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Growth Conditions and Rifampin Susceptibility

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The susceptibility of *Escherichia coli* to rifampin was measured during unlimited growth in rich and poor media and during chemostat growth limited by the carbon source. During batch growth at low turbidities, the susceptibility of the bacteria increased as the growth rate decreased, consistent with the longer time available for drug penetration in the poorer media. During chemostat culture, the bacteria remained highly susceptible or became genetically resistant, dependent on the manner in which the bacteria were exposed to the antibiotic. If the concentration of rifampin was abruptly raised, susceptible cells were replaced by genetically resistant cells. However, if the concentration of antibiotic was raised slowly, the genetically susceptible cells continued to grow. This difference in response of chemostat cultures according to mode of drug administration was attributed to an inducible detoxification of the drug by the bacteria, because the susceptible genotype is maintained only when the concentration of rifampin is increased gradually and when a high population of cells is maintained. Direct evidence for the inactivation of the rifampin from the bioassay of culture supernatants is presented.

An antibiotic might be expected to have variable effects on cultures of genetically susceptible bacteria when the bacteria grow under conditions permitting differing growth rates. This follows, since bacterial physiology and morphology are known to alter in many ways depending on the growth rate attained (13, 14, 20). Population steady-state doubling times can be manipulated within a wide range (in the present study, from 16 min to 14 h) by varying culture techniques from batch growth to slow chemostat culture, and by varying the amount and quality of available nutrient. Most studies of antibiotic effects have been conducted by utilizing bacteria growing in dilute suspension in adequate medium with fast to moderate doubling times (25 to 60 min). In nature, bacteria living under harsher environmental conditions will differ physiologically when compared to rapidly growing bacteria and may, therefore, be expected to differ in susceptibility to antibiotic action.

To begin to assess the importance of the growth rate dependency of susceptibility to antibiotics, we have studied the action of rifampin on *Escherichia coli* under a variety of different culture conditions. Rifampin was chosen as the antibiotic for initial study because it has a single site of action, i.e., in preventing the RNA polymerase from initiating transcription (3, 22, 23). Genetic resistance to rifampin occurs at *rpoB* which causes alteration of the β-subunit of the polymerase so that the enzyme still functions in RNA synthesis, but does not bind any rifamycin derivative (19). Such mutant cells grow normally in the presence of very high levels of rifampin, suggesting that additional effects of the drug are minimal.

We find that *E. coli* ML 308 in dilute batch culture is more susceptible to rifampin when the growth rate is low. This suggests that the dominant growth effect is due to slow penetration of the outer lipopolysaccharide layer (12). In other growth situations, detoxification mechanisms for the rifampin are important.

MATERIALS AND METHODS

E. coli strain ML 308 and its Rif^r mutant were grown in batch culture under forced aeration at 37°C as previously described (8, 10). The minimal medium employed was M9; the carbon source additions are given in the text. In addition, nutrient broth (Difco Laboratories) and a rich medium, designated LBK-glu and consisting of 10 g of tryptone, 5 g of yeast extract, and 2 g of glucose per liter of M9, were used. Chemostat culture was carried out in an apparatus (9) in which all or almost all of the parts wetted by the bacterial culture were made either of Teflon or polycarbonate, thus avoiding problems of wall growth. The medium for chemostat cultures was M9 plus carbon source. When the carbon source was 0.02% galactose, the average turbidity of the chemostat cultures was 0.623 ± 0.050 at 420 nm. This corresponds to 98 μg (dry weight) per ml or 1.5×10^8 bacteria per ml. Viable counts were carried out on an agar designed by Bochner and Savageau (4). The agar mixture consisted of 7.0 g of K_2HOP_4, 3.0 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 2.0 g of proteose peptone (Difco), and 15.0 g of agar (Difco) per liter of deionized, distilled

