ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1996, p. 2152–2159 Vol. 40, No. 9
0066-4804/96/$04.00+0
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Recovery of Active β-Lactamases from *Proteus vulgaris* and RTEM-1 Hybrid by Random Mutagenesis by Using a *dnaQ* Strain of *Escherichia coli*

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***Proteus vulgaris* and RTEM-1 β-lactamases that belong to molecular class A with 37% amino acid similarity were examined to find the relationship between amino acid residues and activity of enzymes. MICs of ampicillin were >2,000 μg/ml for *Escherichia coli* cells producing these enzymes. We have made 18 hybrid genes by substituting the coding region of the *P. vulgaris* β-lactamase gene with the equivalent portions from the RTEM-1 gene. Most of these hybrids produced inactive proteins, but a few hybrid enzymes had partial or trace activity. From one of the hybrid genes (MIC of ampicillin, 100 μg/ml), we recovered three kinds of active mutants which provided ampicillin MICs of 1,000 μg/ml by the selection of spontaneous mutations in a *dnaQ* strain of *E. coli*. In these mutants, Leu-148, Met-182, and Tyr-274 were replaced with Val, Thr, and His, respectively. These amino acids have not been identified as residues with functional roles in substrate hydrolysis. Furthermore, from these hybrid mutants, we obtained a second set of mutants which conferred ampicillin MICs of 1,500 μg/ml. Interestingly, the second mutations were limited to these three amino acid substitutions. These amino acid residues which do not directly interact with substrates have an effect on enzyme activity. These mutant enzymes exhibited lower K_m values for cephaloridine than both parental enzymes.**

The creation of mutant proteins with specific changes in amino acid sequence by site-directed mutagenesis (1) affords a general method for studies of the relationship between their structures and functions. This approach has been applied to many enzymes, including β-lactamases (2, 8, 10, 15, 23, 35, 40, 41).

β-Lactamases (EC 3.5.2.6) constitute a family of enzymes that confer resistance toward β-lactam antibiotics by hydrolyzing them into inactive compounds. Understanding of the catalytic mechanism of β-lactamases would be an important step in the design of improved β-lactam antibiotics or inhibitors of β-lactamases. On the basis of primary structures, β-lactamases were divided into four classes: A, B, C, and D (3, 19). The three-dimensional structures of four class A β-lactamases, RTEM-1 (18, 36), *Staphylococcus aureus* PC1 (14), *Streptomyces albus* G (11), and *Bacillus licheniformis* 749/C (21, 29) have been solved by X-ray crystallography. Because these class A enzymes have similar structures, we tested whether hybrid constructs from two members of class A β-lactamases remain active.

β-Lactamases from *Proteus vulgaris* 5E78-1 of 271 amino acid residues, and RTEM-1 of 263 amino acid residues, that belong to class A were examined to find the role of amino acid residues in the structure and activity of enzymes. The RTEM-1 β-lactamase encoded by the *bla* gene contained in the *Escherichia coli* plasmid, pBR322 (37), has 37% amino acid sequence similarity with the *P. vulgaris* β-lactamase which showed markedly different substrate spectra. The RTEM-1 β-lactamase prefers penicillins to cephalosporins as substrates, whereas the *P. vulgaris* β-lactamase exhibits broad substrate spectra and is able to hydrolyze cefuroxime efficiently which many other enzymes can not hydrolyze (27, 28, 32). We constructed 18 hybrids between these two genes, recovered several active β-lactamases from one of them by random mutagenesis with an *E. coli dnaQ* strain, and characterized them.

MATERIALS AND METHODS

***E. coli* strains, plasmids, and phages.** *E. coli* XLI-Blue {*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lac*) λ⁻ [F′:*proA*⁺ *B*⁺ *lacI*ᵠ *lacZ*ΔM15 Tn*10* (Tetʳ)]} and CJ236 [*dut-1 ung-1 thi-1 relA1*/pCJ105 (Cmʳ)] (20) were used for preparation of M13mp18, M13mp19 vectors and derivatives, and uracil-containing DNA templates for site-directed mutagenesis, respectively. *E. coli* MK148 (HfrC *metB1 lac-3 malA38 tsx-76 relA1 dnaQ49*) was a gift from H. Maki and M. Sekiguchi (Kyushu University) (38). A mutator strain, MK148, contains a chromosomal *ampC* gene which confers an ampicillin MIC of 10 μg/ml. The *ampC* gene on the chromosome was inactivated by inserting the gene conferring kanamycin resistance. This strain was named MKS148. AS2261 derived from C600r⁻m⁻ (F⁻ *thr-1 leuB6 thi-1 hsdS1 lacY1 tonA21 supE44* λ⁻) (31) contains a deletion from the *Xho*I site to the *Hin*dIII site in its chromosomal *ampC* gene (17) which resulted in inactivation of the gene. Bacterial cells were grown at 30 or 37°C in Luria broth. Luria agar plates were supplemented, when needed, with the relevant antibiotics, ampicillin (Meiji), tetracycline (Wako), kanamycin (Meiji), chloramphenicol (Wako), cephaloridine (Shionogi), and cefuroxime (Glaxo), and were used for growth of bacteria harboring plasmids. Nitrocefin was purchased from Glaxo Pharmaceuticals, Ltd.

