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Penicillinases of *Klebsiella pneumoniae* and Their Phylogenetic Relationship to Penicillinases Mediated by R Factors

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On the assumption that the penicillinase determinants on a group of R factors conferring ampicillin resistance have a phylogenetically close relationship to the penicillinase gene of the *Klebsiella* group, the penicillinases from four strains of *K. pneumoniae*, GN69, GN1103R$^-$, GN422, and GN118, were purified 230- to 1,000-fold and compared with the known two R-factor-mediated penicillinases. By gel filtration on Sephadex G-75, the molecular weights were estimated to be 17,400, 18,100, 20,000 and 18,300, respectively, which are slightly lower than those of the R-factor penicillinases. The isoelectric points of the *Klebsiella* penicillinases were not in agreement with those of the R-factor penicillinases. All the enzymes showed a pH optimum between 6.3 to 7.2 and a temperature optimum of 45 C, and those properties, together with behavior towards inhibitors, were about the same as those in the R-factor penicillinases. The substrate specificity and the Michaelis constants of the *Klebsiella* penicillinases for penicillins and cephaloridine were broadly similar to those of the R-factor penicillinases, however, some variations were found even among the four penicillinases of *K. pneumoniae*. The reactivities of the four penicillinases of *K. pneumoniae* with the antiserum against one R-factor penicillinase were tested, and three of the four *Klebsiella* penicillinases were found to be indistinguishable immunologically from both R-factor penicillinases. The remaining *Klebsiella* penicillinase, from GN1103R$^-$, showed an immunological partial homology with the R-factor penicillinases.

It is widely acknowledged that drug resistance in many enteric bacteria depends upon the presence of transmissible R factors. The host ranges of R factors include not only many genera of enteric bacteria but also the genus *Pseudomonas* (16). Many R factors have been studied as to their genetic (3, 31) and physical (10, 23, 34, 41) properties, and also extensive epidemiological surveys for R factors have been made in Britain (3) and in Japan (32). The concept that transmissible R factors are formed by combination between two initially independent genetic elements (3), namely a sex factor and drug-resistant determinant(s), is to a large extent accepted to this day. It is also known that the so-called sex factors are present in a number of enteric bacteria (2, 22, 33), and it is believed that they existed in enteric bacteria long before the occurrence and recognition of R factors.

One of the approaches to the clarification of the evolution of R factors is to identify the origin of the drug-resistant determinant(s). Ampicillin-resistant determinants on R factors may be most useful material for such a purpose because the resistant determinant, in itself, might be a penicillinase gene which can be identified by the characteristics of its penicillinase.

Since the discovery of ampicillin-resistant R factors (4), we have studied the penicillinases produced by *Escherichia coli* strains harboring R factors and have classified those penicillinases into two types, i.e., type I and II, on the basis of their enzymological and immunological properties (14, 39, 45). Recently, we demonstrated the existence of a variant in type I penicillinases. The variant was named type Ib penicillinase, and the original type I penicillinase was renamed type Ia penicillinase (40). The two type I penicillinases were similar to each

other in many enzymological properties and were indistinguishable from each other in immunological properties. However, they differ in molecular weight, isoelectric point, and penicillinase activity confered on the host organisms.

Also, we have compared the penicillinases of R factors with crude preparations of species-specific β-lactamases from various gram-negative bacteria (39). The survey revealed the existence of remarkable similarity between R-factor type Ia penicillinase and the penicillinases from *Klebsiella pneumoniae* in enzymological and immunological properties, and suggested that the genetic determinant of R-factor type Ia penicillinase might have been derived from the *Klebsiella* group.

In this paper we describe further detailed properties of partly purified penicillinases isolated from four strains of *K. pneumoniae*, together with a comparison of these penicillinases with the type Ia and Ib penicillinases previously characterized.

MATERIALS AND METHODS

Bacterial strains. All *K. pneumoniae* strains used were of human origin and resistant to ampicillin. The transferability of the resistance character in these strains was not detected (39), and the ampicillin resistance (or the ability for penicillinase production) was not lost from those strains after storage in cooked meat medium (Eiken Chem Co., Tokyo, Japan) for long periods. The strains were identified according to the criteria of Breed et al. (9) and of Edwards and Ewing (13). Strains GN69, GN118, and GN422 were isolated in Japan in 1965. Strain GN1103 was isolated in Japan in 1966 and harbored an R factor mediating resistance to chloramphenicol, streptomycin, tetracycline, and sulfonamide. During storage of GN1103 in cooked meat medium, a derivative strain which spontaneously lost the R factor was isolated and designated as GN1103R$^-$.

Drugs. Penicillins and a cephalosporin used were benzylpenicillin, ampicillin, phenethicillin, 6-aminopenicillanic acid, and cephaloridine. Penicillins were supplied by Meiji Seika Co., Tokyo, Japan. Cephaloridine was a product of Glaxo Laboratories, England, and kindly provided by Torii Pharmaceutical Co., Tokyo, Japan.