water. After autoclaving, 25 mg of 2,3,5,-triphenyl tetrazolium chloride and 10 ml of 20% galactose solution were added. Rifampin (Lepetit for Calbiochem) dissolved in a 1:1 CH_3OH/H_2O was added for final doses of 10 or 100 μg/ml. Thus, the agar contains a tetrazolium dye in the presence of sufficient buffer so that the colonies capable of fermentation of galactose produce the reduced colored formazan. The nonfermenting cells are colorless because the agar contains low levels of potentially fermentable organic nutrients. Susceptible cells grow only in the absence of rifampin. Genetically resistant cells have the same efficiency of plating on 0 and 10 μg of rifampin per ml, and are 54% as efficient on 100 μg/ml. (A cloned Rif^r organism plates with the same efficiency on plates containing 10 and 100 μg of rifampin per ml. However, the mutants present in cultures of the susceptible parent plate with 54% efficiency on 100 μg/ml plates relative to 10 μg/ml. We have not distinguished between plate mutants, phenocopies, or an additional class of mutation susceptible to 100 μg of rifampin per ml but resistant to 10 μg/ml.) No evidence was found for genetically stable forms giving rise to low-level-resistant colonies plating on 10 but not on 100 μg/ml. Tests for contamination were made by demonstration of the constitutive production of β-galactosidase in amounts characteristic of the strain ML 308 (10, 17).

Batch growth was followed turbidimetrically. Most measurements were made in a flow-through cuvette to remove the variation introduced by removing and replacing a cuvette in its holder. In some cases, the culture was allowed to grow within the constant temperature chamber of the spectrophotometer in a 1- or 10-cm cuvette system in which the growing culture is stirred and aerated in such a way that the air bubbles do not interfere with continuous measurement of the turbidity (21). This system, when used in the Cary model 16 spectrophotometer linked to a Wang 720C computer, was used when very precise growth rates were to be measured. The computer was programmed to calculate the specific growth rate during successive 800-s growth periods. Three absorbance measurements were averaged in each 2-s period, converted into dry weight concentration, and then the natural logarithm of the averages was used in a least-squares regression. The slope of the regression is an estimate of the specific growth rate. Successive values are shown by the same unconnected symbols in Fig. 2 and 3. The solid lines in Fig. 3 show the time course of growth. The specific growth rate goes through zero at the same time as there is a maxima of the dry weight concentration in the conventional plots (Fig. 1 and 2). The measurements were made at 420 or 660 nm, and correction for the absorption of rifampin was made where necessary.

The bioassay for rifampin was carried out in a manner identical to the batch growth studies: very low inoculum and 0.02% galactose in M9.

Reservoir bottles and petri plates containing rifampin were protected from light.

RESULTS

Balanced batch growth at low cell densities. Figure 1 shows the growth response in different batch media to 0, 0.5, 1.0, 1.5, and 2 μg of rifampin per ml. To facilitate comparison, the same symbols are used in each panel of the graph for the same concentration of rifampin. At 2 μg/ml, growth was inhibited but not immediately. The turbidity reached a maximum in 1 to 2 h and subsequently declined in all media. This decline has been observed before (16). The cells do lyse, and the cultures foam on prolonged aeration. It has been reported that the turbidity remains constant from the time that very high concentrations (e.g., 200 μg/ml) of rifampin are added and does not drop (1). We confirm this observation for the first 2 h after addition to cells growing in minimal glucose medium, or in glucose medium enriched with 0.2% Casamino Acids and 1% vitamins. However, there was a subsequent gradual decrease in turbidity. Thus, macromolecular synthesis was stopped very rapidly, cell lysis occurred more slowly at 200 μg/ml.

Quantitative differences in the time to attain turbidity maximum in these and two other media are given in Table 1 for 1 and 2 μg of rifampin per ml. In all of these cases, the bacteria had been in balanced growth in the same medium before drug addition. Although it is difficult to quantitate because of the lysis phenomenon, it is clear that during balanced growth rapidly growing bacteria are more resistant to rifampin than are more slowly growing bacteria.

The measurements reported in Fig. 1 and Table 1 were made with very low bacterial concentrations. This required careful optical measurement and, in some cases, correction for the contribution of the highly colored rifampin. The use of low concentrations of bacteria was mandated by the finding, reported in the next section, that the bacteria can detoxify the antibiotic. We believe that drug inactivation was not significant in any of the experiments presented in Fig. 1 and Table 1.

Even though all the cultures were started at low turbidities, detoxification could have been

TABLE 1. *Susceptibility of bacteria in balanced growth to rifampin*

Medium	Doubling time (min)	Time (h) to turbidity maximum at the following rifampin concn (μg/ml):[a]	
		1	2
LBK-glu	17	GC	GC
Nutrient broth	27	GC	2.0
Glucose-M9	43	GC	1.9
Galactose-M9	56	2.1	1.5
Succinate-M9	65	3.5	1.5

[a] GC, Growth continues indefinitely, but the growth rate may be temporarily less than the drug-free control.

