Resistence of *Escherichia coli* to Penicillins

V. Physiological Comparison of Two Isogenic Strains, One with Chromosomally and One with Episomally Mediated Ampicillin Resistance

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Two essentially isogenic strains of *Escherichia coli* K-12 were compared: D31 had chromosomally and D1-R1 episomally mediated resistance to ampicillin. The two strains had the same ability to form colonies on ampicillin plates, but in other tests they were quite different. In serial dilution tests as well as in exponentially growing cultures, D1-R1 was far more resistant to ampicillin than was D31. The inoculum effect with D1-R1 was large and with D31 was rather small. On plates, D31 was more resistant to penicillin G than was D1-R1. The penicillinase activity of buffer suspended cells against DL-ampicillin was 15 times higher for D1-R1 than for D31, but the two strains showed about the same rate of hydrolysis of penicillin G. With DL-ampicillin as substrate, for D1-R1 the apparent $K_m$ was $1.7 \times 10^{-4}$ M, whereas D31 gave a slightly sigmoid curve with a half-saturation concentration of about $5 \times 10^{-3}$ M. No induction of penicillinase activity was found. When the growth rate was varied by a factor of four, the amount of penicillinase per cell mass was constant in both D1-R1 and D31, whereas in two wild-type strains the amounts of penicillinase increased with increasing growth rates. With exponentially growing D1-R1, ampicillin disappearance started within 3 min, but at low ampicillin concentrations the rate was less than 10% of the rate of hydrolysis by buffer-suspended cells. Before D31 started hydrolysis, there was a lag period that lasted at least one generation and depended on the concentration of ampicillin. After this lag period, the rate of hydrolysis was 10 times higher than that observed with buffer-suspended cells. These differences between growing and non-growing cells indicate that both the chromosomally and the episomally mediated penicillinases are controlled by some products present in growing cells.

*Escherichia coli* can acquire high resistance to penicillins in two different ways, either as the result of several consecutive alterations involving at least two chromosomal genes (H. G. Boman et al., Genet. Res., in press), or by the introduction of an extrachromosomal R factor (6, 19). Both types of resistance are in part due to the production of cell-bound penicillinases. The enzyme made from a chromosomal resistance gene (*ampA*) and the corresponding wild-type protein have been purified (B. Lindstrom and H. G. Boman, Biochem. J. 106:43p, 1968). Purification and characterization of a penicillinase mediated by an R factor (*Rₜₐₚₘ*) has been described by Datta and Richmond (8).

Resistance tests can be performed in liquid media to determine the minimal inhibitory concentration (MIC) and on agar plates with the disc method. However, in genetic experiments it is desirable to define resistance as the maximal concentration of a substance which allows a single cell to form a colony. By this criterion, the R factor-containing strain D1-R1 [the same as strain 711 with R1 of Meynell and Datta (13)] was resistant to a DL-ampicillin concentration of 100 μg/ml. From the parent strain D1, transduction and ethyl methane sulfonate (EMS) treatment gave the *ampA*-containing strain D31 (genetically characterized by Boman et al., Genet. Res., in press), which also was resistant to 100 μg/ml. Except for the resistance genes, the two strains were therefore isogenic. We have now compared the two strains with respect to other resistance determinations, their production of penicillinase in different media, and the kinetics of ampicillin hydrolysis by whole cells. Since penicillin acts only on growing cells, we have compared ampicillin hydrolysis by growing and nongrowing cells of both strains. The results show that the two strains differ markedly in their reactions to penicillins. A preliminary account of this work has appeared (Burman, Nordström,
Materials and Methods