Penicillinases of R factors. Type Ia penicillinase was purified from *E. coli* W3630 harboring R_{GN14} by a modification of the procedures reported previously (45). The enzyme preparation had a specific penicillinase activity of 20,000 U/mg of protein, and its purity was estimated to be more than 20% on the basis of recent data for completely purified type Ia penicillinase. Type Ib penicillinase from *E. coli* ML1410 harboring R_{GN823} was purified by the methods described previously (40). The type Ib penicillinase preparation was almost completely pure.

Preparation of columns. Sephadex G-50 and G-75 (Pharmacia, Uppsala, Sweden) were prepared and packed in columns as recommended by the manufacturer. Carboxymethyl (CM)-cellulose and diethylaminoethyl-cellulose (Brown Co., N.Y.) were washed before use with sodium hydroxide (0.5 N) and hydrochloric acid (0.5 N), and packed as recommened by the manufacturer.

Enzyme assays. Penicillin beta-lactamase (penicillinase) activity was determined iodometrically at 30 C by the method of Perret (37), except that 0.1 M phosphate buffer (pH 6.5) was used instead of 0.2 M phosphate buffer (pH 6.5). Cephalosporin beta-lactamase (cephalosporinase) activity was measured similarly except that cephaloridine was used as substrate and the amount of hydrolyzed cephaloridine was calculated on the basis of 1 mol being equivalent to 2 mol (4 g-atom) of iodine (1). The microiodometric assay method devised by Novick (35) was employed for kinetic measurement of the enzyme reaction.

Penicillinase and cephalosporinase activities were expressed in units; one unit of the enzyme activity was defined as that amount of enzyme which hydrolyzed 1 μmol of benzylpenicillin or cephaloridine, respectively, in 1 h at 30 C in 0.1 M phosphate buffer (pH 6.5) with 8 mM substrate.

Protein determinations. The concentration of protein in column eluates was estimated by measuring the absorbancy at 280 nm. For more accurate estimations, the method of Lowry et al. (27) was used.

Determination of molecular weight. The molecular weights of the penicillinases were estimated by gel filtration through a Sephadex G-75 column by the method of Andrews (5). The enzyme preparation was dissolved in 0.5 ml of 0.15 M phosphate buffer (pH 7.0) containing 10 mg of crystallized ovalbumin (mol wt 45,000; C. F. Boehringer & Soehne GmbH, Mannheim, Germany) and 8 mg of cytochrome C (molecular weight 12,400, C. F. Boehringer & Soehne GmbH, Mannheim, Germany), and loaded onto a Sephadex G-75 column (1.2 by 98 cm) equilibrated with the phosphate buffer. The column was eluted with the phosphate buffer at a flow rate of 2.5 ml/h at 5 C. Elution of protein was followed by measuring the absorbancy at 280 nm with an ultraviolet monitor (Hitach, model QPR83), and fractions (1.0 ml each) of the column effluent were assayed for penicillinase activity. Under the same experimental conditions, the molecular weight of chymotrypsinogen (mol wt 25,000, C. F. Boehringer & Soehne GmbH, Mannheim, Germany) was estimated to be 24,300.

Immunology. Rabbit antiserum against R-factor-mediated type Ia penicillinase was prepared as described previously (39). The effect of the antiserum on the penicillinase activity was examined by the method described previously (39).

Penicillinase preparations were analyzed by micro double immunodiffusion (Ouchterlony technique) (36). The gel contained 1% agar (special agar-Noble, Difco) in physiological saline was prepared as a 1-mm thick layer on a microscope slide. The wells with a diameter of 1.8 mm were removed by using a glass capillary tube. The distance between the central well for the antiserum and the outer wells for penicillinase preparations was 7 mm, and that between each outer well was 1 cm. However, in the case of the penicillinase from GN1103R$^-$, the distance between the outer

wells was reduced to 7 mm. Anti-(type Ia penicillinase)-serum (2.5 to 2.8 μliters) was applied in the central well by two cycles of application; the penicillinase solutions were applied at 0.8 to 4% concentration and their dilutions filled the outer wells. The volume of the penicillinase solution in the outer wells was from 1 to 2 μliters each. Immunodiffusion was carried out for 24 h at 30 C, after which the agar plate was washed by standing for 2 days in physiological saline, and the photograph of the immunoprecipitate was taken.

Disk electrophoretic analysis. Polyacrylamide gel disk electrophoresis was carried out in a tris(hydroxymethyl)aminomethane-glycine buffer system (pH 8.9) by the method described by Aoki et al. (6). About 100 μg of the penicillinase preparation was applied to two identical gels and subjected to the electrophoresis at 2 C for 45 min at 2 mA per tube (0.5 by 7 cm). One of the gels was stained with Amido Black 10B. The position and density of the stained protein bands were measured with a densitometer, model Ozumor-82 (Asuka Manufacturing Co., Tokyo, Japan). For the detection of the penicillinase activity on the gel, the other gel was cut into 2-mm thick slices which were assayed for their penicillinase activity.

