JOURNAL OF BACTERIOLOGY, Jan. 1970, p. 218-231

Vol. 101, No. 1
Printed in U.S.A.

Resistance of *Escherichia coli* to Penicillins

VI. Purification and Characterization of the Chromosomally Mediated Penicillinase Present in *ampA*-Containing Strains

E. BÖRJE LINDSTRÖM, HANS G. BOMAN, AND BARBARA B. STEELE
Department of Microbiology, University of Umeå, S-901 87 Umeå 6, Sweden

Received for publication 18 October 1969

The chromosomally mediated penicillinase present in three strains of *Escherichia coli* K-12 has been purified and characterized. Two of the strains carried the *ampA* gene and the third the wild-type allele. The purification involves release of the enzyme by spheroplast formation, dialysis, chromatography on sulfoethyl cellulose, and chromatography on hydroxylapatite. Enzyme from the two mutants appeared homogeneous in polyacrylamide gel electrophoresis. Enzyme from the wild-type strain gave two bands. Immunologically, the enzymes from all three strains were identical. Ultracentrifugation gave a homogeneous peak with a sedimentation coefficient of 3.4*S*. Gel filtration gave an estimated molecular weight of 29,000. The N-terminal amino acid residue was found to be alanine. Complete amino acid analysis showed a lack of cysteine. Ultraviolet spectra were recorded at three different *p*H values. The extinction coefficient at 280 nm is 21.0 for a 1% solution at *p*H 6.8. The optimal *p*H is 7.3. With enzyme from one of the resistant mutants, the following K_m and turnover number values were obtained: for penicillin G, 12 μM and 2,080; for D-ampicillin, 6 μM and 83; for cephalosporin C, 217 μM and 18,400. The effect of different salts on the enzyme activity was tested. Under many conditions the enzyme was found to be unstable.

It is well known that penicillin resistance in bacteria at least in part is due to the enzymic hydrolysis of the β-lactam ring of the penicillin molecule. Several such enzymes, called penicillinases or penicillin β-lactamases, have been purified and characterized from strains of *Bacillus* and *Staphylococcus* (7). Transformation studies (9) and genetic work on plasmids mediating penicillinases have also been reported for these organisms (26, 33). However, for gram-positive bacteria, it has hitherto been difficult to correlate biochemical and genetic data to an understanding of how penicillin resistance is developed.

Among the gram-negative bacteria, *Escherichia coli* offers unique possibilities for work attempting to correlate biochemical and genetical data (39). Our first genetic study of penicillin resistance showed that mutations in at least two separate loci were giving a stepwise increased resistance to ampicillin (10). We have so far made genetic studies on three classes of resistant mutants which all contain the *ampA* gene (a gene mediating ampicillin resistance; 5, 11, 24). Recently, we also compared strain D31, the most resistant of these mutants (which has chromosomal resistance genes), with an isogenic strain, D1-R1, which carries a penicillinase-producing episome (6).

In this paper we report the purification and characterization of the chromosomally mediated penicillinase present in two mutants, the moderately resistant G11a1, and the highly resistant strain D31, which both contain the *ampA* gene (5, 11). We have also purified the penicillinase from strain G11 containing the wild-type allele of the *ampA* gene. The companion paper by Lindqvist and Nordström (20) describes the purification of the penicillinase mediated by the R factor R1. Preliminary accounts of the location of the chromosomal enzyme and the first purification steps have previously been reported (4, 21).

MATERIALS AND METHODS

Bacterial strains. All strains used were *E. coli* K-12. Strain G11a1 which carries the *amp A1* allele is a spontaneous mutant obtained from the wild-type strain G11 (10). The *ampA1* allele was transduced into strain D2, which afterwards was mutated to the highly ampicillin-resistant strain D3 (5). A second mutation step induced streptomycin resistance in D3, thus producing strain D31 (5). Strains G11a1 and D2 both form single cell colonies on plates containing D-ampicillin concentrations of 15 to 20 μg/ml, whereas strain D31 can form single-cell colonies on plates with D-ampicillin concentrations of 75 to 100 μg/ml (for resistance determinations, see references 6 and 24).

Media and growth conditions. The growth medium

used was made up from the basal medium E of Vogel and Bonner (40), supplemented with 0.2% glucose, 1.3% nutrient broth (Oxoid), and 0.1% tryptone (Difco). Bacteria were usually obtained from 15-liter cultures grown in 5-gallon Pyrex bottles. Larger cultures of 40 liters or more were grown in a Biogen fermentor (American Sterilizer Co., Erie, Pa.). Cultures of 2 to 4 liters grown overnight on a rotary shaker were used as inoculum. Growth temperature was always 37 C. Foaming was prevented by the use of antifoam (Emulsion RD, Midland Silicones Ltd., Readings, England), at a concentration of 0.5 ml/liter of culture. Growth was followed with a Klett-Summerson photoelectric colorimeter by using filter no. W66. The bacteria were harvested at a cell density of approximately 2.5×10^9 cells/ml or 1 mg of protein/ml. Growth was terminated by pouring the 15-liter cultures into a container with 10 liters of ice cubes precooled to −20 C. This procedure cools the culture to 0 C within 1 to 2 min. The chilled bacteria were collected by a Sharples centrifuge (model T-1P) equipped with cooling facilities. The rotor used was type T-E 2004-2 run at the maximal speed of 63,000 × *g* with a flow rate of 600 ml/min. This procedure normally yields about 80 g of bacterial paste from a 15-liter culture. When the Biogen fermentor was used, the culture was divided into 15-liter batches and processed in the same manner. The bacterial cells obtained from a 15-liter culture were washed once by resuspension in 750 ml of 0.01 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer, *p*H 8.0. The bacteria were recovered by centrifugation at 23,000 × *g* for 10 min and were used immediately for spheroplast formation.

Substrates. All penicillin derivatives, including ^{14}C-DL-ampicillin with 2.8 μc/mg, were kindly donated by ASTRA, Södertälje, Sweden. The amount of penicillin in each batch was determined analytically, and remaining material was assumed to be water, for which corrections were made. Cephaloridine, cephalosporin C, and 7-amino-cephalosporanic acid (7-ACA) were obtained from Glaxo Laboratories Ltd., Grennford, Eng.

Other materials. Egg white lysozyme (3× crystallized) was obtained from Sigma Chemical Co., St. Louis, Mo.; crystalline horseradish peroxidase (HRP) and myoglobin (MbI) were kindly donated by K. G. Paul, Umeå; pepsin (from hog stomach, 3× crystallized, molecular weight 35,500) and trypsin (from beef pancreas, 2× crystallized, molecular weight 23,800) were from Mann Research Laboratories; *Bacillus* penicillinase (Neutrapen) was from Sceen Laboratories Pharmaceutical Inc., New York, N.Y.; sulfoethyl cellulose (SE cellulose), substitution degree corresponding to exchange capacity of 0.37 meq/g of dry cellulose, was kindly prepared for us by Mo & Domsjö AB, Örnsköldsvik, Sweden; diethylaminoethyl (DEAE) cellulose, exchange capacity of 0.76 meq/g, was obtained from Bio-Rad Laboratories, Richmond, Calif.; hydroxylapatite used for chromatography was prepared as described by Levin (19); Sephadex G75 was from Pharmacia Fine Chemicals, Uppsala, Sweden; phenylisothiocyanate was from Eastman Organic Chemicals, Rochester, N.Y.; gelatin was from Oxoid.