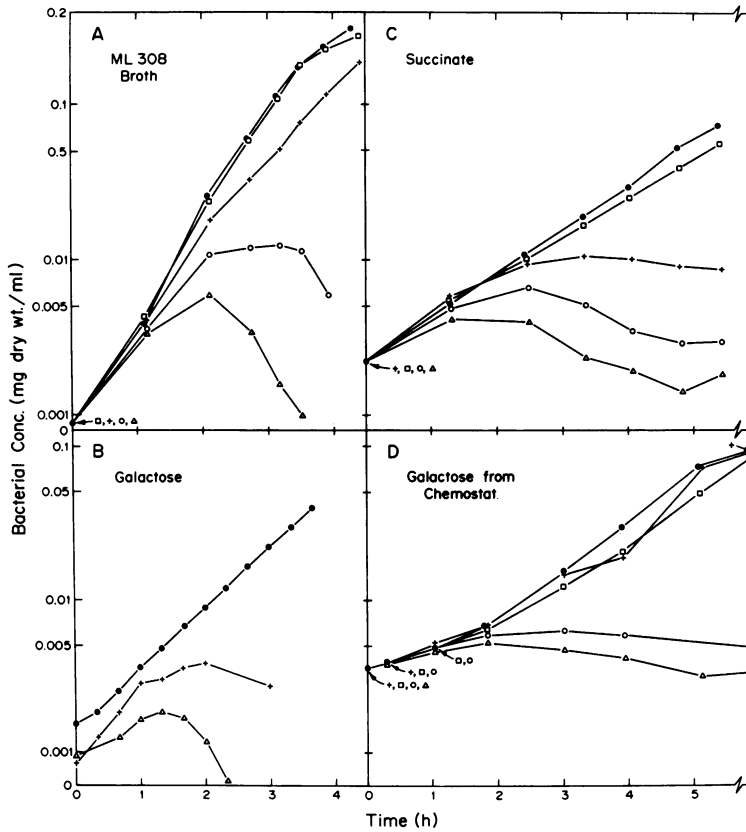

FIG. 1. *(A) Effect of rifampin on E. coli during batch growth in broth containing rifampin. E. coli ML 308 was cultured in nutrient broth at 37°C. Subcultures, diluted 26-fold into the same medium, were made with the following concentrations in micrograms of rifampin per ml: 0 (●), 0.5 (□), 1 (+), 1.5, (○), and 2 (△). Optical density was followed at 660 nm to avoid correction for absorbance by broth and by the rifampin. (B) Growth in galactose-M9 containing rifampin. The same type of experiment is shown as in A, except that the pregrowth and experiment were carried out in 0.02% galactose-M9. (C) Growth in 0.4% succinate-M9 containing rifampin. Growth was followed at 420 nm with correction for the contribution of rifampin to the apparent absorbancy. (D) Effect of rifampin on growth on culture diluted from chemostat in 0.02% galactose-M9. The cells were taken from a 13.2-h doubling time chemostat in the same medium and diluted 26-fold into the same medium employed in chemostat culture with various concentrations of rifampin.*

important in the rapidly growing broth culture of panel A, because cells grow so rapidly in this medium that dry weight concentration rose above 100 μg/ml before there was time for inhibition to become evident. Therefore, an additional experiment was done in which a culture was repeatedly diluted in nutrient broth with 1 μg of rifampin per ml to keep the bacterial concentration low. Growth continued without inhibition.

Balanced batch growth in glucose minimal medium. The solid lines in Fig. 2 show growth curves in 0.2% glucose-M9 medium containing 2 μg of rifampin per ml. At initial densities below 10 μg (dry weight) per ml, a similar growth response was observed to those shown in Fig. 1. With the largest initial inoculum, growth was not substantially impeded by the rifampin, consistent with greater drug detoxification by larger numbers of cells.

Growth measurement in the long light-path apparatus. In an effort to refine the growth measurements, and because of the need to work at low bacterial concentrations, we employed the 10-cm growth apparatus developed in other work (21). Because growth and aeration were carried out within the growth chamber housed directly inside the thermostated spectrophotometer and because the turbidity was automatically recorded by the computer, the precision of the data was greater than with manual operation.

