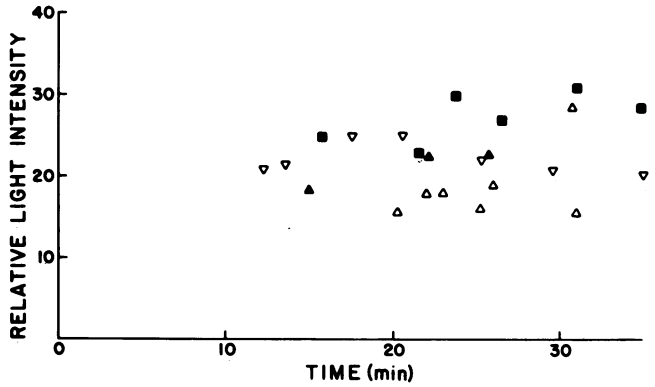

FIG. 3. Percentage of vesicles in cells of component I. The percentage of the total light intensity in the control attributable to component I, computed as indicated in that text, versus time of ampicillin treatment is plotted. Data were taken from four different experiments, using 10 (■, ▲) or 500 (△, base up and base down) μg of ampicillin per ml.

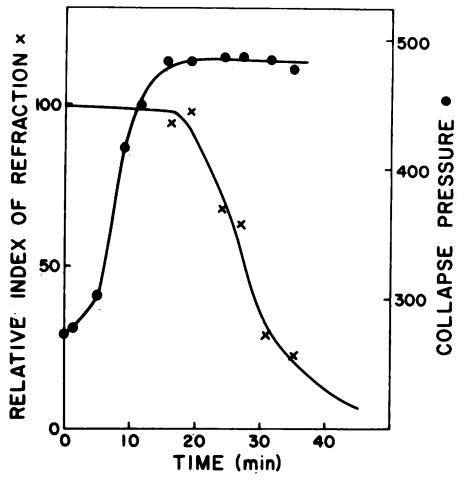

FIG. 4. Loss of cell substance from light-scattering measurements. The experiment represented in Fig. 1 is analyzed here in more detail and includes runs omitted from Fig. 1 for clarity. The leakage of the cytoplasm of the component II cells is shown derived from the decrease in the refractive index of the environment of the vesicles in component II. This was calculated as indicated in Materials and Methods. This last curve should be compared with that indicated by the closed symbols in Fig. 5b.

vesicles. The interpretation of the behavior of the light refracted by component II, however, is more complicated. It is shown below (see Fig. 5) that the turbidity of ampicillin cultures declined more slowly than did the turgor pressure. It follows that the loss of cytoplasm is slow or that the vesicles are released from the cells slowly or not at all or both. As a result, in any case the refractive index of the immediate environment of the vesicles would approach that of the medium very slowly.

It was tentatively assumed that the observed decrease in the light scattered at 90° (cf. Fig. 1C and D) was entirely due to a change in the index of refraction of the immediate surroundings of the vesicles and not to destruction of vesicles. It follows that the small observed decrease in the intensity of scattered light associated with component II shown in Fig. 1 was attributable totally to the lowering of the index of refraction within the cells of component II as leakage proceeded.

The results of such an analysis are shown in Fig. 4. The assumption was made that the decrease in 90° light scatter observed at the end of 35 min of treatment with 10 μg of ampicillin per ml represented partial loss of cytoplasm from the vicinity of the vesicle. From other more protracted experiments extending to 2.1 h, we estimate that the light scatter would have decreased after 35 min 15% more in achieving its final value. The total change, if no vesicles become destroyed, corresponds to the index of refraction of the original cell being 6% higher than the medium. On this basis the index of refraction of the cytoplasm was calculated at intermediate times, converted into percentage of original cytoplasm. The results are in Fig. 4 in the format of Fig. 5b, where the kinetics of loss in turbidity at the same ampicillin concentration was followed. Although this calculation is crude, a comparison of Fig. 4 and 5b shows that the index of refraction changed with the same kinetics as the loss of turbidity. Together they support the conclusions that (i) the loss of turgor before the loss of turbidity was due to an early release of pressure through minute holes in the cell wall (24) followed by the slow leakage of the cell contents; (ii) the index of refraction of the cytoplasm was 6% higher than that of the medium; and (iii) the destruction of vesicles during the time of these experiments is minimal.