Isoelectric point. Isoelectric points were determined by using an ampholine electrofocusing equipment (model 8100; LKB, Stockholm-Bromma, Sweden). The carrier ampholytes used gave a pH gradient spectrum between pH 3 and 10. The electrofocusing was performed by procedures recommended by the manufacturer. The electrofocusing was carried out below 5 C in an electrolysis column of 110-ml capacity. The total amount of the carrier ampholytes in each experiment was 1.2 g. The enzyme protein (1 to 5 mg) was dissolved in about 2 ml of distilled water, and the undissolved material was removed by centrifugation at 10,000 × *g* for 15 min at 2 C. The supernatant fluid was mixed with the solution containing one-third of the carrier ampholytes. A mixed solution (60 ml total) was used as the less-dense solution. The dense solution contained two-thirds of the carrier ampholytes and 28 g of sucrose in 60 ml of total volume. The cathode (bottom) was filled with the solution which consisted of 12 g of sucrose, 0.4 ml of ethylenediaminetetraacetic acid, and 14 ml of distilled water. To prevent contact between the ampholytes and the anode (top), a solution containing 0.1 ml of concentrated phosphoric acid in 10 ml of distilled water was added at the top of the column.

After focusing for 69 to 72 h with a final potential of 400 V, the contents of the column were cut into 2-ml fractions, and each fraction was assayed for its penicillinase activity and pH. The pH of the fractions was measured at 25 C by a radiometer pH meter (Radiometer, Copenhagen, Denmark) with a relative accuracy of ±0.003 pH units.

Assay of resistance to ampicillin. Methods used to determine ampicillin resistance were described previously (14).

Culture and harvesting of organism. The cells of *K. pneumoniae* GN69 were obtained by cultivation in nutrient broth, with procedure described previously (40). For the large-scale cultivation of the other strains, medium E of Vogel and Bonner (44), supplemented with 0.2% glucose, 0.1% tryptone (Difco), and 1% meat extract (Kyokuto Pharmaceutical Industries, Ltd. Tokyo, Japan), was used. The organism was grown overnight in 500 ml of brain heart infusion broth (Difco) at 37 C. The culture was diluted with 9 liters of the enriched medium E and then grown at the same temperature under aeration for 5 h. The organisms were harvested by using a continuous-flow centrifuge. The yield of organisms was about 40 g wet weight.

Purification of penicillinases from K. pneumoniae strains GN69, GN1103R⁻, GN422, and GN118. Except for the case of strain GN118, the crude enzyme preparations were extracted from bacterial cells as follows. The cell pellet was ground with 200 g of alumina per 100 g of cells in a porcelain mortar at 5 C, and the broken organisms were suspended in five volumes of distilled water and centrifuged at 10,000 × *g* for 30 min at 2 C. The enzyme was further extracted from the precipitate by suspending it with distilled water and centrifuging. The combined supernatant fluid was centrifuged again. Dihydrostreptomycin sulfate was added to the supernatant fluid to give 2% (wt/vol) concentration for removal of nucleic acids. After standing for 4 h at 5 C, the precipitate was removed by centrifuging at 10,000 × *g* for 30 min at 2 C. The supernatant fluid was then dialyzed three times against distilled water at 5 C for 16 h each time. After the precipitate which formed during this dialysis was removed by centrifuging at 10,000 × *g* for 30 min at 2 C, the dialyzed solution (the first stage of the purification) was employed for the next stage (stage 2) of the purification. In the case of strain GN118, the bacterial cells suspended in distilled water were disrupted for 5 min below 5 C by an ultrasonic disintegrator (20 kHz), and the procedures for removal of cell debris and nucleic acids were the same as those described above.

Purification of penicillinase from strain GN69 was carried out as follows (see Table 2). For stage 2, the crude enzyme extract obtained from 159 g (wet weight) of bacterial cells was diluted with 0.01 M phosphate buffer (pH 6.0) and applied to a CM-cellulose column (2.5 by 54 cm) equilibrated with the buffer. After the column was washed with about 500 ml of the buffer, the enzyme was eluted with a linear concentration gradient of NaCl at a flow rate of 45 ml/h, and 20-ml fractions were collected. The gradient was constructed from 1 liter of the phosphate buffer and the same volume of the buffer containing 1 M NaCl. The pooled active eluate (360 ml total) was dialyzed against distilled water for 16 h and used for the next stage of the purification. For stage 3, the enzyme solution from stage 2 was freeze-dried and redissolved in 5 ml of distilled water. The enzyme solution was loaded onto a Sephadex G-50 column (3.7 by 54 cm) equilibrated with 0.005 M phosphate buffer, pH 7.0. The enzyme was eluted with the phosphate buffer at a flow rate of 14 ml/h, and the fraction size was 20 ml. Four fractions at the main enzyme peak were pooled and dialyzed against distilled water. For stage 4, the dialyzed enzyme solution from stage 3 was freeze-dried and redissolved in 4 ml of distilled water. The solution was again loaded onto a Sephadex G-50 column (2.6 by 82 cm) equilibrated

