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Synthesis of R-Plasmid-Coded β-Lactamase in Minicells and in an In Vitro System

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β-Lactamase encoded by a small, nontransferring R-plasmid, NTP1, conferring ampicillin resistance to its host bacteria, was purified. NTP1 plasmid-coded β-lactamase was found to be periplasmically located in the host *Escherichia coli* cell, to have a molecular weight of about 25,000, and to show a relatively low activity against oxacillin and methicillin compared with benzylpenicillin. These characteristics indicate that NTP1 plasmid-coded β-lactamase is very similar or identical to the "TEM-type" β-lactamase, which is the most common β-lactamase coded by R-plasmids in enteric bacteria. In minicells containing NTP1 plasmids, at least six plasmid-specific proteins were synthesized, and β-lactamase was synthesized in a greater amount than other plasmid-coded proteins. In a cell-free transcription-translation coupled system from *E. coli*, NTP1 plasmid deoxyribonucleic acid directed the synthesis of several species of plasmid-specific proteins, including active β-lactamase. The in vitro system also showed preferential synthesis of β-lactamase, as was observed in minicells containing NTP1 plasmids.

NTP1 plasmid is a small, nontransferring R-plasmid conferring ampicillin resistance to its host enteric bacterium (2). Because 10 to 20 copies of the plasmid deoxyribonucleic acid (DNA) are present per host cell (25), NTP1 plasmid DNA can be easily prepared in a large quantity and in a pure form. Therefore, it is suitable as a simple model system for the study of control mechanisms involved in plasmid gene expression in an in vitro system.

Since NTP1 plasmid-coded β-lactamase (penicillinase, or penicillin β-lactam amidohydrolase, EC 3.5.2.6) had not been well characterized, it was necessary to purify and characterize this enzyme before initiating studies on the expression of NTP1 plasmid genes. NTP1 plasmid-coded β-lactamase was purified from the host *Escherichia coli* cells. The purified enzyme showed characteristics very similar or identical to the "TEM-type" β-lactamase (7, 8, 11), the most common R-plasmid-coded β-lactamase, which is found in a variety of naturally occurring penicillin-resistant enteric bacteria (11, 14).

To minimize host protein synthesis, which would mask plasmid-specific protein synthesis in the host cells, NTP1 plasmid-specific protein synthesis was studied by using a minicell-producing strain of *E. coli* (1, 23). In R^+ minicells, which contained NTP1 plasmids, at least six plasmid-specific proteins, including β-lactamase, were synthesized.

An in vitro transcription-translation coupled system was prepared from plasmid-free *E. coli* cells, and the ability of NTP1 plasmid DNA to direct the synthesis of plasmid-specific proteins was tested. Proteins synthesized in vitro, including active β-lactamase, were similar in size to those synthesized in minicells containing NTP1 plasmids. In both minicells and the in vitro system, β-lactamase appeared to be synthesized preferentially.

MATERIALS AND METHODS

Bacterial strains. Table 1 shows the bacterial strains used.

Materials. Benzylpenicillin (sodium salt) was a gift from Sigma Chemical Co. Oxacillin and methicillin were given to us by Bristol Laboratories. [^{3}H]thymidine, [^{3}H]phenylalanine, and uniformly ^{14}C-labeled amino acid mixture were purchased from New England Nuclear Corp. Penicillinase and nuclease-free Pronase were obtained from Calbiochem. Other chemicals were from commercial sources.

Purification of β-lactamase. *E. coli* 18R405 carrying NTP1 plasmid was grown in 30 liters of L-broth (21) to mid-log phase (200 Klett units with a green filter). β-Lactamase was released from the cells by the formation of spheroplasts prepared as described by Lindström, Boman, and Steele (20). More than 90% of the total β-lactamase activity of the cells was released. The released β-lactamase was purified by the procedure of Datta and Richmond (8). The procedure included a stepwise elution of the enzyme from

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TABLE 1. *Escherichia coli strains*

Strain	Plasmid carried	Relevant genetic markers
1R713	None	K-12, lac^-, wild type (2)
18R405	NTP1	Same as 1R713 (2)
13R108	A-Δ[a]	Same as 1R713 (2)
χ925	None	$minA^-$ $minB^-$ thr^- leu^- thi^- (23), same as P678-54 of Adler et al. (1)
40R936	NTP1	Same as χ925
D10 F⁻	None	Ribonuclease I⁻, met^- (28)