Determinations of penicillinase activity. Two different methods were used, one based on the hydrolysis of radioactive substrate, the other the iodometric method of Novick (25). In the first case, the reaction mixture contained 7 mM ^{14}C-DL-ampicillin (diluted to a specific activity of 0.28 μc/mg), 0.05 M potassium phosphate buffer (*p*H 7.4), and various concentrations of enzyme. Incubation was performed at 37 C in 0.3-ml microtubes. At different times (usually after 30 and 60 min) portions of 10 μliters were spotted on Whatman 3MM paper which terminated the reaction. The products were separated by using paper chromatography as previously described (6). After staining with ninhydrin, the ampicilloic acid spot was cut out and the radioactivity was counted by using a Nuclear-Chicago liquid scintillation counter. This method was used during the first part of the investigation and has the advantage of being absolutely specific for enzymes which cleave the β-lactam ring.

In the later part of the investigation and for the kinetics described, we used the iodometric method of Novick (25) adapted to the Technicon Autoanalyzer as briefly described before (6). Reference samples of penicilloic acid were prepared by using Neutrapen. The amount of enzyme which hydrolyzes 1 μmole of substrate per min at *p*H 7.4 and 37 C was defined as 1 unit of penicillinase. Routine analyses of penicillinase activities in chromatograms were performed at room temperature and by use of a standard curve converted to units at 37 C. All buffers and reagents were prepared with water redistilled in a quartz apparatus.

Analytical polyacrylamide-gel electrophoresis. Gels, containing 2 ml of 7.5% acrylamide, were made in potassium acetate buffer (*p*H 4.3), and the tray buffer was β-alaninacetate (*p*H 4.7), according to Reisfeld et al. (31). Electrophoresis was performed by the modification of the method of Hjertén et al. (15) with gel of only one pore size. The samples were dialyzed against 0.01 M potassium phosphate buffer (*p*H 6.8). The electrophoresis at 4 C was performed at 25 v and 2 ma per tube for 15 min, followed by 100 v and 10 ma per tube for 2.5 hr. The gels were stained with 1% amidoschwarz in 7% acetic acid for 2 hr and electrophoretically destained in 7% acetic acid by using a current of 15 ma per tube.

Immunological methods. Rabbit antisera were produced against both partially purified and pure enzyme from strain G11a1. With enzyme from step 3, five injections were administered in the marginal ear vein over a period of 3 months. Each injection contained 0.4 to 0.5 mg of protein. With the purified enzyme, the first injection contained 2.5 mg of protein dissolved in 1 ml of 0.15 M NaCl and emulsified with 1 ml of complete Freunds Adjuvant (Difco). It was distributed in the footpads of the rabbit. Each of the following two injections contained 6 mg of enzyme precipitated with alum (17). Antisera were collected about 2 weeks after the last injections.

From the antisera produced against partially purified penicillinase, gamma globulins were precipitated by addition of ammonium sulfate to 37% saturation.

After stirring overnight at 4 C, the precipitate was collected by centrifugation. It was then dissolved in and dialyzed against 17.5 mM potassium phosphate buffer, *p*H 6.5. The solution was then passed through a column of DEAE cellulose pre-equilibrated with the same buffer. Antibodies against the pure enzyme were enriched by using only the DEAE-cellulose step. After elution, the gamma globulin fraction was concentrated by negative pressure in collodion bags from Membranfiltergesellschaft, Göttingen, Germany.

The immunodiffusion tests were done in 1% agarose (kindly provided by S. Hjertén, Uppsala, Sweden) containing 0.05 M Tris-hydrochloride, *p*H 8.6. To each well was added 10 μliters of the preparations analyzed. The concentration of antibodies against pure penicillinase was 3 mg/ml, and against partially purified enzyme about 6 mg/ml. The enzyme preparations contained 1 to 2 mg protein/ml. Purified enzyme from strain D1-R1 (20) was kindly provided by R. C. Lindqvist in this department.

Determination of N-terminal amino acids. The analysis was by the Edman method as used by Eriksson and Sjöquist (12). The phenyl thiohydantoin (PTH) derivatives were tentatively identified by thin-layer chromatography (16) and then by paper chromatography (37). The water phase, which would contain PTH-arginine and PTH-histidine if present, was also examined. Standard mixtures of PTH amino acids (Mann Research Laboratories) were prepared (37) and used as references in chromatography. Spots on the paper chromatograms were located by producing a negative print on Agfa-Gevaert Copyline Contact paper no. C90 by using ultraviolet (UV) light. The spots were eluted in 95% ethyl alcohol at 40 C for 2 hr, and UV spectra were measured on the eluates, using as blank the eluate from a blank area of paper at the same R_F position as the spots. The spectra and the ratios A_{245}/A_{269} were used to distinguish true PTH derivatives from extraneous spots. The amount of PTH-alanine was calculated from the absorbancy at 269 nm, using a molar extinction coefficient of 16,000.

Formation of spheroplasts. The procedure used is based on the method of Repaske (32). However, the higher cell density and the larger volumes used here, and perhaps also strain differences, make it critical to follow the procedure described as step 1 in Results.

RESULTS

Purification procedure for chromosomal penicillinase. Experiments with suspensions of whole cells have demonstrated that ampicillin-resistant mutants carrying the *ampA* gene hydrolyze the β-lactam ring in ampicillin (10). This penicillin β-lactamase activity (in the following referred to as penicillinase) has been purified from a moderately ampicillin-resistant strain, G11a1, and a highly ampicillin-resistant strain, D31, both containing the same allele of the *ampA* gene (5). The following procedures can without any modifications be applied to both of the strains, although most of the data presented are from experiments with G11a1.

Step 1. Release of the enzyme by spheroplast formation. About 80 g of the washed bacterial paste was transferred to a 1-liter graduated cylinder and resuspended at room temperature in a solution of 20% (w/v) sucrose containing 0.03 M Tris-hydrochloride, *p*H 8.0. A complete homogenization of the bacterial suspension is important and a piston fitting to the graduated cylinder was used. The volume of the resuspended bacteria was 675 ml and the bacterial density corresponded to about 6×10^{10} cells/ml. To these resuspended bacteria was added 37.5 ml of 0.1 M ethylenediaminetetraacetate (EDTA) adjusted to *p*H 8.0, followed by rapid mixing with the piston. Immediately thereafter, 37.5 ml of a freshly made solution of lysozyme (1 mg/ml) was added and mixed with the piston. Spheroplast formation was followed by withdrawing 0.1-ml samples, mixing with 5 ml of water, and reading optical density (OD) in a Klett-Summerson photometer. The OD was normally reduced about 80% after 5 min of incubation at room temperature. This indicated that the spheroplast formation was almost complete. Since the spheroplasts are rather fragile, it was found convenient to transfer the cell suspension to centrifuge bottles during the incubation. Before centrifugation the spheroplasts in each centrifuge bottle were stabilized by the addition of 1 M $CaCl_2$ to a final concentration of 0.01 M (38). Mixing was achieved by carefully turning the bottles upside down a few times. The spheroplasts were removed by centrifugation in a refrigerated centrifuge with a precooled rotor at a force of 23,500 $\times$ *g* for 15 min. From this stage on, all further operations were performed at 4 C. Stabilization of the spheroplasts by calcium gave rather well-defined pellets after centrifugation. The supernatant fluid was then collected by decanting carefully. The total volume of the supernatant fluid was about 600 ml. The pellets were discarded.

Since this step provides evidence for the surface location of the penicillinase, as previously shown (4, 21), we have in a smaller size experiment investigated the amount of penicillinase in different fractions recovered during a spheroplast preparation. The results in Table 1 show that during growth all enzyme activity was cell-bound, but that limited amounts of this activity could be released by washing in buffer or sucrose. The data for the total recovery indicates that the release of the enzyme as such increased the total activity.