with 0.01 M phosphate buffer, pH 6.1. The enzyme was eluted with the phosphate buffer at a flow rate of 14 ml/h, and 15-ml fractions were collected. Fractions of the main enzyme peak (45 ml total) were pooled and used for the last stage of the purification. For stage 5, the enzyme solution from stage 4 was applied to a CM-cellulose column (2 by 29 cm) equilibrated with 0.01 M phosphate buffer (pH 6.1), and the column was washed with about 200 ml of the buffer. The enzyme was then eluted with a linear concentration gradient of NaCl at a flow rate of 30 ml/h, and the 15-ml fractions were collected. The gradient was constructed from 500 ml of the phosphate buffer and the same volume of the buffer containing 0.5 M NaCl. The main enzyme peak was eluted around 0.17 M NaCl. Two fractions which exhibited the highest specific enzyme activity per protein unit were collected and dialyzed three times against distilled water at 5 C for 16 h each and freeze-dried.

Penicillinases from *K. pneumoniae* strains GN1103R$^-$, GN422, and GN118 were purified by the procedures described above, but Sephadex G-75 was used for gel filtration because we noticed that Sephadex G-75 was more suitable for the purification than Sephadex G-50. The purification procedures, together with the enzyme activity remaining after each purification procedure, the specific enzyme activity per protein unit, and the percentage recovery, are summarized in Tables 3, 4, and 5.

RESULTS

Resistance to ampicillin and penicillinase activity. Levels of resistance to ampicillin and penicillinase activity in the *Klebsiella* strains used for the experiment are shown in Table 1. Levels of ampicillin resistance in the four strains were closely related to the amount of penicillinase activities. As we suggested previously (39), penicillinase production in *Klebsiella* may be the most important factor for the resistance to ampicillin.

Purification of penicillinases. The purification procedures for the penicillinases from strains GN69, GN1103R$^-$, GN422, and GN118 are summarized in Table 2, 3, 4, and 5, respectively.

TABLE 1. *Penicillinase activities and levels of resistance to ampicillin in four strains of K. pneumoniae*

Strain	Penicillinase activity[a]	Resistance to ampicillin[b]
GN69	55.0	800
GN1103R$^-$	12.1	400
GN422	4.0	50
GN118	2.8	25

[a] Specific activity of penicillinase in sonically disrupted cells (units per milligram of dry weight of bacteria).

[b] The maximum concentration of ampicillin which allows visible growth of bacteria on heart infusion agar plate after 18 h of incubation at 37 C.

Although the penicillinases were purified from their crude extracts approximately 230- to 1,000-fold, the purified enzyme preparations gave several bands on analytical polyacrylamide gel electrophoresis. The purity of the enzyme preparations from strains GN69 and GN118 were roughly estimated to be 60 and 10%, respectively, on the basis of densitometric tracings of the acrylamide gel after staining with Amido Black 10B. The purity of the enzyme preparations from GN1103R$^-$ and GN422 could not be estimated.

Molecular weights and isoelectric points. In a previous paper (40), we demonstrated that there were variations in the values of molecular weight and isoelectric point even for R-factor penicillinases, which are thought phyletically to be very close each other. We were interested in whether such variations also existed among species-specific penicillinases of *Klebsiella*.

The approximate molecular weights of the four penicillinases were determined by gel filtration through a Sephadex G-75 column by the method of Andrews (5) in the presence of cytochrome *c* and ovalubumin as internal standards and chymotrypsinogen as an external standard (Table 6).

The molecular weights of the penicillinases from strains GN69, GN1103R$^-$, and GN118 were similar to one another and estimated to be 17,400, 18,100 and 18,300, respectively. On the other hand, the penicillinase from strain GN422 had a molecular weight of 20,000. This difference in molecular weights between the penicillinase of GN422 and those of the other strains was not great. However, the difference may be significant because the experiments were done using the same column and the same experimental conditions.

Figure 1 shows the isoelectric patterns of the four penicillinases. The penicillinases of strains GN69, GN1103R$^-$, and GN118 have about the same value for their isoelectric points, 7.80, 7.87, and 7.76, respectively. However, the penicillinase of GN422 has an obviously lower value, 7.20. The results indicate that the penicillinase from strains GN69, GN1103R$^-$, and GN118 have similar properties with respect to the physicochemical properties, but the penicillinase from GN422 differs from the others.

When the results were compared with the physicochemical properties of R-factor-mediated penicillinases, it was found that the molecular weight of the penicillinase from GN422 was about the same as those of R-factor type Ia penicillinase (mol wt 20,600; reference 40) and peni-

TABLE 2. *Summary of the purification of penicillinase from K. pneumoniae GN69*

Stage no.	Procedure[a]	Activity recovered (U)	Spec act (U/mg of protein)	Purification	Recovery (%)
1	Grinding with alumina followed by streptomycin treatment and dialysis	855,836	53	1	100
2	Chromatography on CM-cellulose	568,336	1,568	30	66
3	Gel filtration on Sephadex G-50	401,000	2,480	47	47
4	Gel filtration on Sephadex G-50	254,000	7,120	134	30
5	Chromatography on CM-cellulose	103,500	29,200	550	12

[a] Abbreviation: CM, carboxymethyl.