Antibiotics causing biphasic collapse curves. A number of antibiotics were screened for changes produced in the collapse curve. Table 1 summarizes our findings with those antibiotics which produced biphasic curves similar to those produced by ampicillin. They were all cell β-lactam antibiotics with broad affinities for the various PBPs (9). Only antibiotics that caused inhibition of the growth of *A. aquaticus* M158 are listed. All but one of the cell wall growth-inhibiting antibiotics tested resulted in the development of two component collapse curves. The exception was mecillinam, which is known to bind selectively to PBP 2. The significance of this observation is not yet clear.

Mecillinam was tested at three different concentrations, 10, 80, and 500 μg per ml of growing culture. The lowest dose which caused a significant decrease in the growth rate of strain M158 was 10 μg/ml. No effect on growth after 6 h was observed with by 7 μg of mecillinam per ml. The 50%

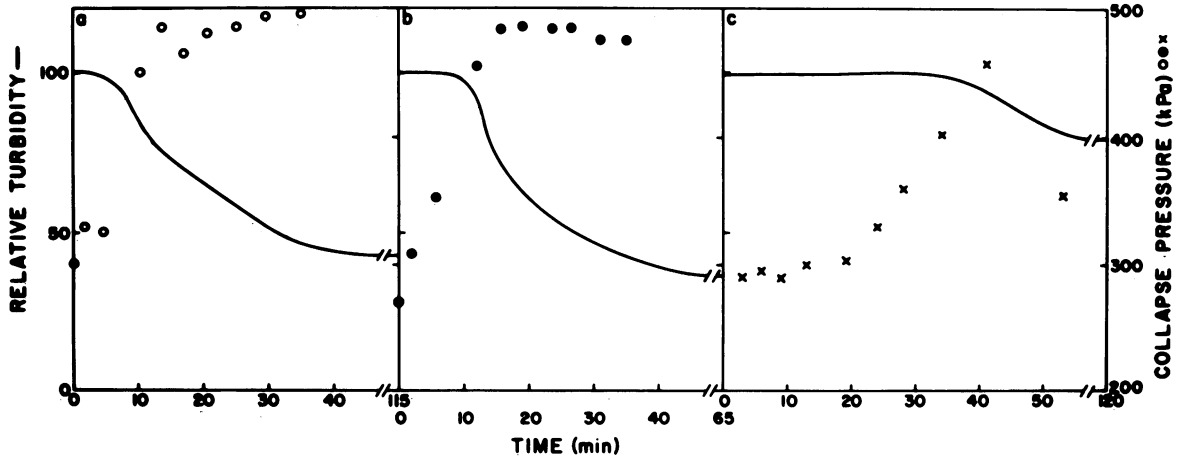

FIG. 5. The 50% collapse pressure and culture turbidity. Three different experiments with three different concentrations of ampicillin (○, 500; ●, 10; ×, 1 μg/ml) are shown. Both the 50% collapse pressure and turbidity after all vesicles in a sample were collapsed were monitored over time. In each case the bulk of the decrease in turgor pressure preceded the decrease in turbidity.

TABLE 1. Conditions producing biphasic collapse curves[a]

Antibiotic (μg/ml)	Mean observation time (min)	Component (kPa)		% Component I	No. of expt
		I	II		
Ampicillin[b]					
10	28	251.75 ± 8.51	481.75 ± 3.45	27.52 ± 5.15	4
500	22	193.00 ± 45.33	469.00 ± 27.60	20.14 ± 4.33	9
Penicillin G[c]					
500,000 IU/ml	35	202.33 ± 38.85	418.33 ± 47.01	16.13 ± 2.72	3
Cephaloridine[d]					
12.5	21	238	462	17.0	1
Cephalexin[e]					
5	23	286	479	33.7	1

[a] From the number of separate experiments a single representative biphasic curve closest to 20 min of treatment with ampicillin is reported here. Several other curves which had broadened slopes were also obtained in each experiment, and most of the experiments had two or more collapse curves with distinct components. Controls run over an 8-month period as part of every experiment had C_a values of 296 ± 23 (n = 63).

[b] 2 μg of ampicillin per ml causes a slowing in the growth rate.