Step 2. Dialysis. The cloudy supernatant fluid from step 1 was dialyzed against distilled water overnight and then for an additional 48 hr against 0.01 M potassium phosphate buffer, *p*H 6.8. After the dialysis, a small precipitate was removed by centrifugation at 23,500 $\times$ *g* for 15 min. The

TABLE 1. *Release of cell-bound penicillinase by spheroplast formation*[a]

Stage	Fractions	Total activity (units)	Recovery	
			Each step	Total
I	(a) Cells suspended in growth medium	25.2	100	100
	(b) Growth medium after centrifugation	<2	<8	<8
II	(a) Cells suspended in wash buffer, Tris-hydrochloride	43.4	100	172
	(b) Wash buffer after centrifugation	5.8	13.3	23
III	(a) Cells resuspended in sucrose	57.5	100	228
	(b) Sucrose after centrifugation	8.7	15.2	34
IV	(a) Cells resuspended in sucrose + EDTA + lysozyme (spheroplasts)	60.0	100	238
	(b) Supernatant fraction after removing the spheroplasts by centrifugation	43.7	72.7	173
	(c) Resuspended spheroplasts in sucrose	6.1	10.2	24

[a] The same batch of cells of strain G11a1 (2 liters of culture) was used throughout the experiment. After stage II, the batch was divided into two parts which were used in stages III and IV, respectively. The total activities given in the table are corrected for this division. Total recovery is given as per cent of total activity in stage Ia. Units of penicillinase are defined as μmoles of penicillin hydrolyzed per minute at 37 C. Penicillin G (2.5 mM) was used as substrate.

volume of the dialyzed extract was between 750 and 900 ml.

Step 3. Chromatography on SE cellulose. The dialyzed extract was applied to a column (30 by 850 mm) containing about 600 ml of SE cellulose. The flow rate was maintained at 40 ml per hr during the entire experiment. The column was pre-equilibrated with 0.01 M potassium phosphate buffer (*p*H 6.8), and elution was first carried out with about 1,000 ml of the same agent. The chromatogram in Fig. 1 shows that a large amount of UV-absorbing material travelled through the column without adsorption. A stepwise change to 0.1 M potassium phosphate buffer (*p*H 6.8) eluted the penicillinase as a well-defined peak. The enzyme activity travelled ahead of the UV-absorbing material, which indicates that this peak contained significant amounts of inactive material. We have previously eluted the enzyme with 0.07 M buffer (4) which often gives a better purification in this step. However, the 0.1 M buffer was selected because it improves the result of the following step.

Step 4. Chromatography on hydroxylapatite. The second peak in Fig. 1, which contained all the penicillinase activity, was applied directly to a hydroxylapatite column 32 by 475 mm pre-equilibrated with 0.1 M potassium phosphate buffer, *p*H 6.8. During this chromatogram, the flow rate was adjusted to 20 ml/hr. Elution was first carried out with 500 ml of the same agent, followed by a linear gradient from 0.1 to 0.25 M potassium phosphate buffer, *p*H 6.8. The chromatogram in Fig. 2 shows that the penicillinase was eluted as a well-defined peak appearing in the first half of the gradient. Two large peaks of inactive material appeared at the end of the gradient.

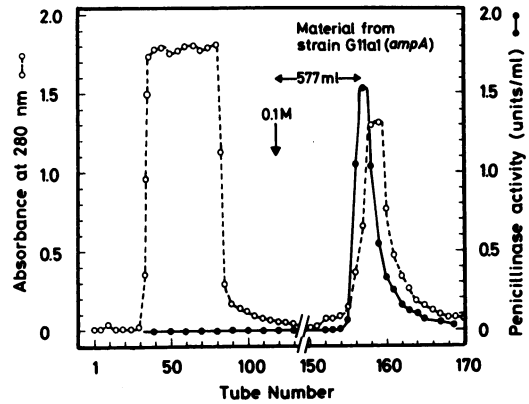


FIG. 1. *Step 3, chromatographic purification of penicillinase from strain G11a1 on sulfoethyl (SE) cellulose pre-equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. The dialyzed extract from step 2 was applied and the column was first eluted with the same buffer. The arrow shows the stepwise change to 0.1 M potassium phosphate buffer (pH 6.8) which eluted the penicillinase activity. Fractions were collected and assayed for protein* (○) *and penicillinase activity* (●).

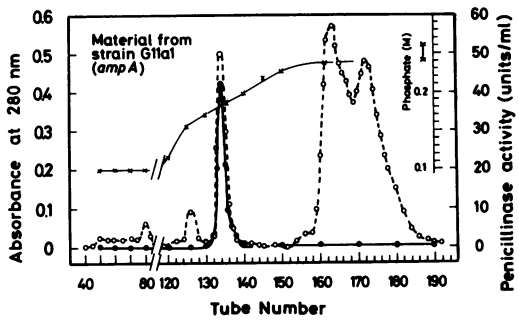


FIG. 2. *Step 4, chromatography of the active material from step 3 on hydroxylapatite. The linear gradient was made of 150 ml of 0.1 M and 150 ml 0.25 M phosphate buffer, pH 6.8. Phosphate concentration* (×) *was determined by conductivity measurements. Fractions were collected and assayed for protein* (○) *and penicillinase activity* (●).

All fractions in the enzyme peak which contained more than 15% of the maximal activity were pooled. After concentration by negative pressure dialysis, the material was used as a purified enzyme source in all further experiments.

The present procedure has been described for the amounts of bacteria obtained from a 15-liter culture. By using the same columns, we have also processed material from 40 liter cultures, and the results of such a purification are summarized in Table 2. The overall recovery of 60 to 80%, as compared to the starting bacteria, is relatively high, but should be related to the fact that more than 100% recoveries were obtained in some of the intermediate steps.

Purification of penicillinase from the wild-type strain G11. We have previously reported that the wild-type strain G11 also contains small amounts of a penicillinase (4, 21). All steps of the preceding purification method can be applied to strain G11. The chromatogram shown in Fig. 3 demonstrates that in step 3 the penicillinase activity travels as a small, well-defined peak together with a very large excess amount of inactive material. A comparison of Fig. 1 and 3 shows that the amount of penicillinase activity in the wild-type strain G11 is at least 10-fold less than that found in the resistant mutant G11a1. The fact that the amount of penicillinase in the wild-type strain G11 is so small has limited the number of experiments attempting to characterize the wild-type enzyme.

TABLE 2. *Summary of purification of Escherichia coli chromosomal penicillinase*[a]

State of enzyme	Total activity	Specific activity	Recovery	No. of times purified
	units	*units/mg*	%	
Cells in growth medium	735	0.02	100	1
Step 1, spheroplast supernatant fraction before dialysis	1,930	1.1	262	55
Step 2, spheroplast supernatant fraction after dialysis	1,800	0.6	245	30
Step 3, enzyme peak after SE-cellulose	1,575	9.2	214	460
Step 4, enzyme peak after hydroxyl apatite	616	88.0	84	4,400

[a] These data are obtained from a 40-liter culture of strain G11a1. Activity was determined with 0.5 mM penicillin G as substrate.