Strains, media and growth conditions. E. coli K-12, strain D1-R1, carrying the R factor R1, and its parent strain D1 [strain 711 of Meynell and Datta (15)] were obtained from N. Datta. The episome R1 is infective and carries resistance to ampicillin, chloramphenicol, sulphonamides, and kanamycin (13). Strain D2 was derived from D1 by introduction of the ampA1 gene (9) by transduction. EMS treatment of D2 and selection on plates with 100 μg of DL-ampicillin per ml gave strain D3. Then D1, D2, and D3 were made resistant to streptomycin, yielding strains D11, D21, and D31 (Boman et al., Genet. Res., in press). Strain D31 is believed to contain ampA1 and another chromosomal mutation, ampB, possibly in close proximity through a transposition denoted ampAB. The resistance of D31 was not infective, but segregated in crosses with sensitive Hfr strains (Boman et al., Genet. Res., in press). Strain G11, which carries the wild-type allele of ampA1 has been characterized elsewhere (K. Nordström, K. G. Ericksson-Grennberg, and H. G. Boman, Genet. Res., in press).

Basal medium E of Vogel and Bonner (20) was used whenever a minimal medium was desired. It was always supplemented with thiamine; for the D strains, it was also supplemented with the required amino acids proline, tryptophan, and histidine, and for G11 with methionine, isoleucine, and valine (25 μg/ml of the L-epimer). The complete medium was LB of Bertani (1) supplemented with medium E (but containing 0.2% glucose). It was solidified with 1.5% agar (LA plates). All experiments were performed at 37°C, and growth was determined by reading optical density with a Klett colorimeter with filter W66.

Materials. DL-Ampicillin (α-aminobenzyl penicillin with the D and L epimers in the ratio 2:3), D-ampicillin G (benzy1-penicillin), and DL-ampicillin labeled with 14C in the carboxyl group of the side chain (2.5 μc/mg) were kindly donated by AB Astra, Södertälje, Sweden.

Determinations of resistance. The bacteria to be characterized by the single-cell test were grown in LB on a rotary shaker and harvested in the exponential phase of growth. About 200 cells were spread on LA plates containing different amounts of ampicillin. The resistance level was defined as the maximal ampicillin concentration permitting 100% cell survival. The method has recently been discussed by Nordström et al. (Genet. Res., in press).

In serial dilution tests, exponentially growing bacteria were diluted and added to test tubes containing 5 ml of LB and different concentrations of ampicillin. After incubation overnight at 37°C, the tubes were scored for turbidity.

Determinations of penicillin-β-lactamase activity. Potassium phosphate buffer, 0.05 M, pH 7.0, was used for suspending cells and for performing the two first assays described.

Isotope assay. The sample to be tested for penicillinase activity was incubated with 14C-ampicillin at 37°C. At intervals, 10-pliter samples from the reaction mixture were spotted on Whatman 3MM paper which was then developed by ascending chromatography for about 4 hr at 4°C. The solvent consisted of 30 ml of n-butyl alcohol, 20 ml of ethyl alcohol, 20 ml of ether, and 30 ml of water. Ampicillin and the reaction product, ampicilloic acid, were localized by ninhydrin (9). The ampicilloic acid spots were cut out and counted in a liquid scintillation counter (System 723, Nuclear-Chicago Corp., Des Plaines, Ill.). One unit of penicillinase was defined as the amount of enzyme that hydrolyzed 1 μmole of ampicillin per min at pH 7 and 37°C.

Automatic iodometric assay. We used Novick's (14) iodometric method, adapted to the Technicon Autoanalyzer (Lindström and Nordström, unpublished data). Since the stalk recommended by Novick precipitates in the tubing, it was replaced by the more soluble Zulkovsky's stalk (Merck, Darmstadt, Germany). It is not necessary to remove bacteria before samples are introduced into the Autoanalyzer. However, aromatic amino acids, especially tyrosine, consume large amounts of iodine, and analysis cannot be performed in the presence of LB or other media which contain such amino acids. The method can detect the hydrolysis of 1 μg/ml (2.5 μmoules/ml) of penicillin. It is possible to increase the sensitivity 10-fold, but the spontaneous hydrolysis of penicillin may then be disturbing. The rate of spontaneous hydrolysis of ampicillin was higher than that found for penicillin G. For each experiment, serial dilutions of ampicilloic acid were used as standards.