TABLE 3. *Summary of the purification of penicillinase from K. pneumoniae GN1103R⁻*

Stage no.	Procedure[a]	Activity recovered (U)	Spec act (U/mg of protein)	Purification	Recovery (%)
1	Grinding with alumina followed by streptomycin treatment and dialysis	364,320	15	1	100
2	Chromatography on CM-cellulose	227,200	425	28	62
3	Gel filtration on Sephadex G-75	8,550	3,450	230	2

[a] Abbreviation: CM, carboxymethyl.

TABLE 4. *Summary of the purification of penicillinase from K. pneumoniae GN422*

Stage no.	Procedure[a]	Activity recovered (U)	Spec act (U/mg of protein)	Purification	Recovery (%)
1	Grinding with alumina followed by streptomycin treatment and dialysis	29,200	2.0	1	100
2	Chromatography on CM-cellulose	18,400	50	25	63
3	Gel filtration on Sephadex G-75	9,885	122	61	34
4	Chromatography on CM-cellulose	7,528	1,017	509	26
5	Gel filtration on Sephadex-G-75	3,136	992	496	11

[a] Abbreviation: CM, carboxymethyl.

TABLE 5. *Summary of the purification of penicillinase from K. pneumoniae GN118*

Stage no.	Procedure[a]	Activity recovered (U)	Spec act (U/mg of protein)	Purification	Recovery (%)
1	Ultrasonic disintegration followed streptomycin treatment and dialysis	77,000	1.1	1	100
2	Chromatography on CM-cellulose	33,600			44
3	Chromatography on CM-cellulose	20,500	67	60	27
4	Gel filtration on Sephadex G-75	8,500	1.100	1,000	11

[a] Abbreviation: CM, carboxymethyl.

cillinase of R_{TEM} (mol wt 21,000; reference 11), and somewhat lower than that of R-factor type Ib penicillinase (mol wt 22,600; reference 40). The molecular weights of the other three penicillinases were lower than those of R-factor-mediated penicillinases. The isoelectric points of the penicillinases from *Klebsiella* strains do not agree with those of both the R-factor type Ia

TABLE 6. *Molecular weights of penicillinases from K. pneumoniae GN69, GN1103R⁻, GN422, and GN118*

Source of enzyme	Mol wt[a]
GN69	17,400
GN1103R⁻	18,100
GN422	20,000
GN118	18,300

[a] The molecular weights of penicillinases from the four *Klebsiella* strains were estimated by gel filtration on a Sephadex G-75 column.

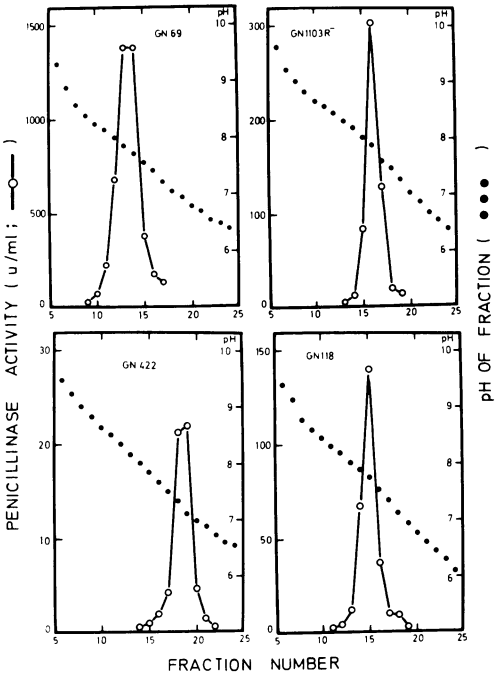

FIG. 1. *Isoelectric patterns of penicillinases from K. pneumoniae GN69, GN1103R⁻, GN422, and GN118. After electrofocusing, the column content was cut into 2-ml fractions, and each fraction was assayed for penicillinase activity* (○) *and pH* (●). *For details see Materials and Methods section.*

and Ib penicillinases, which were reported to be 5.1 and 6.9, respectively (40).

Immunology. We reported previously (39) that the crude penicillinase preparations from the three strains of *K. pneumoniae*, including GN69 and GN422, had their enzyme activities inhibited by the antiserum against type Ia penicillinase of R factor to the same extent as type Ia penicillinase, but the antiserum showed no inhibitory effect on type II penicillinase of R factor and on the species-specific penicillinases from *E. coli* and *Proteus mirabilis*.

We again tested the partly purified penicillinase preparations from the *Klebsiella* strains against the antiserum, together with highly purified type Ia and Ib penicillinases, by the double immunodiffusion technique (Ouchterlony technique).