[a] Single-copy transferring plasmid conferring ampicillin resistance with a molecular weight of 63 × 10^6 (2, 25).

a diethylaminoethyl (DEAE)-cellulose column with 0.3 M phosphate buffer, pH 7.4, and then gradient elution from a second DEAE-cellulose column with 0.03 to 0.05 M phosphate buffer, pH 7.4. This partially purified enzyme preparation was loaded onto a Sephadex G-100 column and eluted with 25 mM phosphate buffer, pH 7.4. The β-lactamase preparation after passage through the Sephadex column showed one major and three minor protein bands by acrylamide-sodium dodecyl sulfate (SDS) gel electrophoresis. Further purification was accomplished by acrylamide gel electrophoresis under nondenaturing conditions, i.e., without SDS and mercaptoethanol. Samples were subjected to electrophoresis by using all sections of a slab gel. One section of the slab gel was stained, and the gel regions in other sections corresponding to the major protein bands were sliced and combined. The material eluted from the combined gel slices with 25 mM phosphate buffer, pH 7.4, contained all the β-lactamase activity of the sample and was almost pure when analyzed by acrylamide-SDS gel electrophoresis (Fig. 1).

β-Lactamase assay. Throughout the purification process, and for assaying β-lactamase activity in cell-free extracts and in vitro systems, a microiodometric assay procedure described by Novick (22) was used. The assay was standardized by using both standard penicillinase (Calbiochem) and sodium thiosulfate. One unit of β-lactamase activity was, as defined by the International Commission on Enzymes, the amount catalyzing hydrolysis of 1 μmol of substrate per min at 30°C in 25 mM phosphate buffer at pH 7.4.

Acrylamide-SDS slab gel electrophoresis. Slab gels (8 cm long and 1.5 mm thick) were prepared as described by Laemmli (18) and contained 12.5 or 15% acrylamide and 0.1% SDS. Electrophoesis was carried out at 12 mA for 1.75 h followed by 30 mA for 2 to 3 h. Usually, nonradioactive marker proteins and radioactive proteins were subjected to electrophoresis in the same slab gel by using different sections. After electrophoresis, the gel was stained to detect marker proteins and then photofluorographed to detect radioactive proteins (4).

Preparation of R⁺ and R⁻ minicells. The minicell-producing strains 40R936 carrying NTP1 plasmid (R⁺) and plasmid-free χ925(R⁻) (Table 1), were grown to a cell density of 250 to 300 Klett units at 37°C in M9 medium (21) plus 0.4% glucose supplemented with 4 mg of Casamino Acids (Difco) per ml and 10 μg of thiamine per ml. Separation of R⁺ and R⁻ minicells from normal cells was achieved by successive sucrose gradient centrifugation as described by Roozen et al. (23). Both R⁺ and R⁻ minicells prepared by this method contained fewer than 10^3 viable cells per 10^9 minicells.

Protein synthesis by R⁺ and R⁻ minicells. Purified minicells were suspended in M9-glucose medium with 40 μg of threonine, 40 μg of leucine, and 10 μg of thiamine per ml to a concentration of 10^9 minicells per ml. Uniformly ^{14}C-labeled amino acid mixture, 20 μCi/ml, was added to the minicell suspension and incubated at 37°C for 30 min. At the termination of protein synthesis, minicells were harvested by centrifugation and lysed with lysozyme and ethylenediaminetetraacetic acid (EDTA) by the procedure of Kool et al. (17), and the radioactive proteins in the lysate were analyzed by acrylamide-SDS slab gel electrophoresis.