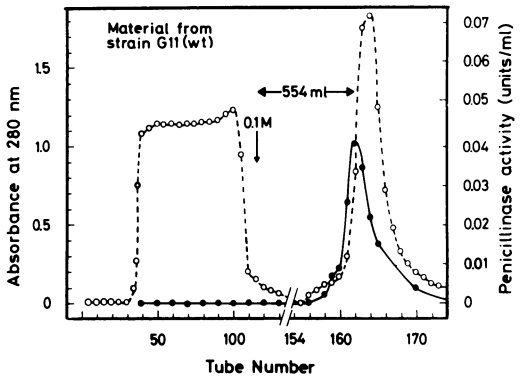


FIG. 3. *Step 3, chromatographic purification of penicillinase from the wild-type strain G11 on SE cellulose pre-equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. The arrow shows the stepwise change to 0.1 M phosphate buffer, pH 6.8. Note that the penicillinase scale is 30 times larger than in Fig. 1. Fractions were collected and assayed for protein (○) and penicillinase activity (●).*

Chemical characterization of the purified penicillinase. Analytical polyacrylamide-gel electrophoresis was used to test the homogeneity of penicillinases purified from strains G11a1 and D31. Figure 4 shows that after step 4 the penicillinases from these strains gave only one band, whereas a number of components could be demonstrated in the material obtained after steps 2 and 3. However, even after step 4, penicillinase from the wild-type strain G11 always produced two bands. The purified preparations were also characterized by immunodiffusion against antibodies obtained with partially purified enzyme as antigen (Fig. 5A). Both parts of Fig. 5 show immunological identity for penicillinases from strains G11, G11a1, and D31. The experiment also included the purified penicillinase mediated by the R factor R1 (Fig. 5B; reference 20). This enzyme gave no precipitation with the antisera prepared against the penicillinase mediated by the chromosomal gene.

Penicillinase from strain G11a1 was examined in the ultracentrifuge. Figure 6 shows the schlieren pattern obtained at four different times. Only one symmetrical peak was obtained and the sedimentation constant was 3.4*S*.

The molecular weight of the penicillinase was estimated by gel-filtration experiments on Sephadex G75 by using, among others, horseradish peroxidase and myoglobin as known references. The results in Fig. 7 show that the penicillinase was eluted between the two references. Interpola-

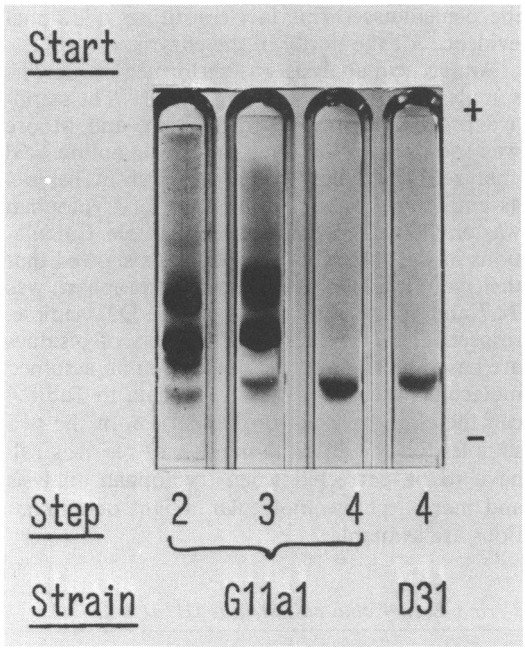


FIG. 4. *Polyacrylamide-gel electrophoresis of material from different steps in the purification of E. coli chromosomal penicillinase. To each gel was layered 100 μliters of the respective samples containing about 100 μg protein and 8% sucrose to increase the density. The electrophoresis was run at 4 C for 2.5 hr at 100 v and 10 ma/tube at pH 4.7.*

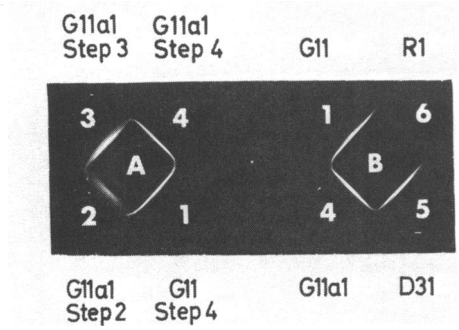


FIG. 5. *Immunodiffusion analysis of different preparations of penicillinase in 1% agarose containing 0.05 M Tris-hydrochloride, pH 8.6. The wells contained (A) antibodies obtained with crude enzyme from strain G11a1 and (B) antibodies obtained with purified enzyme from strain G11a1; (1) enzyme from the wild-type strain G11 after step 4, (2) enzyme from strain G11a1 after step 2, (3) enzyme from G11a1 after step 3, (4) enzyme from G11a1 after step 4, (5) enzyme from strain D31 after step 4, (6) purified enzyme from strain D1-R1 (reference 20).*

tion according to Andrews (2) gave a molecular weight of 29,000 ± 1,000 for the penicillinase.

Determination of the N-terminal amino acid was carried out with the Edman-Sjöquist technique, and the results are summarized in Table 3. The molar amounts of PTH derivatives were calculated (molecular weight of the penicillinase was assumed to be 29,000). The results indicate that alanine was the only N-terminal amino acid in

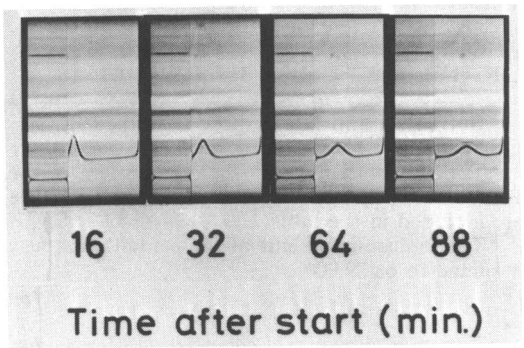


FIG. 6. *Ultracentrifugation andlysis of purified penicillinase from G11a1. Enzyme concentration was 3 mg/ml in 0.01 M potassium phosphate buffer, pH 7.4. The centrifuge used was a Spinco model E. Rotor speed was 245,000 × g and the bar angle was 60°. Sedimentation was from left to right and time was measured from the start of analysis.*

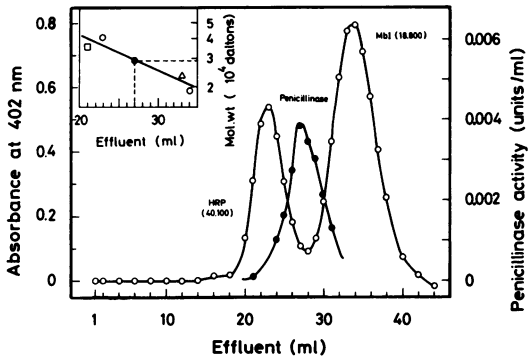


FIG. 7. *Determination of the molecular weight of penicillinase from strain G11a1 by gel filtration on Sephadex G75. The column (1 by 72 cm) was pre-equilibrated with 0.1 M potassium phosphate buffer, pH 7.4. A sample containing crystallized horseradish peroxidase (HRP) and myoglobin (Mbl) dissolved in 200 μliters of purified penicillinase (50 μg) was applied by using upward-flow elution at a flow rate of 30 ml per hr. Fractions were collected every 20 min and assayed for protein (○) and penicillinase activity (●). The inset shows data for pepsin (□) and trypsin (△) obtained in two separate experiments. The interpolated molecular weight for the penicillinase was 29,000.*

TABLE 3. *Determination of N-terminal in Escherichia coli penicillinase from strain D31*

Sample	System[a]	A_{345}/A_{269}	R_F[b]	Moles of alanine per mole of enzyme
Penicillinase[c] from D31	Solvent I	0.47	0.07	0.81
	Solvent II	0.44	0.16	0.71
Reference PTH-alanine	Solvent I	0.45	0.06	
	Solvent II	0.41	0.16	

[a] Solvents I and II are those described by Sjöquist (37).

[b] The R_F value and absorbancy quotient of the PTH-alanine in the standard reference mixture are included in the table.

[c] The molecular weight of the penicillinase was assumed to be 29,000.

the penicillinase. This fact constitutes additional evidence for the purity of the enzyme.

Amino acid analysis was performed on enzyme samples from strains G11a1 and D31. The standard procedure of Spackman, Stein, and Moore was used with a Beckman automatic amino acid analyzer. The primary data are given in Table 4 as μmoles/mg of sample. A peak for tryptophan was only obtained with the D31 sample. Calculations of the amounts of amino acids showed that the material balance without tryptophan was 72.7 and 81.2% for the G11a1 and D31 sample, respectively. The calculated numbers of residues are based on these recoveries and on an assumed molecular weight of 29,000. The data in Table 4 can therefore be used for comparison of the two samples but the absolute number of residues will have to be corrected when tryptophan analysis and more accurate molecular weight determinations are available.