The double diffusion analysis was performed in the presence of the antigen type Ia penicillinase to detect the immunological homology between the tested penicillinases and type Ia penicillinase. The results show immunological identity for type Ia and Ib penicillinases and the penicillinases from strains GN69, GN422, and GN118 (Fig. 2B, C, E, and F), however, the penicillinase from strain GN1103R⁻ gave a precipitation pattern which suggested a partial homology of the penicillinase to R-factor type Ia penicillinase (Fig. 2D). Furthermore, the antiserum demonstrated its inhibitory effect on the activity of the penicillinase from GN1103R⁻. The activity of the penicillinase of GN1103R⁻ was about 40% inhibited by the antiserum, and it is the same extent as the inhibitory effect of the antiserum on the penicillinase of GN69.

Enzymology. The pH activity plots for the penicillinases from strains GN69, GN1103R⁻, GN422, and GN118 with benzylpenicillin as the substrate are shown in Fig. 3. All the penicillinases have about the same optimal pH range between 6.3 to 7.3. The shapes of the plots and

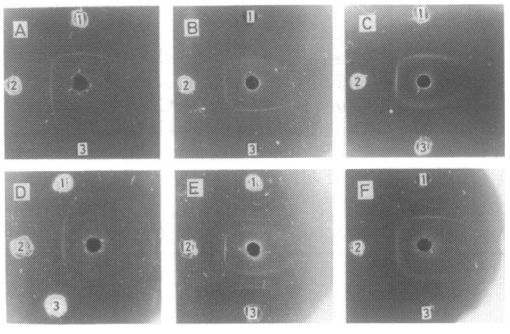

FIG. 2. *Immunodiffusion analysis of penicillinases from K. pneumoniae and E. coli R. The antiserum against type Ia penicillinase of R factor was placed in the central well. The outer well (2) contained the highly purified antigen, i.e., type Ia penicillinase. The other outer wells contained: plate A, type Ia penicillinase (1) and physiological saline (3); plate B, type Ib penicillinase of R factor (1) and its fourfold dilution (3); plate C, penicillinase from GN69 (3) and its threefold dilution (1); plate D, penicillinase from GN1103R⁻ (3) and its twofold dilution (1); plate E, penicillinase from GN422 (1) and its twofold dilution (3); plate F, penicillinase from GN118 (1) and its fourfold dilution (3).*

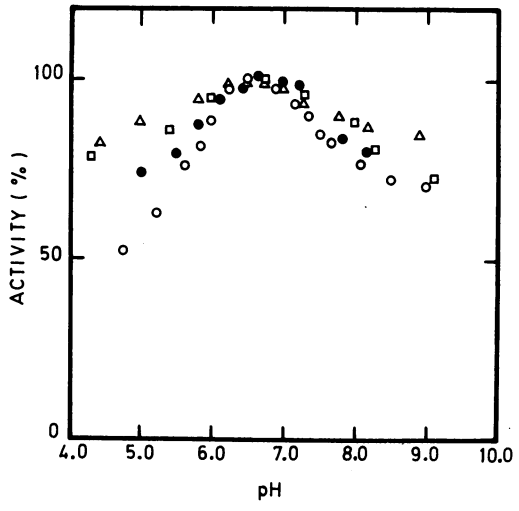

FIG. 3. *Effect of pH on the activities of penicillinases from K. pneumoniae GN69, GN1103R⁻, GN422, and GN118. Acetate buffers (0.1 M) were used for the range pH 4.3 to 5.8, phosphate buffers (0.1 M) for the range pH 5.9 to 7.3, and tris(hydroxymethyl)aminomethane-hydrochloride buffers (0.1 M) for the range pH 7.3 to 9.1. Symbols: O, penicillinase from GN69; Δ, penicillinase from GN1103R⁻; □, penicillinase from GN422; ●, penicillinase from GN118.*

the optimal pH range were very similar to those of type Ia and Ib penicillinases (40, 45).

The temperature activity curves of the four penicillinases are shown in Fig. 4. About 45 C was optimal for all the penicillinases, and this value is about the same as the optimal temperature for type Ia and Ib penicillinases (40, 45). A more rapid decrease in the activity of the penicillinase from GN1103R⁻ was observed at a higher temperature than 50 C, and this heat instability was also observed in the rate of heat inactivation at 60 C. After incubation for 20 min at 60 C in 0.1 M phosphate buffer, pH 6.5, the penicillinases from strains GN69 and GN422 lost 70% of their activities and the penicillinase from GN1103R⁻ lost 90% of its activity. These heat-labile property of the penicillinase from GN1103R⁻, together with the behavior against the antiserum, may suggest that the enzyme from GN1103R⁻ differs somewhat in its tertiary structure from the penicillinases of other strains.