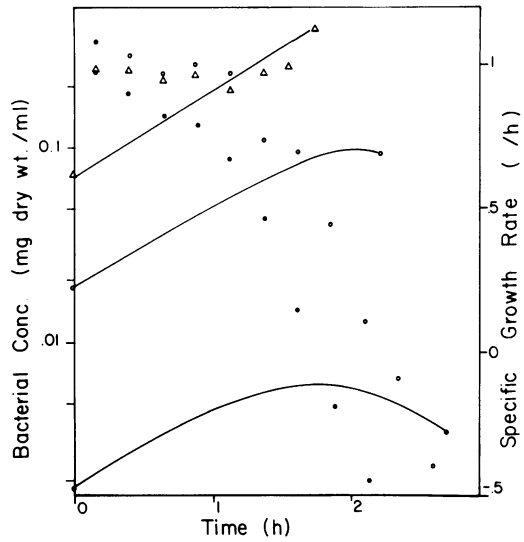

FIG. 2. *Response of ML 308 to 2 μg of rifampin per ml at different initial densities. Growth was followed at 37°C in the aerating cuvette system contained inside the thermostated cuvette compartment. Because growth was monitored by the computer, resulting in minimal error in measurement, the growth curves are drawn as solid lines. Data were collected, on the average, once every 0.6 sec. The computer output also yields the specific growth rate. This is indicated by the symbols and on the righthand ordinate. Each symbol indicates the average specific growth rate over an 800-s interval. The same symbols are affixed to the ends of the growth curve lines for identification.*

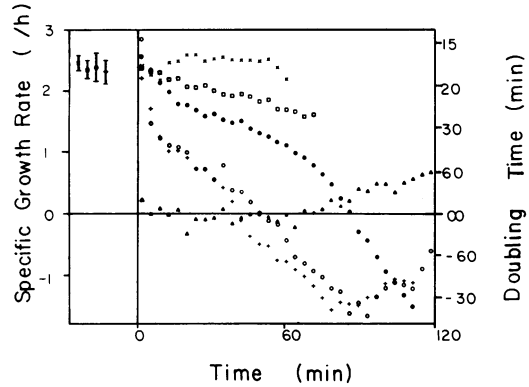

FIG. 3. *Effect of rifampin in medium supporting very fast growth. The data for this experiment were measured in 10-cm light path flow-through aeration cuvette. The turbidity measured by the double-beam spectrophotometer was converted by the on-line computer into the specific growth rate at 200-s intervals. Each successive period is marked with the same symbol. Symbols: X, drug-free control; □, 4 μg of rifampin per ml; ●, 10 μg of rifampin per ml; +, 20 μg/ml. The open circles (○) also correspond to an experiment with 10 μg of rifampin per ml, but the inoculum was taken from a culture in early stationary phase. A 5-min pulse of 80 μg of rifampin per ml followed by 40-fold dilution to 2 μg/ml was the experiment depicted with closed triangles, (▲). The medium used in this experiment contained 10 g of tryptone and 5 g of yeast extract per liter of 0.2% glucose-M9.*

Figure 3 shows results with a rich LBK-glu medium which supports a 16- to 18-min doubling time. In this very rich medium, a rifampin concentration of 4 μg/ml was slightly inhibitory, 10 μg/ml halted growth in 80 min, and 20 μg/ml slowed the specific growth rate to zero in 40 min. Also shown are two experiments demonstrating that penetration is rate determining for toxic activity. The circles show the progressive inhibition of the specific growth rate in the presence of 10 μg of rifampin per ml; the only difference between the open and closed circles is that the inoculum was not growing in the experiment shown with open circles because the parent culture had overgrown and had been starved for 30 min. It can be seen that inhibition developed more quickly than in the rapidly growing control. The second experiment shows the growth of cells treated with 80 μg of rifampin per ml for 5 min and then diluted to 2 μg/ml. Growth ceased immediately and resumed only after a prolonged interval. If after a 5-min pulse the cells were washed, growth reinitiated with a 40-min lag (data not shown).

Steady-state chemostat culture. In chemostat culture, the growth rate is determined by the flow rate and volume of the culture vessel selected by the experimenter. The organisms consume the limiting nutrient as fast as it enters the culture vessel. If an inhibitor is added, the effect can be seen only when it lowers the growth rate below the actual dilution rate of the chemostat. Then there will be a progressive decrease in the bacterial concentration.