TABLE 4. *Amino acid composition of penicillinases from Escherichia coli strains G11a1 and D31*

Amino acid	Samples[a]		Residues per molecule		
			Calculated value		Avg whole no.
	G11a1	D31	G11a1	D31	
	μmoles/mg	*μmoles/mg*			
Lysine	0.42	0.47	17.0	16.8	17
Histidine	0.11	0.11	4.4	3.9	4
Arginine	0.24	0.24	9.5	8.7	9
Aspartic acid	0.63	0.66	25.0	23.4	24
Threonine	0.46	0.52	18.3	18.4	18
Serine	0.40	0.40	15.9	14.2	15
Glutamic acid	0.83	0.88	33.0	31.5	32
Proline	0.46	0.55	18.5	19.8	19
Glycine	0.58	0.62	23.0	22.1	23
Alanine	0.70	0.80	27.9	28.7	28
Valine	0.41	0.48	16.5	17.0	17
Methionine	0.10	0.13	4.0	4.7	4
Isoleucine	0.38	0.46	15.0	16.6	16
Leucine	0.58	0.67	23.0	23.9	23
Tyrosine	0.26	0.33	10.4	11.7	11
Phenylalanine	0.19	0.21	7.5	7.4	7
Tryptophan	—	0.18	—	(6.3)	(6)
Cysteine	<0.003	<0.003	0	0	0
Total (tryptophan excluded)	6.75	7.53	268.9	268.8	267
Ammonia	0.785	0.625			

[a] The sample from D31 was dialyzed against redistilled water before freeze-drying. The sample from G11a1 was desalted on Sephadex G25 equilibrated with 0.1 M acetic acid before freeze-drying. These analyses were kindly performed by J. O. Jeppsson, Umeå (G11a1) and D. Eaker, Uppsala (D31). None of the analyses showed any carbohydrates. The values for G11a1 were obtained after 24 hr of hydrolysis. The values for D31 were average results from 24 and 72 hr of hydrolysis, with the exception of threonine, serine, and tryptophan which were extrapolated to zero hours of hydrolysis.

Agreement between the two analyses is within the normal experimental error for all amino acids except tryptophan. However, the amino acid composition differs from those found for other penicillinases. The number of arginine and lysine residues was 26, whereas Ambler and Meadway (1) recently reported 47 and 39 for a *Staphylococcus* and a *Bacillus* penicillinase, respectively. It should be emphasized that our penicillinase lacks cysteine, as do other known penicillinases (1, 7).

The ultraviolet spectra of the purified enzyme at three different *p*H values is shown in Fig. 8. At *p*H 6.9, the spectrum shows a small shoulder at 290 nm which is somewhat more pronounced than for most proteins. The extinction coefficient at 280 nm was determined as 21.0 for a 1% solution in 0.01 M potassium phosphate buffer, *p*H 6.8. This high extinction coefficient is not in strict agreement with the fact that the amino acid analysis showed only 24 aromatic residues including tryptophan.

Enzymic characterization of the penicillinase. Optimal *p*H was determined with both Tris-hydrochloride and potassium phosphate buffers. The results in Fig. 9 show that potassium phosphate buffer gave almost three times higher activity than did Tris-hydrochloride buffer. The optimum in both cases was found to be *p*H 7.3.

By using both penicillin G (benzylpenicillin) and D-ampicillin (D-epimer of α-aminobenzylpenicillin) as substrate, the K_m values were determined for the purified enzyme from strains G11, G11a1, and D31. Figures 10 and 11 show that the

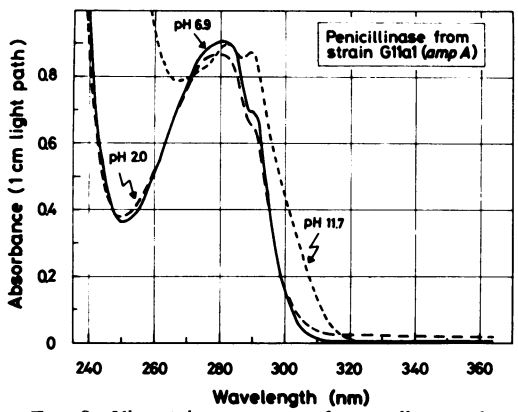


FIG. 8. *Ultraviolet spectra of penicillinase from G11a1 at three different pH values. The enzyme, 0.4 mg/ml, was dialyzed against 0.01 M potassium phosphate buffer, pH 6.9 (solid line). pH values 11.7 (short lines) and 2.0 (long lines) were obtained by using KOH and HCl, respectively.*

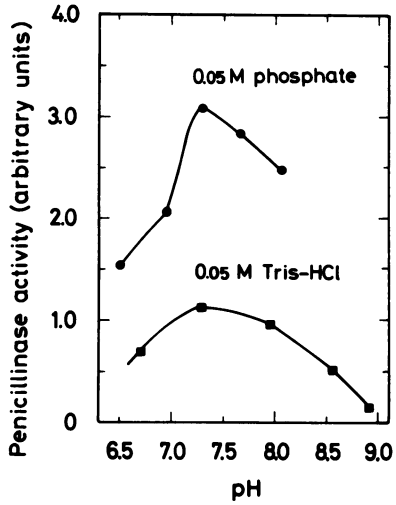


FIG. 9. *Determination of optimal pH for penicillinase from strain G11a1. pH values were obtained by using 0.05 M potassium phosphate buffers (●) and 0.05 M Tris-hydrochloride buffers (■). The substrate was penicillin G.*

enzymes from G11a1 and D31 have similar K_m values for each of the substrates. Table 5 shows K_m and V_{max} values for a number of different penicillin and cephalosporin derivatives. The 7-ACA used gave a decolorization of the starch-iodine complex, presumably as a result of the presence of impurities. For this reason, we could only determine the enzymic hydrolysis of this compound at a concentration of 0.1 mM. By using these conditions, penicillinases from strains G11a1 and D31 gave turnover numbers of 1.6 and 0.9 mmoles of substrate per mmole of enzyme per min, respectively. The corresponding values obtained with cephalosporin C were extrapolated to approximately 600 and 500. These results show that the side chain, consisting of the D-epimer of α-amino adiapic acid, drastically increased the hydrolytic function of the enzyme. For the penicillins, a similar effect was observed with the side chains (Table 5).

Preliminary experiments showed that the enzyme activity was affected by phosphate as well as by magnesium ions. Different salts were therefore tested for their effect on the enzyme activity. Table 6 shows that stimulation of penicillinase activity was obtained with several cations. All the salts listed in Table 6 were also tested without enzyme. Of these, only $ZnCl_2$ gave rise to hydrolysis of penicillin. Since $MgCl_2$ gave no hydrolysis, it can be concluded that Zn^{2+} ions have the ability to catalyze the hydrolysis of penicillin to a significant extent. The concentration de-