Isolation of NTP1 plasmid DNA. NTP1 plasmid-containing strain 18R405 was grown to log phase in 1 liter of L-broth. Isolation of NTP1 plasmid DNA followed the method described by Clewell and Helinski (5). The cells were harvested, washed, and lysed with Brij 58, and cleared lysate was treated with Pronase. The DNA was precipitated with ethanol, resuspended in tris(hydroxymethyl)aminomethane-EDTA buffer, and subjected to CsCl equilibrium banding in the presence of ethidium bromide. CsCl gradient fractions containing the covalently closed circular DNA were pooled and passed over a column of Bio-Rad AG50WX2 (to remove the ethidium bromide) and Bio-Gel A15M to separate remaining ribonucleic acid (RNA) and protein from the DNA. The yield of plasmid DNA, which showed a ratio of absorbancy at 260 nm to absorbancy at 280 nm of about 2.0, was usually 150 μg per liter of culture.

Synthesis of NTP1 plasmid proteins in vitro. The in vitro transcription-translation coupled system used was the same as described previously (29) for the synthesis of T7 phage proteins directed by T7 DNA and contained washed ribosomes and DEAE-cellulose-treated supernatant fraction (S200) from *E. coli* D10 F⁻ (ribonuclease I⁻). NTP1 plasmid DNA, 1.5 μg, was added to 50 μl of reaction mixture with 5 μCi of [^3H]phenylalanine and was incubated at 37°C for 30 min. The products of the in vitro system were analyzed by acrylamide-SDS slab gel electrophoresis. T7 DNA-directed protein synthesis was carried out in parallel to assure the protein-synthesizing activity of the in vitro system and also to provide radioactive T7 proteins as molecular weight markers for the electrophoretic analysis. Sometimes, samples of the reaction mixture were assayed for the β-lactamase activity.

RESULTS

Properties of β-lactamase coded by NTP1 plasmid. NTP1 plasmid-coded β-lactamase was purified from *E. coli* 18R405 cells as described in Materials and Methods. Purified β-lactamase showed a single protein band in acrylamide-SDS gel electrophoresis (Fig. 1).

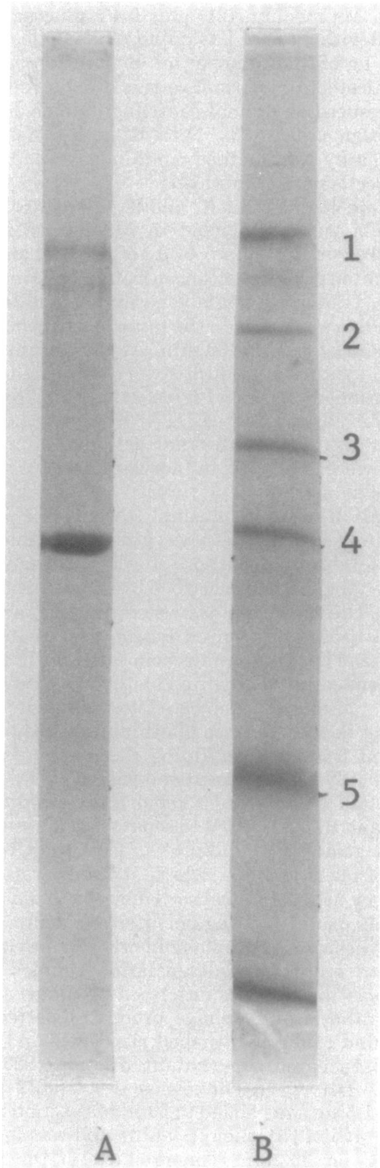

FIG. 1. *Acrylamide-SDS gel electrophoretic pattern of purified NTP1 plasmid-coded β-lactamase. NTP1 plasmid-coded β-lactamase was purified from 18R405 cells as described in Materials and Methods and subjected to acrylamide-SDS slab gel electrophoresis together with marker proteins of known molecular weights. Electrophoresis was run from top to bottom. (A) Purified β-lactamase. (B) Marker proteins: 1, bovine serum albumin (molecular weight 68,000); 2, fumarase (49,000); 3, lactic dehydrogenase (37,000); 4, carbonic anhydrase (29,000); 5, β-lactoglobulin (18,000).*

During the purification process, it was found that most of the β-lactamase activity of the cells (more than 90%) was released by the formation of spheroplasts, indicating that the enzyme is periplasmically located in the cell (26).