When rifampin was added to both the growth chamber and the reservoir bottle at 10 μg/ml (Fig. 4) of a chemostat limited with 0.02% galactose, the optical density decreased 30% and returned to normal within 48 h. The incidence of Rif^r mutants increased from 2×10^{-5}% to 100%.

In contrast, if the drug was added only to the reservoir bottle, and therefore only entered the culture vessel gradually, the turbidity never showed a drastic decrease, and the population always remained predominantly susceptible to the rifampin when tested on plates containing either 10 or 100 μg/ml (Fig. 4).

The organisms from this and similar chemostats were not only genetically susceptible to rifampin when tested on plates, but they were

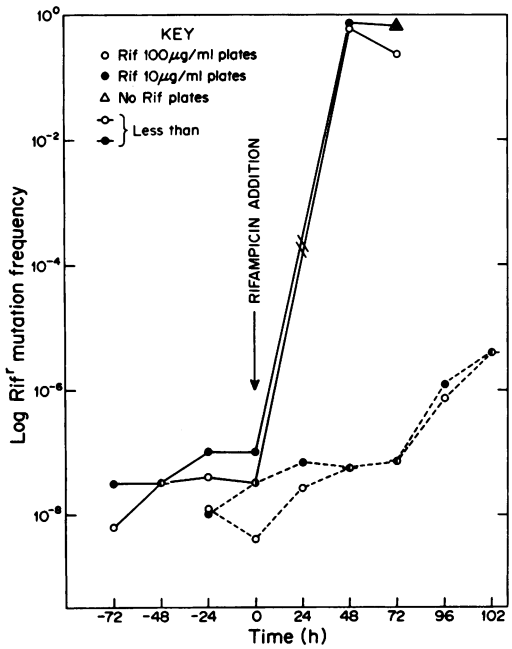

FIG. 4. *Comparison of gradual versus discontinuous addition of rifampin in selecting for Rif^r organisms. The course of development of Rif^r mutations in two chemostat cultures is shown. Both chemostat cultures were grown at 37°C with aeration in 0.02% galactose-M9. The doubling time corresponding to the dilution rate are 14.0 and 12.1 h for the solid and dashed curves, respectively. The mutant frequency, Rif^r/(Rif^s + Rif^r), was calculated from the colonies on plates containing 10 μg of rifampin per ml (closed symbols) or containing 100 μg of rifampin per ml (open symbols). The total concentration of organisms was inferred from turbidity measurements. Rifampin was added at zero time to the reservoir as well as the cultures vessel for the experiment shown in solid lines to a final concentration of 10 μg/ml. In the experiment shown in dashed lines, the same concentration was placed in the reservoir only. Thus, the concentration increased only gradually in the culture vessel with a half time of 12.1 h.*

also susceptible to the drug when tested for growth at low bacterial concentrations in fresh drug. When diluted into challenge doses in batch culture, the growth curves were identical to those shown in Fig. 1D, which present similar growth studies but are from chemostats not exposed to rifampin. Thus, paradoxically, the bacteria grow in a nominal 10 μg of rifampin per ml in the chemostat, but are killed in 2 μg/ml during the immediately ensuing growth test. Therefore, the bacteria from the chemostat cultures were not phenocopies of the drug-resistant Rif^r mutants.

Experiments I and III (Table 2) show the displacement of the susceptible population. Experiment V does not. It is clearly contradictory to the rest of our observations, but the experiment appears valid. Experiment IV shows that, under conditions in which the concentration of cells was low and rifampin at 2 μg/ml was added discontinuously, the susceptible bacteria were able to grow and therefore were more resistant than were the most slowly growing bacteria in the batch experiments of Table 1 and Fig. 1.

The phenomenon of maintenance of susceptibility even in the presence of presumed inhibitory concentrations of rifampin was observed on all chemostat trials in which the drug was gradually introduced and in which the steady-state bacterial concentration was supported with the standard 0.02% galactose in the M9 medium (Table 3). In all these experiments, the majority of the organisms remained susceptible and did not grow indefinitely at high dilution in liquid medium or give a colony on rifampin-containing plates. These results suggest that the bacteria are capable of detoxification of the drug entering their environment as a combined action of a sufficient number of cells.