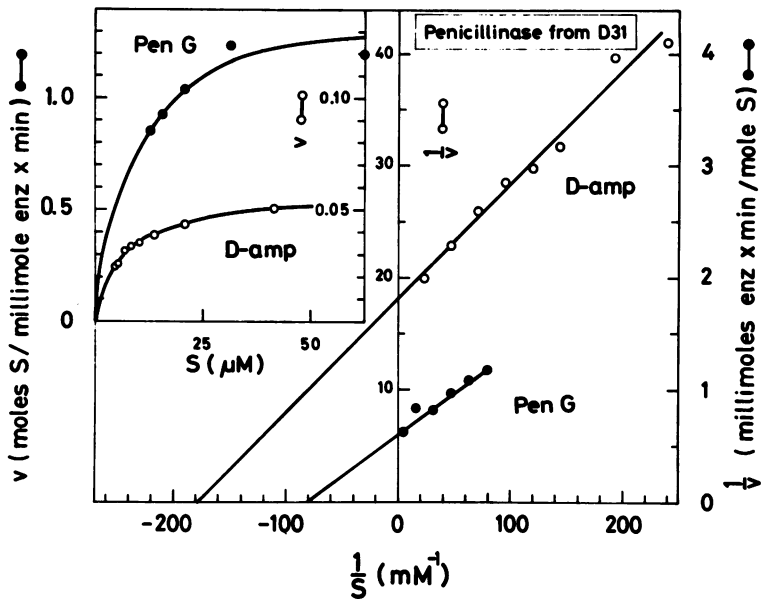


FIG. 10. *Lineweaver-Burk plot for the hydrolysis of* D*-ampicillin (*D*-amp, O) and penicillin G (penG, ●) by enzyme from strain D31. The hydrolysis was performed at 37 C. Note that the scale for penG is ten times larger than for* D*-amp.* K_m *= 12 to 13 μM for penG.* K_m *= 6 to 7 μM for* D*-amp. Inset shows the initial hydrolysis versus substrate concentration. Note that the scale for penG is ten times less than for* D*-amp.*

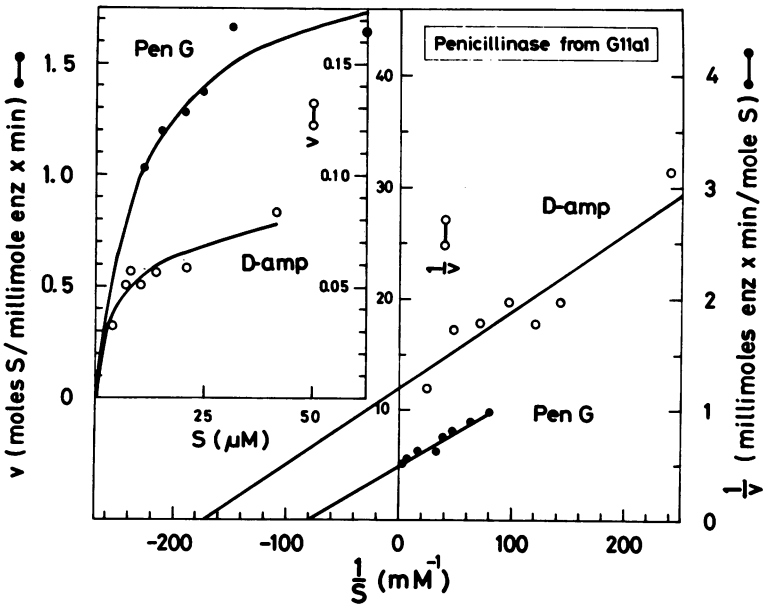
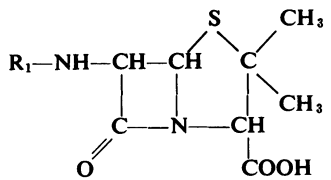
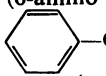
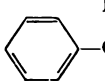
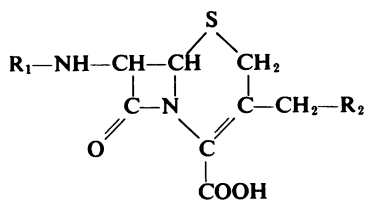
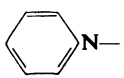


FIG. 11. *Lineweaver-Burk plot for the hydrolysis of* D*-ampicillin (*D*-amp, O) and penicillin G (penG, ●) by enzyme from G11a1. The hydrolysis was performed at 37 C. Note that the scale for penG is ten times larger than for* D*-amp.* K_m *= 12 to 13 μM for penG,* K_m *= 6 to 7 μM for* D*-amp. Inset shows the initial hydrolysis versus substrate concentration. Note that the scale for penG is ten times less than for* D*-amp.*

TABLE 5. *Substrate profile for penicillinase from strains G11, G11a1, and D31*

Substrate			K_m			V_{max} (turnover no.[a])		
Nucleus	Side chains		G11	G11a1	D31	G11	G11a1	D31
	R_1	R_2	μM	μM	μM			
Penicillins 	H— (6-amino penicillanic acid)			830	1,000		23	22
	 —CH_2—CO— (penicillin G)		12	12	12	906	2,080	1,670
	 H L-epimer		—	28	—	—	200	—
	NH_2 D-epimer (ampicillin)		5	6	6	42	83	55
Cephalosporins 	NH_2  D-epimer	CH_3—CO—O—	—	217	190	—	18,400	15,400
	(cephalosporin C)  (cephaloridine)		—	2,500	2,000	—	1,440	1,800

[a] Turnover number is expressed as millimoles of substrate hydrolyzed per millimole of enzyme per minute. Incubation was performed at 37 C. The amount of product formed was determined in the Autoanalyzer at room temperature by using Novick's method (25).

TABLE 6. *Effect of different salts on the penicillinase activity*[a]

Addition	Penicillin hydrolyzed[b]	Relative activity[c]
None	0.35	100
1 mM $MgCl_2$	0.57	163
1 mM $KHSO_4$	0.55	157
1 mM K_2HPO_4	0.50	143
1 mM $CaCl_2$	0.48	137
1 mM EDTA	0.55	157
0.1 mM $ZnCl_2$	0.51	146
0.1 mM $ZnCl_2$ Without enzyme	0.32	92

[a] Substrate was 0.5 mM penicillin G in 0.01 M Tris-hydrochloride, *p*H 7.4. Each sample contained approximately 10^{-4} mM penicillinase and 10 mM concentration of the salt examined with the exception of $ZnCl_2$, in which case 1 mM was used. During assay in the Autoanalyzer, the samples were diluted 10 times by mixing with the substrate solution.

[b] Values expressed as micromoles of penicillin hydrolyzed at 0 C.

[c] Values expressed as percentage of control.

pendence for Mg^{2+} was investigated in a separate experiment shown in Fig. 12. The fact that the optimal concentration corresponds to about 10^5 Mg^{2+} ions per enzyme molecule indicates that magnesium ions either induced a conformational change in the protein or protected the enzyme against denaturation. The stability of concentrated and diluted enzyme solution was therefore studied. Figure 13 shows that a diluted penicillinase sample lost about 40% of its activity during 25 min at 0 C. Gelatin, previously known to protect *Bacillus* penicillinase (28), offered almost full protection but did not increase the activity to the same extent as Mg^{2+} ions. Also D-ampicillin gave a temporarily increased activity (Fig. 13).

DISCUSSION

The location of the chromosomal penicillinase. Malamy and Horecker (22) first demonstrated that spheroplast formation can liberate a cell-bound enzyme and that these types of experiments provide evidence for a surface location of the activity. The method has later been applied to several other enzymes as recently reviewed by Heppel (14). The results in Table 1 show that during normal growth all the penicillinase in strain G11a1 was cell-bound and that spheroplast formation gave an almost complete release of the enzyme. This also applies to the *ampAB* strain D31 (5), for the enzyme mediated by the R factor R1 (20), and for some other *E. coli* strains (23). However, some of the chromosomally mediated penicillinase present in *ampA* strains is excreted during growth when the strain also carries the *ampB* mutation (K. Nordström, L. G. Burman, and K. G. Eriksson-Grennburg, J. Bacteriol., *in press*).