Bioassay on plates with Sarcina lutea. The method of Kavanagh (10) was used to determine the disappearance of ampicillin during growth in LB medium. Each 9-cm petri dish contained 6 ml of Penassay Base Agar (Difco). The test organism, S. lutea ATCC 9341 (obtained from U. Forsgren), was grown in tryptose broth to a density of 300 Klett units. A 1-ml amount of this culture was mixed with 100 ml of Penassay Seed Agar (Difco), and 3 ml was added to each plate. The culture samples containing ampicillin were filtered through 23-mm filters (Ca5, Membranfilter, Göttingen, Germany); paper discs (diameter, 5 mm) were soaked in the filtrates and applied to the agar surface. Zones of growth inhibition were measured after 18 hr of incubation at 30°C and were compared to those obtained with known standards used in the same experiment. Ampicillin concentrations of 2 to 25 μg/ml could be determined with this method.

Results

Determinations of resistance by different methods. The two isogenic strains used in this study were selected for comparison, since both of them could form single-cell colonies on plates with a maximal DL-ampicillin concentration of 100 μg/ml. We first investigated to what extent resistance on solid medium corresponded to resistance in liquid medium. Exponentially growing cultures of D1-R1 and D31 were diluted at
zero-time into a number of subcultures containing about 10^6 cells/ml and different concentrations of ampicillin. Incubation was continued, and the optical density was recorded. Figure 1 shows that by this test the R factor-carrying strain D1-R1 was much more resistant than the ampAB strain D31. Growth inhibition was observed at 30 µg/ml for D31, but only at 300 µg/ml for D1-R1. When viable count was determined (not shown), it was found that cell division was slightly inhibited at lower concentrations of D1-ampicillin. Lysis, recorded as a decrease in optical density, was observed at 40 µg/ml and 1,300 µg/ml for D31 and D1-R1, respectively.

Another standard method for determination of resistance is the serial dilution test. Tubes with 5 ml of LB and various concentrations of ampicillin were inoculated with different numbers of cells and incubated overnight. Figure 2 shows that by this test also D1-R1 was much more resistant to ampicillin than D31. With small inocula (fewer than 10^6 cells/ml), the maximal concentration permitting growth was 100 µg/ml for D1-R1 but was only 20 µg/ml for D31. These figures represent the resistance of the individual cells. With larger inocula, the tolerated concentration of ampicillin increased markedly for D1-R1, but only slightly for D31. In this case, it is the survival of the population rather than that of single cells that is determined. However, it should be stressed that tests of this type with large inocula are difficult to interpret. The inoculum effect is generally attributed to the presence of penicillinase. Production of a large amount of penicillinase reduces the penicillin concentration, and growth resumes. The results given in Fig. 2 therefore suggest a higher penicillinase activity in D1-R1 than in D31 cells. At high concentrations of ampicillin, most of the cells died initially, and the survivors later grew out to a dense population. After such an experiment with a large inoculum, we have tested the surviving cells and found them to have the same resistance as the initial population.

A summary of the resistance determinations is

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**FIG. 1.** Growth curves of D31 and D1-R1 cells in the presence of ampicillin. The cells were grown in LB on a rotatory shaker at 37 C. At zero-time (at 25 Klett units = 10^6 cells/ml), ampicillin was added as indicated in the figure.
given in Table 1. Ampicillin is a potent antibiotic against E. coli and most other gram-negative bacteria, whereas penicillin G is much less inhibitory. The two strains were therefore also compared with respect to their resistance to penicillin G and the pure D epimer of ampicillin. The results in Table 1 show that, by the single cell test on plates, the ampAB strain D31 was considerably more resistant to penicillin G than was D1-R1.