A test of this hypothesis is to lower the concentration of galactose and thus the number of bacteria that can engage in detoxification. In such experiments (Table 3), with the gradual addition of rifampin at 10 μg/ml, the chemostats washed out and the susceptible population was never able to maintain itself. In experiment VI with 0.01% galactose, the turbidity level was quickly reestablished, but in experiment VII and 0.002% galactose only after a very low period, 70 h (43 nominal doubling times), did the turbidity increase. However, it increased to less than one-tenth the expected value from the galactose concentration, and the population plated as partially susceptible. We did not establish the nature of the organisms that did eventually grow.

In experiment VIII (Table 3), the rifampin concentration was changed discontinuously with small additions to both the reservoir and the growth chambers. Not until the increment from 4 to 6 μg/ml did the chemostat temporarily show a decrease in turbidity. When the normal turbidity was reestablished, a predominantly susceptible population was observed, but one with a much enriched proportion of Rif^r mutants. In this case, we did show that the susceptible population was very efficient at rifampin detoxification by the test described in the next section. The major point is that chemostat cultures with a lower cell concentration are less able to resist rifampin.

Bioassay of the potency of rifampin exposed to bacterial cultures. Control experiments showed that the rifampin present in the reservoir bottle had not lost its potency even

TABLE 2. *Chemostat experiments with discontinuous introduction of rifampin*

Expt no.	Rifampin dosage (μg/ml)	Chemostat doubling time (h)	Initial washout	Mutant frequency of resulting population[a]
0.02% Galactose-M9				
I	10	14.0	Yes[b]	103
II[c]	10	12.7	No	3×10^{-4}
0.02% Glucose-M9				
III	10	8	Yes	59
0.002% Galactose-M9				
IV	2	13.3	No	$<2 \times 10^{-5}$
V	6	2.14	No	2×10^{-5}

[a] Mutant frequency is the ratio to the total number of cells inferred from turbidity measurements, i.e., 100 $Rif^r/(Rif^r + Rif^s)$.

[b] Yes, Cases in which the turbidity of the chemostat effluent dropped below 30% of its predrug value.

[c] Induced for 30 min with rifampin at 1 μg/ml before the level in the culture vessel and the reservoir was brought to 10 μg/ml.

TABLE 3. *Chemostat experiments with gradual introduction of rifampin*[a]

Expt. no.	Rifampin dosage (μg/ml)	Chemostat doubling time (h)	Initial washout	Mutant frequency of resulting population (%)
0.02% Galactose-M9				
I	10	12.1	No	5×10^{-4}
II	10	1.0	No	7×10^{-4}
III	2	13.2	No	7.2×10^{-5}
IV	2	1.8	No	6.1×10^{-5}
V	1	13.4	No	3×10^{-5}
0.01% Galactose-M9				
VI	10	1.6	Yes	42
0.002% Galactose-M9				
VII	10	1.6	Yes	62
VIII	2[b]	13.3	No	$<2 \times 10^{-5}$
	4	1.7	No	$<1 \times 10^{-4}$
	6	1.7	Yes	5×10^{-1}
IX	2[b]	11.4	No	$<5 \times 10^{-6}$
	4	11.4	No	1×10^{-5}

[a] See footnotes to Table 2.

[b] Rifampin was added by means of several small-step additions of 2 μg/ml. In each step, rifampin was added to both the culture vessel and the reservoir.

during the longest period (several days) that elapsed between the time the rifampin was added to the medium and the time it entered the chemostat. Nor was it significantly destroyed in room light at 37°C with aeration under the conditions in which we culture bacteria. On the other hand, the potency of the rifampin leaving the bacterial growth chamber was reduced relative to that entering the chemostat (Fig. 5), whereas the supernatant of culture withdrawn directly from the culture vessel was essentially devoid of activity. Thus, the detoxification was, to a degree, a reversible one.

The selective disadvantage of the Rif^r allele. A necessary control for all the chemostat experiments is the measurement of the relative rates of growth of Rif^r and Rif^s organisms in the absence of rifampin. A preliminary experiment was performed, testing our stock of ML 308 against a stock derived from a rifampin-resistant derivative by mixing them together in the proportions of 1,000:1 in the growth chamber of a 12-h chemostat. The Rif^r organism grew 2.5% faster than did its parent. Since the rifampin-resistant polymerase should be less effective than the wild type, this result is unexpected and suggests the possibility of random genetic drift in picking the clone of Rif^r organism actually tested.