Plasmid pSU2719 (25) was obtained from F. de la Cruze, and plasmids pHSG397 and pHSG299 (39) were purchased from Takara Shuzo.

Plasmid DNA was prepared by the alkaline lysis method, and cloning techniques were based on those of Maniatis et al. (24). The preparation of *E. coli*-competent cells and subsequent transformation with plasmid DNA were performed by the method of Morisson (30).

Restriction endonucleases, T4 DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were obtained from Takara Shuzo.

A plasmid pTEM1 was constructed by cloning the 919-bp *Ssp*I-*Dra*I fragment from pBR322 (37) into the vector pHSG397 at the *Hin*cII site.

pKT10 plasmid carrying the kanamycin and tetracycline resistance genes as selective markers was constructed from pKA13 (5) and pBR322 (37) plasmids. The *Eco*RI-*Acc*I fragment containing the tetracycline resistance gene from pBR322 was inserted into the *Eco*RI-*Acc*I sites of the pKA13 vector, and the constructed plasmid was named pKT10. The 6,300-bp *Xho*I fragment from the *P. vulgaris* 5E78-1 DNA carrying the β-lactamase gene was cloned at the *Xho*I site

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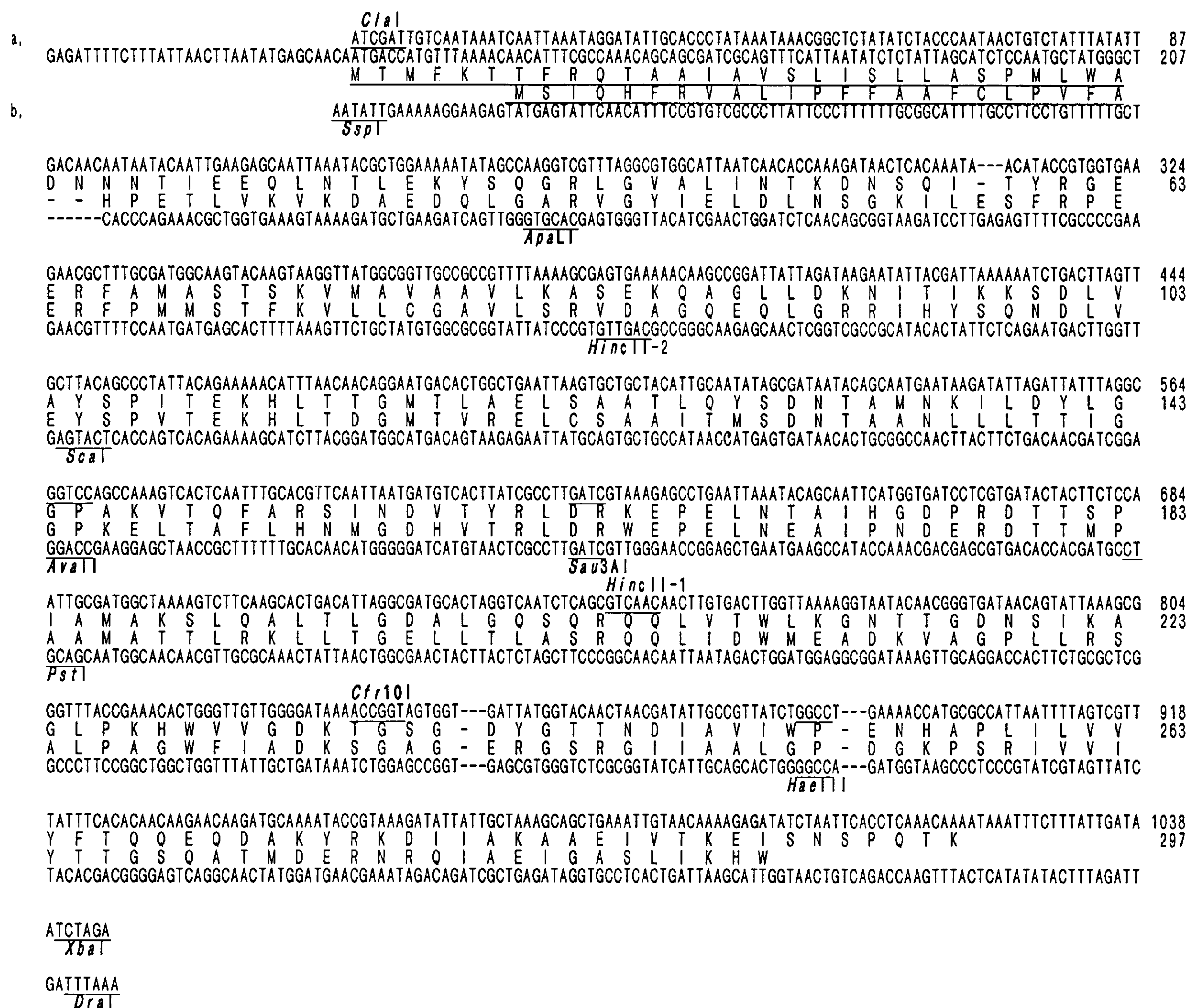