A microscopic comparison was made of D1-R1 and D31 grown on LA plates with different amounts of penicillin. At lower concentrations, both strains produced normal, motile cells. At the highest permissible concentration, the cells of D1-R1 still seemed to be normal, whereas D31 produced filaments, often with sectional enlargements ("bubbles"). This indicates that the lethal action of penicillin may be somewhat different in the two strains.

Amount of penicillinase in cells pregrown at different rates. That penicillin kills only growing cells is an old observation (17). It has also been shown that, for a penicillin-sensitive strain, the time to penicillin-induced lysis is shorter the faster the cells grow (2). More recently, we have found that differences in growth rate produced three- to fivefold differences in the amounts of penicillinase in ampA-containing strains, whereas strains containing both ampA and ampB showed only minor variations (3). We therefore wanted to characterize the new strains in the same way. Strains G11, D11, D21, D31, and D1-R1 were grown in the absence of penicillin on a rotatory shaker in five different media which yielded growth rates of 0.5 to 2.0 doublings per hour. All cultures were harvested at the same optical density (4 × 10^6 cells/ml in LB), washed once in 0.9% NaCl, and then assayed for penicillinase activity. In Fig. 3a, penicillin G was used as substrate since in wild-type strains the activity against ampicillin was too low to be measured (cf. Table 1). In Fig. 3b, the substrate was 14C-DL-ampicillin (see Material and Methods). A comparison of the figures shows that the resistant strains D21, D31, and D1-R1 had much more penicillinase activity than the two wild-type strains. The penicillinase activity of G11 and D11, as well as of the ampAI strain D21, increased two to four times when the growth rate was increased by a factor of four. As pointed out previously (3), this correlation indicates that the enzyme has a growth rate-dependent function in the normal biosynthesis of the cell. D31 contained more penicillinase than D21 at all rates of growth. The ampAB strain D31 can therefore be regarded as having a constant overproduction of the enzyme. The R factor-carrying strain D1-R1 contained 15 times more penicillinase than D31. The absence of regulation of the penicillinase activity in D1-R1 is reasonable, since the episome is completely dispensable.

Both strains were tested for ampicillin induction of the penicillinase. D1-R1 and D31 cells were grown in the presence of 100 and 20 μg of ampicillin per ml, respectively, and cells were harvested at various times and assayed for penicillinase activity. No change in enzyme level was observed,

<table>
<thead>
<tr>
<th>Determination</th>
<th>D31 (chromosomal resistance genes)</th>
<th>D1-R1 (episomal resistance genes)</th>
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<tbody>
<tr>
<td>Growth experiments with DL-ampicillin^b</td>
<td>30</td>
<td>300</td>
</tr>
<tr>
<td>Growth rate decreased (μg/ml)</td>
<td>40</td>
<td>1,300</td>
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<td>Single-cell-colony formation on plates with</td>
<td></td>
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<tr>
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<tr>
<td>Penicillin G (μg/ml)</td>
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<td>100</td>
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<tr>
<td>Buffer-suspended cells</td>
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<td></td>
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<tr>
<td>Apparent $V_{max}$ with D-ampicillin (units/mg of cells, dry wt)</td>
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<td>0.047</td>
</tr>
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<td>Apparent $K_m$ with D-ampicillin (mm)</td>
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<td>0.17</td>
</tr>
<tr>
<td>Apparent $K_m$ with penicillin G (mm)</td>
<td>5^a</td>
<td>0.10</td>
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</tbody>
</table>