[c] 50,000 IU of penicillin G per ml was the lowest concentration that always arrested the increase in turbidity of a culture.

[d] Cephaloridine arrested the increase in turbidity immediately with 5 μg/ml, but slowed the growth rate >50% with as little as 500 ng/ml after 3 h.

[e] Cephalexin at 1 μg/ml had no effect on growth rate as measured by turbidity in 4 h. On the other hand, 5 μg/ml decreased the growth rate by >50% after 2 h.

collapse pressure was obviously not dose dependent and rose by about 121 ± 14.6 kPA above the control for the three experiments. The SD of the collapse curves as an indication of the broadness of the curve was 119 ± 11.5 kPA, compared to 105 kPa for the controls to which no drug was added. This suggests that variability exists in the turgor pressure changes produced by mecillinam, but two distinct components were never observed. The 90° light intensity decreased an average of 13% after 1 h regardless of mecillinam concentration, suggesting that leakage of cytoplasm occurred at a rate independent of drug concentration.

Antibiotics other than β-lactams. A variety of antibiotic inhibitors of macromolecular synthesis were also tested. The results obtained with those which had growth-inhibiting effects on strain M158 are shown in Table 2. The turgor increased in chloramphenicol- and rifampin-treated cultures, whereas it decreased with kanamycin, streptomycin, neomycin, and chlortetracycline. In addition, two drugs, nalidixic acid and spectinomycin, showed no significant change in the collapse pressure or broadness of the curve. In none of these cases was there any evidence of a two-component system.

Interaction of ampicillin and nalidixic acid. Nalidixic acid at 10 μg/ml did not by itself change the collapse pressure or the SD of the collapse curve. When 500 μg of ampicillin per ml was added after 26 min of treatment with 10 μg of nalidixic acid per ml, a biphasic curve developed. After 26 min of ampicillin (and 52 min of nalidixic acid) treatment, component I had a 50% collapse pressure of 217 kPA and component II had one of 489 kPA. The vesicles of component I contributed 27% of the light-scattering intensity compared with that of the culture before either drug was added. Thus the cells, although prevented from DNA synthesis by the nalidixic acid, did not alter the proportions of the two turgor pressure classes.

Evidence for homogeneity of vesicles. When sucrose at a final concentration of 1 osmolal was added to an ampicillin-treated culture after the two components had developed, the biphasic nature of the collapse curve was lost and the midpoint of the collapse curve was 434 kPA, similar to 407 kPA obtained for non-ampicillin-treated cells to which 1 osmolal sucrose was added or to the values reported previously for turgor-free vesicles (15). The same result was obtained when 500,000 IU of penicillin per ml was used (403 kPA). The collapse curves of cultures to which sucrose had been added were not as broad as with either the ampicillin or penicillin alone. The collapse pressure ± SD for a culture treated with sucrose alone was indistinguishable from that calculated for the component II of cultures treated with either penicillin and sucrose or ampicillin and sucrose. The SDs of the component II collapse curves were 92 and 94 kPa for ampicillin and penicillin treatment and 97 kPa for the entire collapse curve of the control. This experiment was done to eliminate the possibility that the biphasic curves

TABLE 2. Effect of inhibitors of macromolecular synthesis on collapse pressure

Effect/antibiotic	Treatment		50% Collapse pressure (kPa)		Ratio[a]
	Time (min)	Concn (μg/ml)	Treated	Nontreated control	
Collapse pressure lowered					
Chloramphenicol	113	12.5	234	310	1.25
	139	2,500	262	310	4
	26	50	227	286	5
	20	100	238	276	10
Rifampin	73	5	259	303	5
	70	5	224	310	5
Collapse pressure raised					
Kanamycin	120	80	379	321	16
Streptomycin	169	5	345	317	1
Neomycin	182	12.5	396	296	2.5
Chlorotetracycline	204	50	414	293	
Collapse pressure not altered					
Nalidixic acid	62	100	321	314	
Spectinomycin	85	0.5	306	287	
	261	40	324	293	

[a] Ratio is the quotient of the dose used relative to the dose that just caused a detectable lowering of the growth rate within 2 h.

obtained were produced by some average difference in the properties of the vesicles within the two classes of cells and not by differences in the turgor pressure response of different groups of cells to the antibiotics.