Consequently, we repeated this experiment,

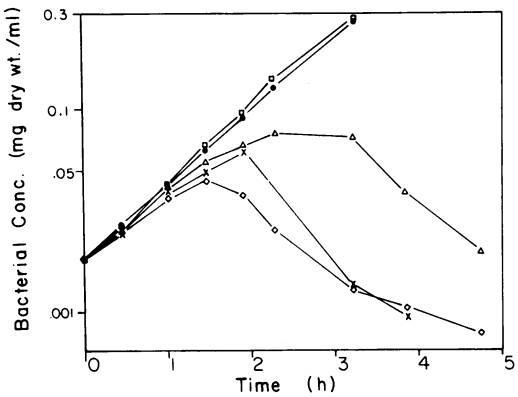

FIG. 5. *Detoxification of rifampin. ML 308 was grown in a galactose-limited chemostat at a low level of carbon source (0.002%). The doubling time was maintained at 11.4 h. After 80 h, 2 μg of rifampin per ml was introduced abruptly into the culture vessel and the reservoir. After 63 h, the rifampin concentration was raised to 4 μg/ml abruptly; 23 h later, the supernatant of the chemostat and of a sample of the overflow collected on ice were tested with ML 308 naive to rifampin. At this time the level of Rif^r organisms was 1.3 × 10^{-7} of the Rif^s populations. Visual evidence of the detoxification was provided by the bleaching of the highly colored rifampin in the culture vessel and the redevelopment of color in the effluent. This experiment was conducted with chemostat listed as experiment IX (Table 3). Symbols: ●, control; △, 2 μg of rifampin per ml; ◇, reservoir 4 μg/ml; □, supernatant from culture vessel; X, supernatant from collection tube kept on ice.*

taking precautions to avoid such drift. The ML 308 stock was streaked, and all of one colony was used to start a culture. All of that culture was used to start a chemostat culture (doubling time, 10 h). As soon as the turbidity became steady, a "pulse" of rifampin (10 μg/ml) was added to the culture vessel alone. The dose was chosen to cause a temporary drop in turbidity, such that when the drug had been washed out and/or detoxified and the turbidity had returned to normal, the incidence of Rif^r would be increased approximately 1,000-fold. In actuality, the incidence rose from 2.3 × 10^{-6}% to 1.1%. This chemostat culture was then followed for 166 h. Value of the slope of the linear regression of the logarithm of the mutant frequency versus time corresponded to a selective disadvantage of 12.6 ± 0.5% per generation. This experimental design minimized genetic variation by using all the descendants of a single cell for the culture and thus minimizing the number of generations in which mutants of any kind could be formed and accumulate in the culture. Secondly, the Rif^r organisms selected by the pulse of rifampin were derived from approximately 200 independently arising mutations that had occurred during the growth from a single cell. Therefore, the competition was between a collection of rifampin-resistant mutations arising independently (and mostly in the absence of the drug) rather than a particular mutant selected during a single-cell isolation.

DISCUSSION

Gram-negative organisms are much more resistant to rifamycins than are gram-positive organisms; e.g., the minimal inhibitory concentration of rifampin for *E. coli* is 1 to 10 μg/ml and for *Staphylococcus aureus* is 0.025 μg/ml (2), even though the RNA polymerase in vitro is inhibited in both cases by rifampin at 0.01 μg/ml (2, 22, 23). This difference suggests that penetration of the outer membrane of gram-negative cells must be very slow. In fact, gram-negative cells with defective envelopes are more susceptible to a variety of antibiotics (11) including rifampin. For example, strain χ^d 1776 of *E. coli* strain K-12 specially developed in the laboratory of Roy Curtiss for plasmid research has three lesions in different parts of the outer membrane structure and is susceptible to 0.04 μg of rifampin per ml, only four times the concentration inhibiting RNA polymerase. Measurements of penetration rate with wild-type organisms are consistent with the time required for growth to stop, if it is assumed that saturation binding of the polymerase in vivo is required to stop growth. Thus, our findings are consistent with that of White and Lancini (24) that 5 min is sufficient to saturate the RNA polymerase contained within the bacteria when exposed to rifampin at 20 μg/ml; we found that it takes 10 to 20 times longer for bacterial cultures to reach stop growth when exposed to one-tenth the dose (2 μg/ml).