FIG. 1. Nucleotide sequences and comparison of the deduced amino acids of (A) *P. vulgaris* and (B) RTEM-1 β-lactamases (37). The signal peptides are underlined. Horizontal lines within the sequences represent apparent deletions. Amino acid residue numbers are according to the numbering of Ambler (3, 4). The nucleotides of the *P. vulgaris* β-lactamase gene are numbered from the *Cla*I site. The sequences that correspond to the restriction sites are underlined.

in the kanamycin resistance gene of pKT10 by selecting ampicillin-resistant colonies of *E. coli* AS2261. Analysis of the complete nucleotide sequence of the coding regions revealed two possible open reading frames starting from nucleotide position 121 and 127, respectively. The mature protein of 271 amino acids started at position 208 and terminated with stop codon TAA at position 1021 to 1023 with a predicted molecular mass of 29,522 Da (Fig. 1). Plasmid pVUL1 was constructed by subcloning the 1,039-bp *Cla*I-*Xba*I fragment encompassing the β-lactamase gene into the *Cla*I-*Xba*I sites of pHSG397. The nucleotide sequence of the *P. vulgaris* 5E78-1 β-lactamase gene and the deduced amino acid sequence of the enzyme differ at several positions compared with those of *P. vulgaris* K1 (40), B317 (9), and RO104 (33). This is due to divergence among the strains. The sequence of the β-lactamase gene (*blaB*) from *P. vulgaris* 5E78-1 has been submitted to the GSDB/DDBJ/EMBL/NCBI nucleotide sequence database with accession number D37831.

Oligonucleotides and site-directed mutagenesis. Site-directed mutagenesis was performed by the Kunkel method (22) with M13mp18 or M13mp19 vectors. Oligonucleotides were synthesized by Hokudo Company. For various substitutions of the coding region of the RTEM-1 β-lactamase with the equivalent portions from the *P. vulgaris* β-lactamase, several common restriction sites were created in the RTEM-1 or the *P. vulgaris* β-lactamase gene by site-directed mutagenesis. Site-directed mutagenesis employing the primer oligonucleotides (Table 1) was performed separately to introduce an *Apa*LI, a *Hinc*II, a *Sca*I or a *Pst*I site in the *P. vulgaris* β-lactamase gene at the same positions as in the RTEM-1 gene. An *Ava*II and a *Hinc*II sites were separately introduced in the RTEM-1 β-lactamase gene by oligonucleotides 5 and 6, respectively (Table 1). The wild-type RTEM-1 β-lactamase gene contains an *Ava*II site at the same position but with a sequence different from that in the *P. vulgaris* β-lactamase gene. Digestion of the mutant sequences with the restriction enzymes confirmed the creation of the new restriction sites.

In some hybrid genes (pVTV-LH1, pVTV-HS1, and pVTV-SA1), the *Hinc*II-1 site was removed by site-directed mutagenesis with the sequences 5′-ATC-TCA-GCG-*CCA-ACA-ACT-T-3′ as a primer. The asterisk indicates a silent mutation that removes a *Hinc*II-1 site (Fig. 1).