Substrate specificity of the NTP1 plasmid-coded β-lactamase is shown in Table 2. An extract from 18R405 cells containing NTP1 plasmids showed a high absolute activity of β-lactamase against benzylpenicillin but relatively low activities against isoxazolyl penicillin derivatives such as oxacillin and methicillin. The substrate specificity of this enzyme agrees with that of a class of R-plasmid-coded β-lactamase, termed "TEM-type" (11, 14). β-Lactamase activity from an extract of 13R108 cells, which contain a large transferring plasmid, "A-Δ" (Table 1), conferring ampicillin resistance, was relatively low compared with that of 18R405 cells but showed a similar substrate specificity (Table 2). Since the β-lactamase gene of the large transferring plasmid is believed to be derived from NTP1, this difference is probably a reflection of the copy numbers of the two plasmids (gene dosage), NTP1 plasmid being a multicopy plasmid whereas the large plasmid is present as a single-copy plasmid in the 13R108 cell (2, 25).

The molecular weight of NTP1 plasmid-coded β-lactamase was estimated by Sephadex G-100 gel filtration. From the elution volumes and known molecular weights of marker proteins, a molecular weight of about 23,000 was assigned to the β-lactamase. This value agrees with that of the TEM-type β-lactamase reported previously (8, 26). Acrylamide-SDS gel electrophoresis, under denaturing conditions, gave a molecular weight value slightly larger, about 27,000, for the β-lactamase, but indicated that the enzyme is a monomeric protein (Fig. 1).

The characteristics described above, i.e., periplasmic location, substrate specificity, and the molecular weight of the enzyme, strongly suggest that the NTP1 plasmid-coded β-lactamase is very similar or identical to the TEM-type β-lactamase, which appears to be the most com-

TABLE 2. *Substrate specificity of NTP1 plasmid-coded β-lactamase*

Strain	Plasmid carried	β-Lactamase activity (mU/10^9 cells)[a]		
		Benzylpenicillin	Oxacillin	Methicillin
1R713	None	32 (2)	15 (1)	14 (1)
18R405	NTP1	1,366 (100)	222 (16)	195 (14)
13R108	A-Δ[b]	285 (21)	38 (3)	37 (3)

[a] β-Lactamase was assayed by use of cell-free extracts obtained by sonic treatment, as described in Materials and Methods. The numbers in parentheses show the relative activity (percent), with β-lactamase activity of strain 18R405 against benzylpenicillin taken as 100%.

[b] See Table 1.

mon R-plasmid-determined β-lactamase among the enteric bacteria (11, 14).

Protein synthesis in R^+ and R^- minicells. R^+ minicells (containing NTP1 plasmids) and R^- minicells (plasmid-free) were prepared from minicell-producing strains 40R936 and χ925, respectively. Protein synthesis by minicells was carried out in the presence of radioactive amino acids with the use of 10^9 minicells suspended in 1 ml of medium at 37°C for 30 min. In R^- minicells protein synthesis continued at a slow and linear rate for about 60 min and then leveled off. R^+ minicells showed a much higher rate of protein synthesis than R^- minicells, and the synthesis continued for at least 90 min.

Figure 2 shows a fluorogram of acrylamide-SDS slab gel electrophoretic patterns of labeled proteins synthesized by minicells in the presence of ^{14}C-labeled amino acids. R^- minicells synthesized three major proteins with molecular weights of about 38,000, 34,000, and 23,000 (Fig. 2C, protein bands H1, H2, and H3, from top to bottom). These proteins are probably host proteins coded by host messenger RNAs (mRNA's) with very long half-lives. This result is in agreement with a previous report which described host proteins synthesized in R^- minicells (19). In R^+ minicells containing NTP1 plasmids, at least six additional proteins were synthesized in addition to the proteins synthesized in R^- minicells (Fig. 2B, protein bands designated as A1 to A6 from top to bottom). One of the NTP1 plasmid-specific proteins, A3 protein in Fig. 2B, migrated in the gel the same distance as purified β-lactamase from 18R405 cells (marker protein no. 4 in Fig. 2A). This protein is assumed to be β-lactamase and is the predominant protein synthesized in R^+ minicells. Estimated molecular weights of NTP1 plasmid-specific proteins synthesized in R^+ minicells are listed in the legend of Fig. 2.