Since the synthesis of the penicillinase must occur inside the cell membrane, it is reasonable

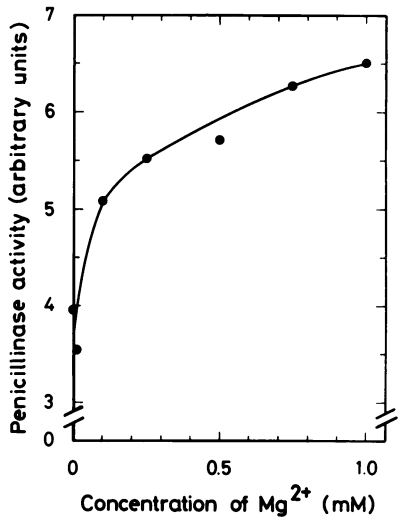


FIG. 12. *Effect of magnesium chloride on the activity of penicillinase from G11a1. The enzyme, dialyzed against 0.01 M Tris-hydrochloride, pH 7.4, was added to the same buffer containing different concentrations of Mg^{2+}. PenG (0.5 mM) was used as substrate. Assay was at 0 C.*

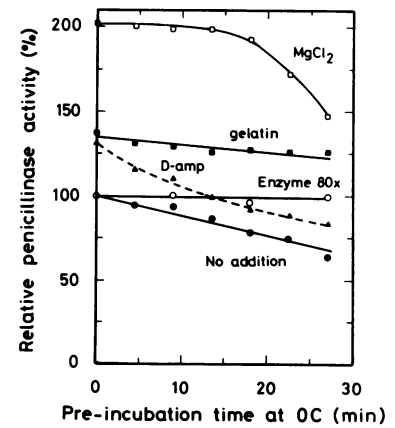


FIG. 13. *Effect of preincubation at 0 C on penicillinase activity. The enzyme, dialyzed against 0.01 M Tris-hydrochloride, pH 7.4, was added to the same buffer supplemented with: □, 10 mM $MgCl_2$; ■, 0.1% gelatin; ▲, 0.2 mM D-amp; ●, no addition; ○, undiluted enzyme, no additions. Assay after preincubation was at 0 C. Substrate was 0.5 mM penG.*

that a fraction of the enzyme activity is retained within the spheroplasts; this is evident from the data in Table 1. However, our value is significantly lower than the percentage found to be intracellular after formation of protoplasts in *Bacillus licheniformis* (35).

Comparison with other penicillinases. The molecular weight of 29,000 found for the chromosomal penicillinase from *E. coli* is of the same order of magnitude as that reported for penicillinases produced by several strains of *Staphylococcus* and *Bacillus* (7). However, in strains of these organisms the penicillinases are inducible and often excreted. It has not been possible to induce the formation of the cell-bound enzyme in *E. coli*. Penicillinases in *Mycobacteria* are also cell-bound and noninducible (18), and they may therefore resemble those in gram-negative bacteria.

Datta and Richmond (8) were the first to purify a penicillinase from *E. coli*, namely the enzyme mediated by the R factor R_{TEM}. Their enzyme, the R-factor enzyme purified by Lindqvist & Nordström (20), and the cephalosporinase produced by *Enterobacter cloacae* (13) have molecular weights between 14,000 and 21,000. This is considerably lower than the 29,000 found for the penicillinase present in *ampA*-carrying strains of *E. coli*. Judging from these data and the known amino acid composition, there is no obvious phylogenetic relationship between the penicillinases from different organisms or episomes in the *Enterobacteriaceae*. Evidence has recently been reported which indicates that the situation could be the reverse in the case of the staphylococcal penicillinases (36). Evolutionary aspects of the structure of purified penicillinases were recently discussed by Ambler and Meadway (1) and by Pollock (29, 30).

All penicillinases previously examined (1, 7) as well as the enzyme from our strain G11a1 and D31 have been shown to lack cysteine (Table 4). With respect to this property, as well as surface location, molecular size, and K_m, our penicillinase resembles the transport protein recently reviewed by Pardee (27).

The correlation between penicillinase activity and resistance to penicillin. By using penicillin G as substrate, the turnover number of our penicillinase is 10- to 100-fold lower than that found for other purified penicillinases (29). Furthermore, the activity against penicillin G was more than 20 times higher than that found against D-ampicillin, despite the fact that both G11a1 (10) and D31 (5) were selected as mutants resistant to DL-ampicillin. The fact that D-ampicillin was hydrolyzed more slowly than the L-epimer can be related both to the finding that the D-epimer is considerably more active than the L-epimer in lysing growing cells (3) and to the well-known fact that the cell wall of *E. coli* contains numerous D-amino acids (34). However, cephalosporin C, which also contains a D-amino acid residue as side chain, gave a V_{max} which was 220 times that obtained for D-ampicillin. In this respect our penicillinase resembles the β-lactamase from *E. cloacae* (13).

Considering the high activity against cephalosporin C, the enzymes could be called "β-lactamase" rather than "penicillinase." However, since it is possible that neither name correctly describes the biological function we have continued to use the term penicillinase.

The yields of purified penicillinase from bacteria grown in rich medium indicate that strain D31 contains about three times more penicillinase than G11a1, which in turn seems to contain at least 10 times more activity than its parent strain G11. However, we have previously shown that the amount of penicillinase for wild-type strains and for *ampA* mutants is directly proportional to the growth rate of the bacteria. This observation has suggested a possible role for the enzyme in the biosynthesis of cell walls (4, 6). Studies of D31 indicate that the amount of enzyme in this strain is constant at different growth rates and that the capacity of the cell-bound enzyme to hydrolyze penicillin is not sufficient to explain the resistance of growing cells (6). When the substrate dependence was investigated for the cell-bound penicillinase in D31 by using DL-ampicillin, the curve was found to be slightly sigmoid, and the substrate concentration for half saturation was estimated to be about 5 mM (6). This is 1,000-fold higher than the K_m for D-ampicillin and the purified enzyme (*see* Table 5). Another factor to be emphasized is that the recovery after the release of the enzyme was more than 100% (*see* Table 1). The stimulatory effect of large amounts of magnesium and phosphate ions indicates that the polypeptide chain is not in the correct conformation for optimal penicillinase activity. Together these observations indicate that our purified penicillinase may not represent a biologically complete unit. It is possible that an effector is missing (6) or that we have purified a subunit of a larger enzyme complex.

The problems of the penicillinase. The characterization of the penicillinase described in this paper has left two main questions unanswered. (i) Is the *ampA* gene the structural gene for the penicillinase which has been purified? (ii) What is the natural function(s) of this penicillinase, which is mediated by a chromosomal gene present also in a wild-type strain?

That the *ampA* region contains at least the

structural information for the penicillinase is indicated by the following fact: strain D31 was constructed to contain the *ampA1* allele from G11a1 (5). The subsequent finding that the enzymes from the two strains are indistinguishable is thus consistent with the assumption that the *ampA* region contains the structural information for the penicillinase.

The mutant G11a1 produced at least 10 times more penicillinase than the wild-type strain. To account for this finding, assumptions must also be made as to the effect of the mutation(s) on the regulation of the structural gene. From the present data it is difficult to conclude whether the wild-type strain produces a penicillinase identical to that of the mutants. However, neither immunological tests (Fig. 5) nor K_m values for two substrates (Table 5) showed any differences between enzyme prepared from strains G11, G11a1, and D31. However, the enzyme prepared from G11 gave two bands in gel electrophoresis and its turnover number was about half of the value obtained for the two other strains. The present data, therefore, do not permit definite conclusions concerning the relationship between the penicillinase and the *ampA* gene.

The second question about the natural function of the penicillinase is still open. We have previously presented a working hypothesis according to which penicillinase is an allosteric enzyme involved in cell wall biosynthesis (4). Since the results here do not substantially support or disprove the hypothesis, we intend to continue and search the bacterial cell for components which can combine with the enzyme or interfere with its catalytic activity.