^a Inoculum size about 10 cells/ml.
^b Ampicillin added to an exponentially growing culture at a cell density of 10^8 per ml (cf. Fig. 1).
^c Since the curves were sigmoid, no Michaelis constant could be determined, but the half-saturation constant was of the order indicated.
Penicillinase activity of whole cells at various substrate concentrations. The penicillinase determinations so far described were performed at high concentrations of ampicillin. Experiments were therefore performed to determine the rate of hydrolysis as a function of the substrate concentration. Cells were grown on the rotatory shaker in LB, harvested by filtration, and resuspended in phosphate buffer. Figure 4 shows the rates of hydrolysis of ampicillin at different substrate concentrations (note the different scales for the two strains). At low ampicillin concentrations, the penicillinase activity of D1-R1 was about 250 times that of D31. The maximal rate was 15 times higher for D1-R1 than for D31, and the strains also differed qualitatively. The strain with the episcopal resistance, D1-R1, showed normal Michaelis-Menten kinetics with a low apparent Michaelis constant (0.17 mm). However, the ampAB carrying strain D31 gave a slightly sigmoid curve, with a half-saturation concentration of about 5 mm, which is 50 times the concentration required to lyse a growing culture of D31. Thus, the D31 enzyme seems to be very inefficient for the hydrolysis of ampicillin.

We also compared the ability of both strains to hydrolyze D-ampicillin and penicillin G. Using buffer-suspended cells (as above), we found the apparent $V_{\text{max}}$ to be about the same with penicillin G for both strains and for D1-R1 with D-ampicillin (see Table 1). However, D31 showed a 37 times lower apparent $V_{\text{max}}$ with D-ampicillin than with penicillin G. This was not linked to any change in the apparent Michaelis constant.

Hydrolysis of ampicillin by exponentially growing bacteria. The experiments described above
were performed with buffer-suspended cells. This condition is not lethal, since penicillins kill only actively growing bacteria. Földes and Boman (unpublished data) earlier observed that growing ampA strains showed a lag period before the onset of ampicillin hydrolysis. It was therefore desirable to investigate to what extent the present strains use their penicillinase during growth in the presence of ampicillin. The experiments were analogous to those shown in Fig. 1 but were performed at low concentrations of ampicillin. At different times, samples were withdrawn and filtered free from cells; the decrease of ampicillin in the medium was measured by disc assay with S. lutea. Figure 5 shows the results obtained at two different ampicillin concentrations. The ampAB strain D31 hydrolyzed ampicillin, but only after a lag period, whereas with D1-R1 ampicillin destruction started at once. Some experiments with 14C-ampicillin gave the same result and showed that ampicilloic acid was formed. The disappearance of ampicillin shown in Fig. 5 was therefore due to penicillin-ß-lactamase activity.

The rate of ampicillin disappearance at various concentrations was determined in several experiments like those in Fig. 5. The results are shown in Fig. 6 where the velocities plotted were obtained from the initial period of hydrolysis. Since the cells were growing and since, with D31, hydrolysis started at different times for different concentrations of ampicillin, it was necessary to correct for the increase in cell density. The ampAB strain D31 gave a slightly sigmoid curve of the same type as that in Fig. 4. To facilitate a comparison between growing and nongrowing cells of both strains, the left part of Fig. 4 was inserted showing the corresponding curves for buffer-suspended cells (lines lacking experimental points).

![Fig. 6. Initial rates of Dl-ampicillin hydrolysis by exponentially growing cultures of D1-R1 (○) and D31 (○) at different concentrations of ampicillin. The activities were corrected to correspond to 10^6 cells per ml. For comparison, the left part of Fig. 4 was inserted showing the corresponding curves for buffer-suspended cells (lines lacking experimental points).](http://jb.asm.org/)

![Fig. 5. Hydrolysis of ampicillin by cultures of D1-R1 (○) and D31 (○) growing exponentially in LB. At zero-time (cell density 10^6 per ml), ampicillin was added. The ampicillin concentration was determined, using bioassay with Sarcina lutea as test organism.](http://jb.asm.org/)
treatment of D1-R1, a small amount of the R-factor enzyme was released from the cells. D1-R1 was grown logarithmically in LB, exposed to ampicillin for a short time, and filtered. The cell-free filtrates were then incubated, and the formation of 14C-ampicilloic acid was followed. Figure 8 shows that the rate of hydrolysis represented about 40% of the value obtained with growing cells. However, this rate was less than 5% of the penicillinase activity of buffer-suspended cells. At the end of the experiment, the growing cells in Fig. 8 were harvested and resuspended in buffer, and their penicillinase was determined. The result showed that the penicillin-treated cells contained almost the same amount of penicillinase as a normal culture of D1-R1. The limited amount of penicillinase in the filtrate was destroyed by heating at 65 C for 10 min.