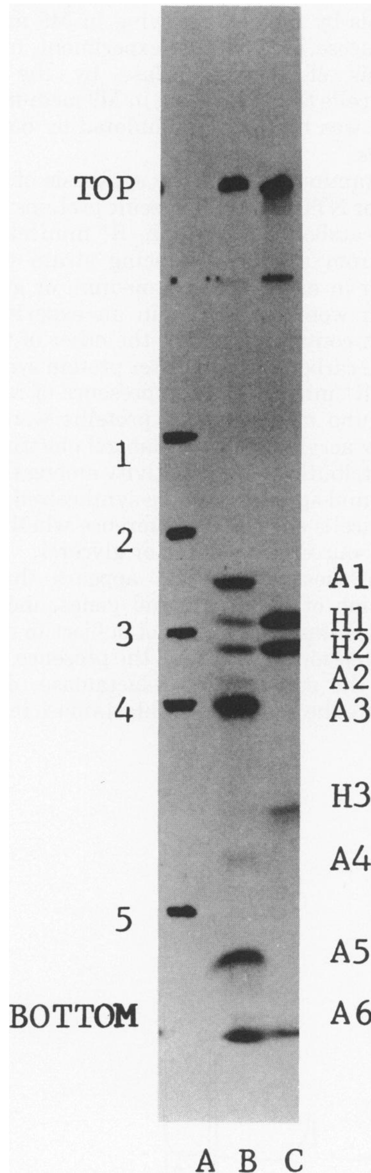

FIG. 2. Acrylamide-SDS slab gel electrophoretic patterns of proteins synthesized in R^+ minicells carrying NTP1 plasmid and in R^- minicells. R^+ and R^- minicells were separated from respective minicell-producing cells, 40R936 and χ925, as described in Materials and Methods. Minicells, 10^9 per ml, were incubated in M9-glucose medium in the presence of ^{14}C-labeled amino acid mixture at 37°C for 30 min, and labeled proteins were analyzed by slab gel electrophoresis as described in Materials and Methods. After the electrophoresis, the slab gel was stained to detect marker proteins and then fluorographed as described in Materials and Methods. ELectrophoresis was from top to bottom. (A) Positions of stained marker proteins (marked by ink to indicate positions): 1, bovine serum albumin (68,000); 2, fumarase (49,000); 3, lactic dehydrogenase (37,000); 4, NTP1 plasmid-coded β-lactamase (27,000); 5, β-lactoglobulin (18,000). (B) ^{14}C-labeled proteins in R^+ minicells containing NTP1 plasmids. (C) ^{14}C-labeled proteins in R^- minicells. Total radioactivity in samples A and B was adjusted to be about the same. Proteins synthesized in R^- minicells were designated as H1, H2, and H3, and their molecular weights were estimated to be about 38,000, 34,000, and 23,000, respectively. NTP1 plasmid-specific proteins synthesized in R^+ minicells were designated as A1, A2, A3, A4, A5, and A6, and their molecular weights were estimated to be about 42,000, 30,000, 27,000, 20,000, 15,000, and 12,000, respectively. Protein bands H3 and A6 are not clearly visible in this figure, but they are detectable in Fig. 3. Radioactive materials at the top and bottom ends of the gel were unknown but disregarded.

To quantitate relative amounts of NTP1 plasmid-specific proteins, fluorograms of radioactive proteins synthesized in R$^+$ and R$^-$ minicells were traced with a densitometer. Figure 3 shows a densitometer tracing of a fluorogram similar to that shown in Fig. 2. Comparison of proteins synthesized in R$^+$ and R$^-$ minicells clearly shows that at least six proteins designated as A1 to A6 are NTP1 plasmid-specific proteins and that at least six proteins designated as A1 to A6 are NTP1 plasmid-specific proteins designated as H1, H2, and H3.

The result shows that the amount of NTP1 plasmid-specific protein A3, which corresponds in size to purified β-lactamase, is greater than that of other plasmid-specific proteins except A5 protein and suggests a preferential synthesis of protein A3, presumably β-lactamase.