Our results show that very rapidly growing cells are more resistant than more slowly growing bacteria in balanced batch growth. Quantitation of this effect is not straightforward because of the possible effects of the physiology of bacterial growth on cellular susceptibility to drugs and because of the lysis of the cells. Moreover, we cannot separate experimentally the time for penetration from changes in surface to volume ratio as cell size changes in response to growth rate (20). Both of these factors tend to protect differentially more rapidly growing bacteria.

There are other factors that tend to protect more slowly growing bacteria. For example, the cell envelopes of slowly growing cells may be thicker and more impermeable than those of more rapidly growing cells. Another factor is the

possible variation in the reserve level of RNA polymerase in bacteria growing at different rates. With greater reserves, higher inhibitor concentrations should be needed to slow growth, since cell regulation mechanisms should activate the reserves to compensate for the applied inhibitors. The number of enzyme molecules actually working during balanced growth has been estimated by Dennis and Bremer (6) and the RNA polymerase content has been measured by Ishihama et al. (7). From these studies it is clear that most of the enzyme is reserve (50 to 80% of total) at moderate growth rates. From our previous study of chemostat culture (15), there is very little increase in the number of working polymerase molecules even in the slowest chemostat culture studied here. Extrapolating from the data of Ishihama et al. (7), we estimate that 88% of the RNA polymerase at the slowest growth rate studied is reserve.

Although the present study demonstrates a detoxification system that protects dense cultures of organisms, it is much less effective in dilute cultures. We have not measured the degree that this system protects isolated cells by destroying the rifampin that enters the cell. In summary, however, of the effects in dilute batch culture, the observed increase in susceptibility to rifampin with decrease in growth rate of *E. coli* is primarily due to the increased time available for drug penetration.

Inducible detoxification systems are known for a number of antibiotics (5). Based on experiment II (Table 2), in which exposure of a chemostat culture for 30 min to rifampin at 1 μg/ml allowed it to withstand 10 μg/ml, we conclude that the rifampin detoxification system is probably inducible.

The factors of wall thickness, RNA polymerase reserves and detoxification protection of susceptible cells discussed above may be more important for bacteria growing slowly under nutrient limitation than when growing faster in the unlimited environment of batch culture. When the galactose concentration of the chemostat medium was lowered, resulting in a culture of low density, and the rifampin was introduced discontinuously into both the culture vessel and the reservoir, the bacteria were more resistant than predicted from the faster batch culture studies. Thus, in experiment IV of Table 2, growth of susceptible cells with a doubling time of 13.3 h continued indefinitely in the presence of 2 μg of rifampin per ml in the chemostat, whereas growth of cells with a prior doubling time of 1.1 h in succinate-M9 was blocked (Fig. 1).

Part of the increased resistance of chemostat cultures may be accounted for by an additional phenomenon expected to occur during chemostat-limited growth. If a growth inhibitory concentration of a drug is introduced into a chemostat, the organisms will tend to be unable to utilize the limiting nutrient. Then, as the concentration of organisms in the culture decreases slightly due to washout, an increase in the concentration of free nutrient in the chemostat will result. This causes a stimulation of growth by increased availability of the previously limiting nutrient and tends to counteract the growth inhibitory action of a toxic or antibiotic substance. This amelioration should be increasingly effective as the speed of the growth permitted in the chemostat decreases. This effect cannot account for the total increase in resistance in chemostat cultures, since the appearance of resistant mutants does not show this growth rate dependency. Instead, the susceptible organisms of the 1-h chemostat of experiment II (Table 3) appear to be able to maintain themselves against a gradual challenge of 10 μg of rifampin per ml just as well as the organisms of the 12.1-h chemostat of experiment I.

These findings and the loss of bioassayable rifampin activity in the supernatant of chemostat cultures lead to the conclusion that detoxification can be important in chemostat culture, particularly when the bacterial density is high and the dilution rate low, and at high cellular densities in batch culture. Because of detoxification and the slowness of drug penetration, under natural conditions the susceptibility of this organism to this antibiotic would be highly variable, depending on prior growth conditions, cell densities, and time course of drug appearance in the environment. This variability may be much more general, with implications for the study of procaryote evolution and for antibiotic therapy.

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