Interaction of chloramphenicol and ampicillin. When 50 or 100 μg of chloramphenicol per ml was added to an ampicillin-treated culture either 12 min before or 14 min after the addition of ampicillin, the collapse curve broadened at 20 min after the ampicillin was added, as was observed in the absence of chloramphenicol. The two components, however, were less clearly delineated. Although precise determination of the 50% collapse pressures of the two components was not possible, it was clear that component II did not develop completely. It can, nevertheless, be concluded that the activity of ampicillin which produces the two turgor pressure classes is not immediately dependent on protein synthesis.

Effects on the ampicillin response of added Mg^{2+}. There have been a number of reports in the literature about the role of Mg^{2+} in preventing antibiotic-induced loss of turbidity (see reference 19). Consequently, measurements were made of collapse curves of ampicillin-treated cells to which appropriate concentrations of Mg^{2+} were added at different times before measurement. It was expected that, if some aspect of the development of the two component system were due to the same factors that affect turbidity, then an effect on the shape and time course of development of the biphasic curves should be observed. However, it was found that Mg^{2+} did not affect either the timing or the shape of the two-component curves. It did, however, shift all measurements of collapse pressure upward slightly. This was also true in the control without antibiotic. Since Mg^{2+} is an osmolyte, we expected an increase in 50% collapse pressure of 10 to 15 kPa from our previous results (15), but this increase cannot be interpreted to have involved a prevention of autolysis.

CCCP-ampicillin interaction. When CCCP was added before or after 500 μg of ampicillin per ml, the development of a biphasic curve was prevented seemingly by loss of the turgor of the cells of component I. The mean collapse pressure rose to a significantly higher value than that measured with CCCP alone or in the control to which no drug was added (data not shown).

DISCUSSION

Many responses of properly cloned cultures are heterogeneous. In addition to the effects of mutations arising in the population, there are many changes which affect a significant portion of the population. This variability within the culture may have important evolutionary and ecological significance (12). Important examples are the cases of spores that develop during balanced growth of cultures of bacilli (21), *Salmonella* cells which switch their flagellin type (28), Mu phages which switch their host range, etc. To this list can now be added the cellular response of *A. aquaticus* to a class of antibiotics affecting wall growth.

A similar heterogeneity in response to antibiotic treatment is apparent in the recent work from the laboratory of Labischinski (18) with *Staphylococcus aureus*. Other instances appear in recent literature. For example, *Escherichia coli*, at a low pH and low ampicillin concentration after treatment for 1 to 4 h, exhibited a plateau at which more than 10% survived (33), but then dropped to only 1 in 10^4 surviving. The critical conclusion to be drawn from our new studies is that the division into the two-component classes had already been made by the bacteria during their prior growth; that is, cell commitment had occurred before we treated the culture with antibiotic. Of course, the biphasic nature of the response could only be measured some few minutes afterwards, i.e., when the turgor pressure of some cells has increased and that of the majority has decreased.

When this biphasic response was first discovered, the first inference was that ampicillin blocks wall growth, and then the pressure builds up with continuing metabolism and uptake of solutes. Eventually the cell ruptures, resulting in loss of turgor pressure. In this view, component I would simply be the precursor of component II. This interpretation can be fully rejected, based on the kinetic studies of Fig. 1 to 3. Although the data for times up to 35 min are shown in Fig. 3, qualitative data were also obtained at 2.1 h, but the resolution is not satisfactory because some vesicles may be destroyed in such cultures. The data presented, however, clearly show that component I cells do not decrease as component II cells arise. Rather it appears that the component II cells lose their turgor without an appreciable transient increase. The component I cells respond by a rapid rise to a turgor pressure that then remains stable for many hours. The turgor pressure by definition is the result of the cell response to the difference in the osmotic pressure of the cytoplasm versus that of the suspending medium. The shift to a higher turgor observed in component I means that the tension in the peptidoglycan wall must increase. How is this possible when wall enlargement is blocked, while biosynthetic and other metabolic processes presumably are continuing? It can be conjectured that component I cells have elicited a special regulatory response. There are several mechanistic possibilities: first, the cells may control mechanisms to down-regulate macromolecular synthesis and the accumulation of solutes from the medium; second, they may be able to control leakage of the cytoplasmic constituents to a significant and perhaps safe degree; and third, they may develop a stronger wall that resists rupture, although the tension builds up to a high level. This last possibility seems to be the case with *Streptococcus mutans* (22). The cells do not lyse and the wall resists rupture, but RNA and protein syntheses are gradually blocked.