The Michaelis constants for four penicillins and cephaloridine are given in Table 7 together with the relative activities (V_{max} relative) at substrate saturation for five substrates. The Michaelis constants were obtained from the Lineweaver-Burk plots (26). When the values obtained are compared, general similarities are observed among the penicillinases of those *Klebsiella* strains. However, they are not identical with one another in these enzymological properties, and there are some differences such as that in the behavior of the enzyme from GN422 to cephaloridine. The relative V_{max} value of the penicillinase from GN422 for cephaloridine is significantly lower than those of the other penicillinases. During the purification of the penicillinase of GN422, it was observed that the crude enzyme extract exhibited a value of 42 for a relative V_{max} for cephaloridine, but the partially purified enzyme preparation lost markedly its "cephalosporinase activity." The cephalosporinase activity was not restored by addition of Zn^{2+} known to be a co-factor of a cephalosporinase of *Bacillus cereus* (38). On the other hand, 1 mM disodium ethylenediaminetetraacetic acid also had no effect on the cephalosporinase activity of the crude enzyme preparation. Jack and Richmond (21) reported that the iodometric assay of cephalosporinase activity for the enzyme preparation from *E. coli* 071 was affected by the amount of contaminating protein and that the ratio of cephalosporinase activity to penicillinase activity increased after some purification. Although there are possibilities that strain GN422 possesses two beta-

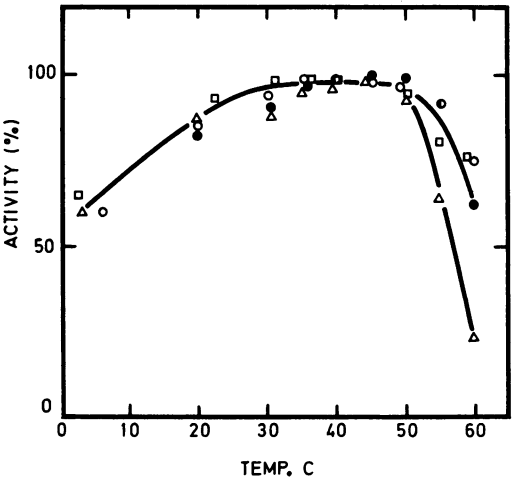

FIG. 4. *Effect of temperature on the activities of penicillinases from K. pneumoniae GN69, GN1103R⁻, GN422 and GN118. The enzyme solution in 0.1 M phosphate buffer, pH 6.5, containing 8 mM benzylpenicillin as substrate was incubated for 10 min at indicated temperature, and reaction rates were determined iodometrically. Symbols: O, penicillinase from GN69; Δ, penicillinase from GN1103R⁻; □, penicillinase from GN422; ●, penicillinase from GN118.*

TABLE 7. *Substrate profiles for penicillinases from K. pneumoniae strains and for type I penicillinases of R factor*

Substrate	K_m (μM)						Relative rate of hydrolysis[a]					
	GN69	GN1103R⁻	GN422	GN118	Ia[b]	Ib[c]	GN69	GN1103R⁻	GN422	GN118	Ia[b]	Ib[c]
Benzylpenicillin	11	15	28	22	27	24	100	100	100	100	100	100
Ampicillin	27	40	31	56	30	32	178	193	168	176	115	112
Phenethicillin	21	12	41	9	32	14	58	57	73	45	33	27
6-Aminopenicillanic acid	111	192	294	152	222	200	71	87	75	93	87	89
Cephaloridine	133	256	108	193	400	500	49	61	8	54	130	111

[a] Rates of hydrolysis of the six substrates are expressed as the percentage of hydrolysis of benzylpenicillin.
[b] Ia: Type Ia penicillinase. Data taken from previous paper (45).
[c] Ib: Type Ib penicillinase. Data taken from previous paper (40).

lactamases (penicillinase and cephalosporinase) or contaminating protein affects on the cephalosporinase activity, the reason for this observation is still obscure.

When the penicillinases from *Klebsiella* strains were compared with R-factor type Ia and Ib penicillinases in these enzymological properties (Table 7), some differences were also seen between the penicillinases of the two groups. However, taking the significant differences observed among species-specific penicillinases from *E. coli*, *Klebsiella*, and *P. mirabilis* or between type I and type II penicillinases of R factors into consideration (11, 12, 14, 17, 24, 39, 42, 43), the variation and the difference observed among the penicillinases of *Klebsiella* and type I penicillinases in these enzymological properties are seen not to be great.

A number of inhibitors and ions whose effect on the activities of type Ia, Ib and II penicillinases of R factors had been previously tested (40, 45), were examined. The methods for measuring inhibitory effect of the compounds were previously described (45). Similar to type Ia and Ib penicillinases, the penicillinases of *Klebsiella* were significantly inhibited their activities by iodine (Table 8). The penicillinase from GN1103R⁻ was more sensitive to iodine. Cupric and ferrous ions exhibited weak inhibitory effect on the activities of the enzymes at a concentration of 1 mM. *p*-Chloromercuribenzoate, urea, disodium ethylenediaminetetraacetic acid, $ZnSO_4$, and $CaCl_2$ had no effect on the activities of all the penicillinases at a concentration of 1 mM, and also 20 mM NaCl exhibited no effect. The behaviors of the penicillinases from *Klebsiella* strains toward the tested compounds were similar to type Ia and Ib penicillinases of R factors (40).