Control of NTP1 plasmid-coded β-lactamase synthesis. The results presented above observed with R$^+$ minicells indicated a possible control mechanism which results in a preferential synthesis of β-lactamase compared with that of other plasmid-coded proteins. The expression of some R-plasmid genes which code for antibiotic-modifying enzymes has been shown to be subject to catabolite repression (10, 27). However, catabolite sensitivity of β-lactamase gene in an R-plasmid has not been known. Therefore, the effect of carbon source and cyclic adenosine 3′,5′-monophosphate (cyclic AMP) on the synthesis of β-lactamase in strain 18R405 carrying NTP1 plasmid was examined.

It was found that the differential rate of β-lactamase synthesis and the specific activity of β-lactamase in 18R405 cells carrying NTP1 plasmids were essentially the same whether the cells were grown in M9 medium with glucose or glycerol as the sole source of carbon. Also, addition of cyclic AMP or 5′-AMP did not increase the differential rate of β-lactamase synthesis by the cells growing in M9 medium with glucose. In a parallel experiment, induced synthesis of β-galactosidase by the same 18R405 cells (*lac*$^+$) growing in M9 medium with glucose was markedly stimulated by both nucleotides.

To examine whether the synthesis of any of the other NTP1 plasmid-specific proteins is subject to catabolite repression, R$^+$ minicells prepared from minicell-producing strain 40R936 growing in either glucose medium or glycerol medium were suspended in an experimental medium containing one or the other of the respective carbon sources. After protein synthesis by the R$^+$ minicells in the presence of radioactive amino acids, labeled proteins were analyzed by acrylamide-SDS slab gel electrophoresis. Distribution of radioactivity among the major plasmid-specific proteins synthesized by the R$^+$ minicells showed no difference whether the carbon source was glucose or glycerol.

From these results, it appears that the expression of NTP1 plasmid genes, including the β-lactamase gene, is not subject to catabolite repression. In addition, the presence of penicillin, the substrate of β-lactamase, did not stimulate the synthesis of β-lactamase in strain 18R405.

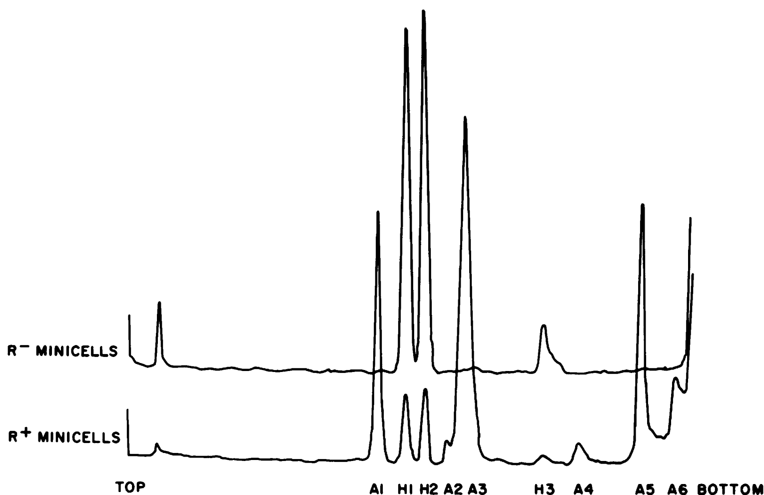

FIG. 3. *Densitometer tracing of fluorograms showing acrylamide-SDS gel electrophoretic patterns of proteins synthesized in R$^+$ and R$^-$ minicells. Fluorograms similar to that shown in Fig. 2 were traced by a densitometer. Protein bands were designated as indicated in Fig. 2.*