The loss of turgor in component II cells represents the breaching of the cell wall, the leakage of cell constituents, and presumably death. The experiments presented in Fig. 4 and 5 demonstrate that the pressure drops before the bulk of the cell constituents are lost from the cell, causing the eventual decrease in turbidity and in the index of refraction of the environment of the vesicles. This disparity of rates suggests that the number of "holes" created by the antibiotic treatment is few and that the total surface area they occupy is small. It is obvious that a fluid-filled container can lose its turgor completely with the egress of very little of the essentially incompressible cytoplasmic fluid. Loss of contained substances will take a longer time, because diffusion of the cell materials through the diffusion barrier will be slow. It is relevant to consider the case of the T-even bacteriophages. Each virus punctures a hole about 2 nm in radius during entry. Cell substance, such as UV-absorbing material, is released to the medium in easily measurable amounts (1). It can be presumed that turgor falls. The viruses carry a gene to repair the hole, although of course the cell is destined eventually to be lysed by the virus-coded lysozyme.

All of the β-lactam antibiotics tested, except mecillinam, gave a clear biphasic response. Although the PBPs of different organisms differ, there is a similarity of size and function (4) in gram-negative organisms. This suggests that, since mecillinam is specific in binding uniquely to PBP 2, this

substance, which is needed for cell elongation (9), is not part of the response that separates the two populations of cells even though it does affect turgor pressure. We were not able to test properly inhibitors of cell wall synthesis not related to the penicillins. Cycloserine and D-alanine did not affect the growth rate of *A. aquaticus*.

The phenomenon of biphasic response may be related to the general nature of penicillin's bacteriocidal character and the tolerance response. Three theories have been proposed for the bacteriocidal nature of penicillin action. Weidel and Pelzer (32) proposed that hydrolytic activity, essential for normal wall enlargement, continues after a synthetic step is blocked. This leads to rupture, lysis, and, in the presence of an osmotic stabilizing agent, protoplast formation. Shockman (27) and Blumberg and Strominger (2) enlarged on this hypothesis, adding that the poorly cross-linked wall resulting from penicillin action was too weak to resist the strain. This has recently been emphasized with studies of *S. faecium* (7). On the other hand, Tomasz (29) focused on the phenomenon of tolerance. This is the circumstance which occurs when the penicillin retains its bacteriostatic activity but loses its bacteriocidal effect. There are many cases in which tolerance occurs. It is still obscure why the natural populations are not all tolerant, but the fact is that they are not. Furthermore, antibiotics are not always needed to produce cell "autolysis." *S. pneumoniae* on plates lyse when the colony matures; *Bacillus subtilis* cultures lyse when they become anaerobic, etc. Tomasz suggests that blockade of the PBPs leads to small local lesions as the result of activation of hydrolases. These lesions lead to rupture of the plasma membrane and lysis (6, 30). For recent discussions of these possibilities, see references 9 and 25.

In the introduction, a second possible cause for heterogeneity observed in our experiments was mentioned. That is, perhaps at some stage of the cell cycle, such as constriction, failure of PBP action is lethal. There is some evidence that different PBPs may function interchangeably. More critically, it has been demonstrated that the bioenergetics of the process of stressed-wall formation must be different in regions of the cell where constriction is taking place (11). It has been shown that there is a sensitive period before cell division at which a pulse of penicillin is more lethal (19). This, plus the recent observation of Tuomanen (31), i.e., that only newly formed PBPs function, could explain the heterogeneity of response in the population. We have not pursued this point with *A. aquaticus* because, as noted before (22), the cell cycle of this organism is not very regular.