Delayed ampicillin hydrolysis during growth of strain D31. The experiments with growing cells described in Fig. 6 showed that the R-factor strain D1-R1 started to hydrolyze ampicillin immediately after its addition. However, the ampAB strain D31 had a more complex response, and disappearance of ampicillin began only after a lag period that was found to depend on the concentration of ampicillin (Fig. 9). The concentration range was limited by the insensitivity of the bioassay and by the lysis of D31. In all experiments with growing cells, the lag period lasted at least one generation. No lag was observed with buffer-suspended cells. To investigate whether a limited pretreatment with ampicillin could raise the resistance of D31, growing cells were exposed to 20 μg of ampicillin per ml for a time which was longer than the corresponding lag period (cf. Fig. 9). The culture was then diluted to 10^9 cells/ml and tested for resistance as in Fig. 1. It was observed that cells which were pretreated with ampicillin had a slightly reduced resistance to ampicillin compared to untreated cells. Despite the now available penicillinase activity, the pretreatment did not provide the cells with any increase in resistance.

DISCUSSION

Determination of penicillin resistance. This study was planned as a comparison of two strains, one with chromosomally mediated resistance and one with resistance mediated by an R factor. The two strains were selected because they showed the same ability to form single-cell colonies on agar plates containing ampicillin. The results in Table 1 show that in other tests the R factor-containing strain D1-R1 was considerably more resistant to

![Fig. 7. Lineweaver-Burk plot for the hydrolysis of ampicillin by growing cells (○) and buffer-suspended cells (●) of D1-R1; v0 is the initial rate of ampicillin hydrolysis (μmoles per 10^9 cells per min) and S the concentration of ampicillin (μm).](http://jb.asm.org/)

![Fig. 8. Hydrolysis of ampicillin by exponentially growing cells of D1-R1 (○), by cell-free filtrate (△), and by the same filtrate preincubated at 65 C for 10 min (□). The reaction was followed by counting the 14C-ampicilloic acid formed. At a cell density of 10^8 cells/ml, 14C-ampicillin was added to a final concentration of 20 μg/ml. After 10 min, the culture was divided, and one part was filtered free from cells. One part of the filtrate was heated as indicated. The heated and the nonheated filtrates were incubated at 37 C together with the remaining culture.](http://jb.asm.org/)
ampicillin than was D31. However, the ampAB strain D31 was more resistant to penicillin G than was D1-R1. Table 1 also shows that resistance on solid medium is poorly correlated to resistance in liquid medium. The method for determining resistance must therefore be selected according to the purpose for which resistance data are needed. Since genetic experiments are based on selective properties of single cells, colony formation must be used if resistance studies are to be correlated with genetic data. For clinical purposes, it may be more appropriate to study the resistance of populations by serial dilution tests rather than the resistance of single cells. When the bioassay with paper discs (see Materials and Methods) was applied to D1-R1 and D31, it was found that both strains were too resistant to give meaningful results with the method.

Mechanism of penicillin resistance. Penicillin resistance is generally attributed solely to the production of penicillinase. This has been studied extensively in some gram-positive organisms like Bacillus, which can excrete large amounts of extracellular penicillinases (5). To correlate the enzymatic properties of exopenicillinases with the resistance of the bacteria, Pollock (15) introduced the term "physiological efficiency," which is defined as the ratio between the maximal velocity ($V_{\text{max}}$) and the Michaelis constant ($K_m$). Preliminary determinations have shown that the purified enzymes from D31 and from D1-R1 have Michaelis constants for D-ampicillin which are of the same order (Lindqvist and Nordström, to be published). The apparent $V_{\text{max}}$ of buffer-suspended cells may be misleading, and, since the R1 enzyme is not yet pure, $V_{\text{max}}$ for the two enzymes cannot be compared. However, "physiological efficiency" does not seem to be especially applicable to our strains with cell bound penicillinase, and it does not take into account the differences between actively growing and buffer-suspended cells. It can also be concluded that in our strains penicillinase can hardly be the full explanation for the resistance.