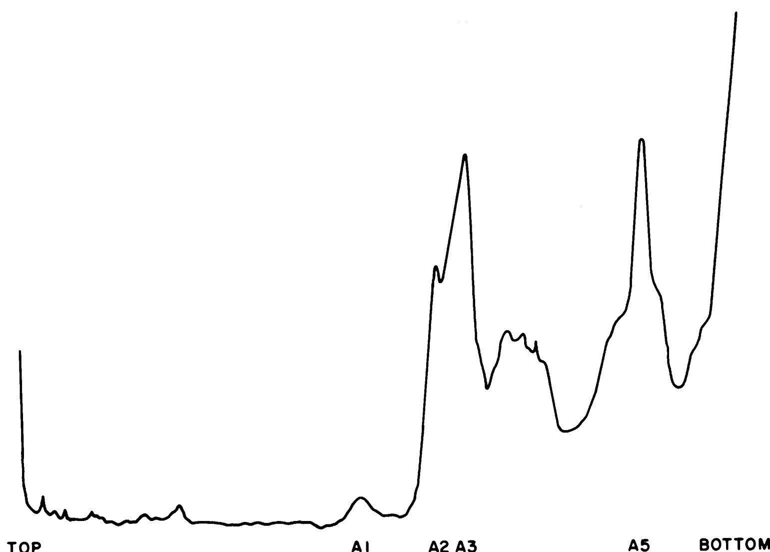

FIG. 4. NTP1 plasmid DNA-directed protein synthesis in vitro. NTP1 plasmid-specific protein synthesis was carried out in an in vitro transcription-translation coupled system at 37°C for 30 min as described in Materials and Methods. The reaction mixture, in a total volume of 50 μl, contained 1.5 μg of NTP1 plasmid DNA and [^{3}H]phenylalanine (5 μCi). Labeled proteins were analyzed by acrylamide-SDS slab gel electrophoresis, and the gel was fluorographed. The fluorogram was traced with a densitometer. From the positions of marker proteins and the proteins synthesized in R^+ minicells, which were subjected to electrophoresis in the same slab gel by using different sections, proteins synthesized in the in vitro system were designated as A1, A2, A3, and A5.

In vitro synthesis of β-lactamase directed by NTP1 plasmid DNA. Purified NTP1 plasmid DNA was used to direct the synthesis of plasmid-specific proteins in an in vitro transcription-translation coupled system prepared from plasmid-free *E. coli* F^- (D10 F^-, ribonuclease I^-) cells (28). Figure 4 shows that NTP1 plasmid DNA was capable of directing the synthesis of several proteins, and at least some of them (designated as A1, A2, A3, and A5 in Fig. 4) were similar in size to those synthesized in R^+ minicells containing NTP1 plasmids (see Fig. 2 and 3). Radioactive materials migrating in the gel between A3 and A5 proteins were regarded as host proteins synthesized as a result of the presence of residual endogenous mRNA in the in vitro system. Gel electrophoretic patterns of proteins synthesized in vitro were often diffused because the samples applied to the gel contained a large amount of nonradioactive proteins from the in vitro system.

The A3 protein corresponds to purified β-lactamase in electrophoretic mobility (molecular weight of about 27,000). Furthermore, β-lactamase assay of the plasmid DNA-directed in vitro system revealed a β-lactamase activity of about 100 mU per μg of NTP1 plasmid DNA after a 30-min incubation. This value is approximately 1% of the β-lactamase productivity of 18R405 cells in vivo if one uses a figure of 15 copies of NTP1 plasmid per cell (25), a plasmid molecular weight of 5.6×10^6 (25), and an enzyme production rate of 1,366 mU of β-lactamase per 10^9 cells per doubling (Table 2).

DISCUSSION

β-Lactamases determined by a variety of enterobacterial R-plasmids have been characterized and classified into two major groups (11). One type, the so-called TEM-type (11, 14), is exemplified by the enzyme specified by plasmid RTEM (now termed R6K); the other type is characterized by its ability to hydrolyze oxacillin and is called O-type (or OXY-type).

The properties of β-lactamase encoded by NTP1 plasmid correspond well with those of TEM-type β-lactamase in many respects. NTP1 plasmid-coded β-lactamase is mostly (more than 90%) periplasmically located in the host cell, and its molecular weight is smaller than that of O-type β-lactamase. Purified β-lactamase from the host cells carrying NTP1 plasmids has a molecular weight of about 23,000 (gel filtration) to 27,000 (acrylamide-SDS gel electrophoresis), and this value agrees well with that of TEM-type β-lactamase determined by other R-plasmids (8, 11). NTP1 plasmid-

coded β-lactamase shows a high absolute activity, over 1,000 mU per 10^9 cells, against benzylpenicillin, whereas its activity against oxacillin and methicillin is low, again demonstrating a good resemblance to the TEM-type β-lactamase (11). From these characteristics, it is almost certain that NTP1 plasmid-coded β-lactamase is a TEM-type enzyme. However, a final determination must depend on a demonstration that the β-lactamase gene of NTP1 plasmid has a base sequence homology with that of the TEM-type β-lactamase gene of other plasmids.