In *Staphylococcus aureus* it has been shown that the developing cross wall has pockets that presumably contain lytic enzymes that are caused to function during cell division (5, 24). Penicillin lysis is attributed to a special morphogenetic wall defect as the result of the cell separation process not being adequately preceded by wall growth. But it is evident that certain antibiotics with preferences for different PBPs respond differently. For *E. coli* it has been shown in many studies (3, 10) that cephaloridine has a preference for PBP 1 and causes cell lysis, while mecillinam has almost an exclusive preference for PBP 2 and causes ovoids considerably fatter than normal rod-shaped cells to form and cephalexin has a preference for PBP 3 and leads to filament formation.

Finally, the method used here together with improvements now in their final stages of development will allow us to study accurately the turgor pressure at the single-cell and cell population levels. The methods must be used with the understanding that they are limited by the variability in the properties of both the vesicles and the cells. Within these constraints, it will now be possible to attack many fundamental and long-standing problems.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM 34222 from the National Institutes of Health.

We thank Donna Mahnke, Paul Demchick, Linda Kwiatkowski, and Cheryl Boone for assistance and advice.

LITERATURE CITED

1. **Adams, M. H.** 1959. Bacteriophages, p. 164–168. Interscience Publishers, New York.
2. **Blumberg, P. M., and J. L. Strominger.** 1971. Inactivation of D-alanine carboxypeptidase by penicillins and cephalosporins is not lethal in Bacillus subtilis. Proc. Natl. Acad. Sci. USA **68:** 2814–2817.
3. **Burdett, I. D. J., and R. G. E. Murray.** 1974. Septum formation in *Escherichia coli*: characterization of septal structure and the effects of antibiotics on cell division. J. Bacteriol. **119:**303–324.
4. **Curtis, N. A. C., D. Orr, G. W. Ross, and M. G. Boulton.** 1979. Competition of β-lactam antibiotics for the penicillin-binding proteins of *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella aerogenes*, *Proteus rettgeri*, and *Escherichia coli*: comparison with antibacterial activity and effects upon bacterial morphology. Antimicrob. Agents Chemother. **16:**325–328.
5. **Geisbrecht, P., H. Labischinski, and J. Wecke.** 1985. A special morphogenetic wall defect and the subsequent activity of "murosomes" as the very reason for penicillin-induced bacteriolysis in staphylocci. Arch. Microbiol. **141:**315–324.
6. **Handwerger, S., and A. Tomasz.** 1985. Antibiotic tolerance among clinical isolates of bacteria. Rev. Infect. Dis. **7:**368–386.
7. **Higgins, M. L., M. Ferrero, and L. Daneo-Moore.** 1986. Relationship of shape to initiation of new sites of envelope growth in *Streptococcus faecium* cells treated with β-lactam antibiotics. J. Bacteriol. **167:**562–569.
8. **Holland, B. I.** 1987. Genetic analysis of *E. coli* division clock. Cell **48:**73–102.
9. **Höltje, J.-V., and U. Schwarz.** 1985. Biosynthesis and growth of the murein sacculus, p. 77–119. *In* N. Nanninga (ed.), Molecular cytology of *Escherichia coli*. Academic Press, Inc. (London), Ltd., London.
10. **Kitano, K., and A. Tomasz.** 1979. *Escherichia coli* mutants tolerant to beta-lactam antibiotics. J. Bacteriol. **140:**955–963.
11. **Koch, A. L.** 1961. Some calculations on the turbidity of mitochondria and bacteria. Biochim. Biophys. Acta **51:**129–441.
12. **Koch, A. L.** 1987. The variability and individuality of the bacterium, p. 1606–1614. *In* F. C. Neidhardt, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
13. **Koch, A. L., and I. D. J. Burdett.** 1984. The variable T model for gram-negative morphology. J. Gen. Microbiol. **130:**2325–2328.
14. **Koch, A. L., and E. Ehrenfeld.** 1968. The size and shape of bacteria by light scattering measurements. Biochim. Biophys. Acta **165:**262–273.
15. **Koch, A. L., and M. F. S. Pinette.** 1987. Nephelometric determination of turgor pressure in growing gram-negative bacteria. J. Bacteriol. **169:**3654–3663.
16. **Konopka, A. E., J. C. Lara, and J. T. Staley.** 1977. Isolation and characterization of gas vesicles from *Microvillus aquaticus*. Arch. Microbiol. **122:**133–140.
17. **Konopka, A. E., J. T. Staley, and J. C. Lara.** 1975. Gas vesicle assembly in *Microcyclus aquaticus*. J. Bacteriol. **122:**1301–1309.
18. **Labischinski, H., H. Maidhof, M. Franz, D. Krüger, T. Sidow, and P. Giesbrecht.** 1988. Biochemical and biophysical investigations into the cause of the penicillin-induced lytic death of staphylococci: checking predictions of the murosome model, p. 242–257. *In* P. Actor, L. Daneo-Moore, M. L. Higgins, M. R. J. Salton, and G. D. Shockman (ed.), Antibiotic inhibition of bacterial cell surface assembly and function. American Society