TABLE 8. *Effect of iodine on the activities of penicillinases of K. pneumoniae strains and type I penicillinases of R factor*

Concn (mM)	Inhibition (%)					
	Penicillinase of *K. pneumoniae*				Penicillinase of R factor	
	GN69	GN1103R⁻	GN422	GN118	Ia[a]	Ib[b]
0.5	68	79	63	64	53	99
0.05	35	71	50	57		
0.01	8	68		11		

[a] Ia: Type Ia penicillinase. Data taken from previous paper (45).
[b] Ib: Type Ib penicillinase. Data taken from previous paper (40).

DISCUSSION

Matsumoto and Tazaki (29, 30) analyzed the genetic linkage map of *K. pneumoniae* by means of a conjugation system. The proposed circular genetic map of *K. pneumoniae* is similar to that of *E. coli* or *Salmonella typhimurium*. Recently, we have confirmed that the penicillinase determinant of the donor strain is located on the chromosome and the penicillinase from the strain is the same as the penicillinase of GN69 in enzymological and immunological properties (28). The four *Klebsiella* strains used here were not capable of transferring their penicillinase genes to *E. coli* strains, although in our experience known R factors are transferred from those *Klebsiella* strains to *E. coli* strains or vice versa. The *Klebsiella* strains have not lost the penicillinase genes after long-time storage in cooked meat medium in which the extrachromosomal R factor, whether being transferable or not, was usually lost from host organisms at a considerable frequency. We consider that the penicillinases examined here are "species-specific chromosomal penicillinases" of *Klebsiella*.

The penicillinase from strain GN422 is different from the others with respect to its molecular weight, isoelectric point, and cephalosporinase activity. The enzyme is very similar in its profile of substrate specificity to the penicillin-

ase synthesized by *K. aerogenes* 418 originally reported by Hamilton-Miller (17) and recently by Jack and Richmond (21). The penicillinase from strain GN1103R$^-$ differs from the others in immunological property, and also exhibited more sensitivity than the others in heat treatment and iodine treatment. It may be concluded that the penicillinases from strains GN69, GN422, and GN1103R$^-$ were, strictly speaking, not identical with one another although they are very similar to one another in many properties. Hamilton-Miller (18) classified penicillinases from 11 strains of *K. aerogenes* into three groups on the basis of their substrate specificity and affinity for competitive inhibitors, and suggested variation in *Klebsiella* penicillinases. In the present paper, it was revealed that variations also existed in their physicochemical and immunological properties.

The purpose of this investigation was to explore the properties of species-specific penicillinase of the *Klebsiella* and to compare them with those of penicillinases whose synthesis are mediated by R factors. We expected that the results from such an investigation would give a clue to assume the origin of the penicillinase gene (ampicillin-resistant determinant) on R factor if the enzyme reflects the characters of its corresponding gene. As a result, it was revealed that the penicillinases from *Klebsiella* strains and R-factor type Ia and Ib penicillinases were similar with respect to their pH optimum, temperature optimum, heat stability, behavior to inhibitors, and cross-reactivity with the antiserum against type Ia penicillinase. Some differences in approximate molecular weight, substrate specificity, and the Michaels constants (K_m) for penicillins and cephaloridine were found between the penicillinases of *Klebsiella* and type I penicillinases. However, the differences in those properties appear not to be greater than the variations observing among the penicillinases of *Klebsiella* or between R-factor type Ia and Ib penicillinases.

As reported previously (40, 45), type Ia and Ib penicillinases are markedly different from type II penicillinase in pH optimum, temperature optimum, heat stability, behavior to inhibitors such as chloride ion, substrate specificity, and K_m of penicillins and cephaloridine, and also the antiserum against type Ia penicillinase does not cross-react with type II penicillinase. Those differences were also seen between type I penicillinase and the penicillinases from *E. coli* (39, 42) or *P. mirabilis* (39). The differences between the penicillinases of R factors and beta-lactamases from other enterobacteria such as *Escherichia freundii*, *Aerobacter* (*Enterobacter*), *Serratia*, *Arizona*, and *Proteus* (except *P. mirabilis*) are greater, because those β-lactamases are generally inducible cephalosporinases (7, 8, 15, 19, 20, 39). Nonsimilarity in enzymological and immunological properties and in approximate molecular weight between the penicillinase mediated by the chromosomal gene of *E. coli* and the penicillinase of the R factor R1 were demonstrated by Lindqvist and Nordström (24) and Lindström et al. (25). The penicillinase of R1 and R_{TEM} appear to be classified into our type I penicillinase from their profiles of substrate specificity and other enzymological properties. Recently, Dale and Smith (11) reported the apparent identity of R1- and R_{TEM}-mediated penicillinases. It is thought that the differences in some properties between the *Klebsiella* penicillinases and type I R-factor penicillinases are smaller than those between penicillinases from different species or between type I and type II R-factor penicillinases. It is an attractive assumption that the penicillinase genes which mediate the synthesis of type I R-factor penicillinases originated from the bacterial chromosomes in some strains belonging to the *Klebsiella* groups, and the multiformity in type I penicillinases may be attributed to the variation in some properties of the *Klebsiella* penicillinase.

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