ACKNOWLEDGMENTS

We thank Anita Lindström for technical assistance. Hugo Thelin at Astra kindly helped us with purification of the stereoisomer of ampicillin. We also thank G. Johansson and G. Blomquist for the ultracentrifugation work.

This investigation was supported by grants from The Swedish Natural Science Research Council (Dnr 2453) and The Swedish Cancer Society (Nr 68:44).

EDITOR'S NOTE

Delays in postal transmittal were responsible for delay in publication of this paper. The manuscript was received for review on 16 June 1969. It was essentially acceptable for publication on 13 August 1969.

LITERATURE CITED

1. Ambler, R. P., and R. J. Meadway. 1969. Chemical structure of bacterial penicillinases. Nature **222**:24–26.
2. Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. **91**:222–233.
3. Boman, H. G., and K. G. Eriksson. 1963. Penicillin induced lysis in *Escherichia coli*. J. Gen. Microbiol. **31**:339–352.
4. Boman, H. G., K. G. Eriksson-Grennberg, J. Földes, and E. B. Lindström. 1967. The regulation and possible evolution of a penicillinase-like enzyme in *Escherichia coli*, p. 366–372. *In* V. V. Koningsberger and L. Bosch (ed), Regulation of nucleic acid and protein biosynthesis. B.B.A. Library, vol. 10. Elsevier Publishing Co., Amsterdam.
5. Boman, H. G., K. G. Eriksson-Grennberg, S. Normark, and E. Matsson. 1968. Resistance of *Escherichia coli* to penicillins. IV. Genetic study of mutants resistant to D,L-ampicillin concentrations of 100 μg/ml. Genet. Res. (Cambridge) **12**:169–185.
6. Burman, L. G., K. Nordström, and H. G. Boman. 1968. Resistance of *Escherichia coli* to penicillins. V. Physiological comparison of two isogenic strains, one with chromosomally and one with episomally mediated ampicillin resistance. J. Bacteriol. **96**:438–446.
7. Citri, N., and M. R. Pollock. 1966. The biochemistry and function of β-lactamase (penicillinase). Advan. Enzymol. **28**:237–323.
8. Datta, N., and M. H. Richmond. 1966. The purification and properties of a penicillinase whose synthesis is mediated by an R-factor in *Escherichia coli*. Biochem. J. **98**:204–209.
9. Dubnau, D. A., and M. R. Pollock. 1965. The genetics of *Bacillus licheniformis* penicillinase: a preliminary analysis from studies on mutation and inter-strain and intra-strain transformations. J. Gen. Microbiol. **41**:7–21.
10. Eriksson-Grennberg, K. G., H. G. Boman, J. A. T. Jansson, and S. Thorén. 1965. Resistance of *Escherichia coli* to penicillins. I. Genetic study of some ampicillin-resistant mutants. J. Bacteriol. **90**:54–62.
11. Eriksson-Grennberg, K. G. 1968. Resistance of *Escherichia coli* to penicillins. II. An improved mapping of the *ampA* gene. Genet. Res. (Cambridge) **12**:147–156.
12. Eriksson, S., and J. Sjöquist. 1960. Quantitative determination of *N*-terminal amino acids in some serum proteins. Biochim. Biophys. Acta **45**:290–296.
13. Hennessey, T. D., and M. H. Richmond. 1968. The purification and some properties of a β-lactamase (cephalosporinase) synthesized by *Enterobacter cloacae*. Biochem. J. **109**:469–473.
14. Heppel, L. A. 1967. Selective release of enzymes from bacteria. Science **156**:1451–1455.
15. Hjertén, S., S. Jerstedt, and A. Tiselius. 1965. Some aspects of the use of "continuous" and "discontinuous" buffer systems in polyacrylamide gel electrophoresis. Anal. Biochem. **11**: 219–223.
16. Jeppsson, J. O., and J. Sjöquist. 1967. Thin-layer chromatography of PTH amino acids. Anal. Biochem. **18**:264–269.
17. Kabat, E. A., and M. M. Mayer. 1961. Preparation of antigens for immunization and injection schedules, p. 871–872. *In* Experimental immunochemistry, 2nd ed., Charles C Thomas Publisher, Springfield.
18. Kasik, J. E., and L. Peacham. 1968. Properties of β-lactamases produced by three species of mycobacteria. Biochem. J. **107**:675–682.
19. Levin, Ö. 1952. Column chromatography of proteins: Calcium phosphate, p. 27–32. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. V. Academic Press Inc., New York.
20. Lindqvist, R. C., and K. Nordström. Resistance of *Escherichia coli* to penicillins. VII. Purification and characterization of a penicillinase mediated by the R-factor R1. J. Bacteriol. **101**:232–239.
21. Lindström, E. B., and H. G. Boman. 1968. Purification of penicillinase from wild type and two ampicillin resistant mutants of *Escherichia coli*. Biochem. J. **106**:43p.
22. Malamy, M., and B. L. Horecker. 1961. The localization of alkaline phosphatase in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **5**:104–108.
23. Neu, H. C. 1968. The surface localization of penicillinases in *Escherichia coli* and *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. **32**:258–263.
24. Nordström, K., K. G. Eriksson-Grennberg, and H. G. Boman. 1968. Resistance of *Escherichia coli* to penicillins. III. *ampB*, a locus affecting episomally and chromosomally

mediated resistance to ampicillin and chloramphenicol. Genet. Res. (Cambridge) 12:157–168.
25. Novick, R. P. 1962. Micro-iodometric assay for penicillinase. Biochem. J. 83:236–240.
26. Novick, R. P. 1967. Penicillinase plasmids of *Staphylococcus aureus*. Fed. Proc. 26:29–38.
27. Pardee, A. B. 1968. Membrane transport proteins. Science 162:632–637.
28. Pollock, M. R. 1963. The differential effect of actinomycin D on the biosynthesis of enzymes in *Bacillus subtilis* and *Bacillus cereus*. Biochim. Biophys. Acta 76:80–93.
29. Pollock, M. R. 1967. Origin and function of penicillinase: a problem in biochemical evolution. Brit. Med. J. 4:71–77.
30. Pollock, M. R. 1968. The range and significance of variations amongst bacterial penicillinases. Ann. N. Y. Acad. Sci. 151:502–515.
31. Reisfeld, R. A., U. J. Lewis, and D. E. Williams. 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195:281–283.
32. Repaske, R. 1958. Lysis of gram-negative organisms and the role of versene. Biochim. Biophys. Acta 30:225–232.
33. Richmond, M. H. 1968. The plasmids of *Staphylococcus aureus* and their relation to other extrachromosomal elements in bacteria. Adv. Microbiol. Physiol. 2:43–88.
34. Salton, M. R. J. 1964. The bacterial cell wall. Elsevier Publishing Co., Amsterdam.
35. Sargent, M. G., B. K. Ghosh, and J. O. Lampen. 1968. Localization of cell-bound penicillinase in *Bacillus licheniformis*. J. Bacteriol. 96:1329–1338.
36. Sawai, T., S. Mitsuhashi, and S. Yamagishi. 1968. Comparison of the chromosomal and extrachromosomal genetic determinants controlling *Staphylococcal* penicillinase production. Jap. J. Microbiol. 12:531–533.
37. Sjöquist, J. 1960. Determination of amino acids as phenyl thiohydantoin derivatives. Biochim. Biophys. Acta 41:20–30.
38. Tabor, C. 1962. Stabilization of protoplasts and spheroplasts by spermine and other polyamines. J. Bacteriol. 83:1101–1111.
39. Taylor, A. L., and C. D. Trotter. 1967. Revised linkage map of *Escherichia coli*. Bacteriol. Rev. 31:332–353.
40. Vogel, H. J., and D. M. Bonner. 1956. Acetyl-ornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. 218:97–106.