for Microbiology, Washington, D.C.
19. **Leduc, M., R. Kasra, and J. van Heijenoort.** 1982. Induction and control of the autolytic system of *Escherichia coli*. J. Bacteriol. **152**:26–34.
20. **Mychajlonka, M., T. D. McDowell, and G. D. Shockman.** 1980. Inhibition of peptidoglycan, ribonucleic acid, and protein synthesis in tolerant strains of *Streptococcus mutans*. Antimicrob. Agents Chemother. **17**:572–582.
21. **Piggot, P. J.** 1985. Sporulation of *Bacillus subtilis*, p. 73–108. *In* D. A. Dubnau (ed.), The molecular biology of the bacilli, vol. 2. Academic Press, Inc., New York.
22. **Pinette, M. F. S., and A. L. Koch.** 1987. Variability of the turgor pressure of individual cells of the gram-negative heterotroph *Ancylobacter aquaticus*. J. Bacteriol. **169**:4737–4742.
23. **Pinette, M. F. S., and A. L. Koch.** 1988. The biophysics of ampicillin action on a gas-vacuolated gram-negative rod, p. 157–163. *In* P. Actor, L. Daneo-Moore, M. L. Higgins, M. R. J. Salton, and G. D. Shockman (ed.), Antibiotic inhibition of bacterial cell surface assembly and function. American Society for Microbiology, Washington, D.C.
24. **Reinicke, B., P. Blümel, H. Labischinski, and P. Giesbrecht.** 1985. Neither the enhancement of autolytic wall degradation nor an inhibition of the incorporation of cell wall amterial are pre-requisites for penicillin-induced bacteriolysis in staphylocci. Arch. Microbiol. **141**:309–314.
25. **Rogers, H. J., P. F. Thurman, and I. D. J. Burdett.** 1983. The bactericidal action of β-lactam antibiotics on an autolysin-deficient strain of *Bacillus subtilis*. J. Gen. Microbiol. **129**:465–478.
26. **Schwarz, U., A. Asmus, and H. Frank.** 1969. Autolytic enzymes and cell division of *Escherichia coli*. J. Mol. Biol. **41**:419–429.
27. **Shockman, G. D.** 1963. Amino acid deprivation and bacterial cell wall synthesis. Trans. N.Y. Acad. Sci. **26**:182–195.
28. **Silverman, M., and M. Simon.** 1983. Phase variation and related systems, p. 537–538. *In* J. A. Shapiro (ed.), Mobile genetic elements. Academic Press, Inc., Orlando, Fla.
29. **Tomasz, A.** 1974. The role of the autolysins in cell death. Ann. N.Y. Acad. Sci. **235**:439–449.
30. **Tomasz, A.** 1979. The mechanisms of the irreversible antimicrobial effects of penicillins: how beta-lactams antibiotics kill and lyse bacteria. Annu. Rev. Microbiol. **33**:113–137.
31. **Tuomanen, E.** 1986. Newly made enzymes determine ongoing cell wall synthesis and the antibacterial effects of cell wall synthesis inhibitors. J. Bacteriol. **167**:535–543.
32. **Weidel, W., and H. Pelzer.** 1964. Bag-shaped macromolecules—a new outlook on bacterial cell walls. Adv. Enzymol. **26**:193–232.
33. **Yourassowski, E., M. P. Van der Linden, M. J. Lismont, F. Crokaert, and Y. Glupczynski.** 1985. Correlation between growth curve and killing curve of *Escherichia coli* after a brief exposure to suprainhibitory concentrations. Antimicrob. Agents Chemother. **28**:756–760.