Regulation of the penicillinase activity. It has been shown that in bacteria the total amount of protein per cell mass remains constant during variations in growth rate (12). However, with increasing growth rates the cells become larger and the number of ribosomes is increased (12). This means that the amount of protein not associated with ribosomes or the cell surface should be expected to decrease with increasing growth rate, and this has in fact been demonstrated for the enzyme isocitrate lyase (11). We have shown (Fig. 3a) that in the absence of penicillin the amount of wild-type penicillinase present in strains D11 and G11 increased with increasing growth rate. Figure 3a and b show that in D21 the enzymemediated by the mutated ampA gene was regulated in the same way. These experiments therefore suggest that the penicillinase has a normal function which is of importance for the growth rate of the bacteria. Since the relevant experiments were performed with washed and buffered suspended cells, there is no reason to believe that the activity determinations were influenced by low molecular weight compounds. Figure 3 should therefore represent variations in the amounts of enzyme and probably indicates a regulation on the gene level of the enzyme content. It has previously been suggested that penicillinases have a "normal" function in cell wall biosynthesis and that allelic genes specify the sites of penicillin sensitivity and penicillin resistance (3, 9). We have recently found several lines of evidence indicating that mutations to penicillinase resistance are accompanied by changes in the surface layers of the bacteria (Broman et al., Genet. Res., in press; Nordström and Burman, in preparation).

Figure 6, 7, and 9 demonstrate that active growth has a profound influence on the penicillinase activity of the bacteria. During normal growth, the penicillinase activity of both of the strains was cell-bound, and the amount released in Fig. 8 corresponds to less than 5% of the activity of buffer-suspended D1-R1. In both of the strains, spheroplast formation released almost all of the enzyme activity (Lindström and Boman, Biochem. J. 106:43p, 1968; Lindqvist and Nordström, to be published). This demonstrates that both the chromosomally and the episomally mediated enzymes are located outside the cell membrane. Therefore, permeability barriers associated with the cell membrane cannot explain the differences between growing and nongrowing
cells. Since at least 80 to 90% of the penicillinase is outside the cell membrane, the possible existence of "latent" enzyme molecules inside the cells can hardly explain why D31 during growth has a lag in the hydrolysis of ampicillin (Fig. 9). It is also difficult to explain the inhibition of the episomal enzyme during growth (Fig. 7) by postulating a permeability barrier to "latent" enzyme molecules.

The most likely explanation for the differences between growing and nongrowing cells is the hypothesis that the penicillinase activity is under the control of some low molecular weight metabolites which probably are associated with cell wall biosynthesis (3). Since the episomal enzyme was inhibited and the chromosomally mediated enzyme was activated (Fig. 6), the existence of both positive and negative effectors may be suggested. To establish this, it will be necessary to isolate the metabolites and to demonstrate that they affect the activity of the purified enzymes.

Evolutionary aspects. Watanabe (18, 19) suggested that R factors have evolved from chromosomal genes, and the evolutionary aspects of bacterial penicillinases were recently discussed by Pollock (16). Both R factors and F factors mediate the formation of pili (4), and, since cell to cell contact is necessary for infection, it is reasonable to conclude that infectious epismes carry information concerning the cell surfaces. It can therefore be argued that the evidence connecting chromosomally mediated penicillin resistance with cell wall biosynthesis (Boman et al., Genet. Res., in press) supports the concept that R factors have evolved from chromosomal genes for cell surface information.

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LITERATURE CITED