Recent work has shown that the TEM β-lactamase gene is sometimes located on a sequence of plasmid DNA (about 3×10^6 molecular weight), called a "transposon" or "translocon" (TnA), which can be translocated from one replicon to another (6, 12–14, 24). It was shown that this same sequence is present on many naturally occurring R-plasmids of different incompatibility groups that specify the TEM-type β-lactamase (14). These findings suggest that the original R-plasmid which was isolated from *Salmonella typhimurium* and from which NTP1 plasmid was derived (2) could have received the TnA sequence by translocation from another plasmid.

In support of these assumption, it is of particular interest that both NTP3 (confers ampicillin and sulfonamide resistance) and NTP4 (confers resistance to ampicillin, streptomycin, and sulfonamide) plasmids (25), resulting from recombination between the NTP1 (ampicillin) and NTP2 (streptomycin and sulfonamide) plasmids and containing the β-lactamase gene, have molecular weights about 3×10^6 larger than that of the parental plasmid NTP2 (25). This suggests the possibility that NTP3 and NTP4 plasmids were derived by a translocation of the TnA sequence from NTP1 to NTP2 rather than by a conventional recombination.

Plasmid-specific protein synthesis in minicells offers an excellent system for studying the expression of plasmid genes and their control, since the synthesis of host RNA and protein is minimized in minicells separated from the normal cells. As presented in this paper, NTP1 plasmid-containing minicells produced at least six plasmid-specific proteins in addition to a few host proteins which were also produced in plasmid-free R^- minicells. Among the plasmid-specific proteins, one protein corresponding to β-lactamase by its mobility in electrophoresis was produced in a greater amount than most other proteins. This suggests the presence of some control mechanism which regulates a preferential expression of the β-lactamase gene.

It has recently been shown that the synthesis of an R-plasmid protein associated with tetracycline resistance is negatively regulated and tetracycline itself acts as an inducer of the control system (30). It is also known that β-lactamase synthesis in *Staphylococcus* is inducible by penicillin (3). However, in enteric bacteria, plasmid-determined β-lactamase synthesis is not stimulated by the presence of penicillin (3). On the other hand, it has been shown that the production of certain plasmid-determined proteins that confer antibiotic resistance to the host *E. coli* cells is subject to catabolite repression. These include resistance to streptomycin (10), chloramphenicol (10), and kanamycin (27). It is interesting to mention that the synthesis of colicin E1, which is determined by ColE1 plasmid, is also sensitive to catabolite repression (15). ColE1 is another small, nontransferring, multicopy plasmid and possesses similar properties to the NTP1 plasmid, including the fact that the replication of both plasmid DNAs depends on the presence of DNA polymerase I (*polA* gene) in the host cells (9, 16). However, neither the synthesis of NTP1 plasmid-coded β-lactamase nor of othe NTP1 plasmid-specific proteins appears to be controlled by catabolite repression, as described in this paper.

In an in vitro transcription-translation coupled system prepared from F^- cells of *E. coli*, NTP1 plasmid DNA directed synthesis of several proteins with electrophoretic mobilities similar to the major NTP1 plasmid-specific proteins produced in R^+ minicells containing NTP1 plasmids. In addition, we were able to show an activity of β-lactamase for the protein synthesized in the in vitro system. Furthermore, our in vitro system appears to show preferential synthesis of β-lactamase as observed in R^+ minicells.

The results obtained from the protein synthesis in minicells and the in vitro system suggest that only limited portions of the 5.6×10^6 molecular weight NTP1 plasmid DNA sequence are involved in coding for all plasmid-specific proteins. If one assumes that each protein band in the gel electrophoretic analysis represents one protein, one can estimate that only a stretch of DNA sequence equivalent to a molecular weight of about 2.4×10^6 is required to code for all plasmid-specific proteins (Fig. 2 and 3). Since β-lactamase gene is included in the TnA sequence, it is tempting to speculate that the β-lactamase gene has an independent transcriptional promoter which is able to express the β-lactamase gene after translocation of the TnA sequence into other replicons and that this promoter may have a high affinity to RNA

polymerase for the preferential transcription of β-lactamase gene. We are currently investigating this possibility.

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