Construction of hybrid genes. After creation of common restriction sites, we constructed 11 hybrid plasmids, pVT-H2, pTV-H2, pVT-S1, pTV-S1, pTV-U1, pVT-P1, pTV-P1, pVT-H1, pTV-H1, pVT-E1, and pTV-E1, by substituting the coding region of various pTEM1 with the equivalent portions from the *P. vulgaris* β-lactamase gene (Fig. 2A). Other seven hybrid plasmids, pTV-L1, pVTV-LH1, pVTV-HS1, pVTV-SA1, pVTV-AP1, pVTV-PH1, and pVTV-HC1, were derived from the above hybrids (Fig. 2B). All hybrids were constructed into the plasmid pHSG397. Because the vector pHSG397 contains an *Apa*LI and a *Sca*I site, hybrid genes (pVT-S1, pTV-S1, pTV-L1, pVTV-LH1, pVTV-HS1, and pVTV-SA1) were constructed using the vector pSU2719 or pHSG299, for lack of *Apa*LI site or *Sca*I site, respectively, and were then transferred to pHSG397.

We constructed the hybrid pVTV-HC1 by PCR with the pVT-H1 hybrid. The two primers which we used for PCR method were (a) 5′-ACC-GGC-TCC-GG

TABLE 1. Chemically synthesized mutant primers

Oligonucleotide no. and sequence (5′→3′)[a]	Gene	Introduced new restriction site	Replaced residues[b]	Ampicillin MIC (μmol/ml)[c]
1. AAA-ATA-TAG-C(GG-T)(GC-A)CG-TTT-AGG-CGT	*P. vulgaris*	*Apa*LI	Q41G, G42A	400
2. GTT-TTA-AAA-GCG-(GTT)-(GAC)-AAA-CAA-GCC-G	*P. vulgaris*	*Hinc*II-2	S84V, E85D	>2,000
3. GAC-TTA-GTT-(GAG)-TAC-(TGC)-CCT-ATT-AC	*P. vulgaris*	*Sca*I	A104E, S106C	200
4. ACT-TTT-AGC-CAT-CGC-T(GC-A)GG-AGA-AGT-AGT	*P. vulgaris*	*Pst*I	I184A	>2,000
5. CGA-TCG-GAG-GTC-CGA-AGG-AGC-3′	RTEM-1	*Ava*II	No change	>2,000
6. TAG-CTT-CCC-GTC-AAC-AAT-TAA-3′	RTEM-1	*Hinc*II-1	No change	>2,000
7. ACC-GGC-TCC-GGA-TTT-ATC-AG	RTEM-1	*Acc*III	No change	>2,000

[a] The sequences that correspond to the restriction sites and amino acid replacements are underlined and in parentheses, respectively.
[b] Amino acid residue numbers are according to the numbering of Ambler (3, 4).
[c] MICs of ampicillin for the *E. coli* AS2261 cells harboring *P. vulgaris* and RTEM-1 β-lactamase genes were >2,000 μg/ml.

A-TTT-ATC-AG-3′ to introduce a blunt-end-digested *Acc*III site (underlined) in the RTEM-1 fragment (the *P. vulgaris* β-lactamase gene contains a blunt-end-digested *Cfr*10I site at the same position) and (b) 5′-CGA-TAA-TAC-AGC-A AT-GAA-TA-3′ in the *P. vulgaris* fragment. The PCR fragment was digested by *Ava*II and *Acc*III, and then substituted with the equivalent portions from the *P. vulgaris* β-lactamase gene at *Ava*II-*Cfr*10I sites.

Isolation of active mutants from hybrids. Constructed plasmids containing hybrid β-lactamase genes represented in Fig. 2A and B were transformed into a *dnaQ* mutant of *E. coli*, MKS148. *E. coli* strains carrying the temperature-dependent *dnaQ49* allele are strong mutators at 37°C (16). Colonies transformed with desired plasmids were picked and inoculated into test tubes containing 5 ml of L broth, and the cultures were incubated at 37°C overnight. The 0.2-ml cultures from each tube were then spread on chloramphenicol plates containing various concentrations of ampicillin (5 to 1,500 μg/ml). The plates were incubated at 30°C. Plates containing the highest ampicillin concentration permissible for growth of *E. coli* were selected; all colonies on the surface were collected, and their plasmids were isolated. The isolated plasmids were transformed into *E. coli* AS2261, and *E. coli* cells were inoculated onto plates containing chloramphenicol and ampicillin and were incubated at 37°C. Some of the colonies grown were selected for sequencing and for inspection of mutations.

DNA sequencing. The nucleotide sequences of the plasmid DNA containing hybrid genes were determined by the Sanger dideoxynucleotide chain termination method (34) with the Sequenase II sequencing kit (United States Biochemical Corp., Cleveland, Ohio).

Measurement of bacterial susceptibility to antibiotics. MICs for *E. coli* AS2261 harboring plasmids were determined at 30°C with inocula of 10^5 CFU on agar plates containing various concentrations of β-lactam antibiotics.

β-lactamase purification. *E. coli* cells harboring the wild-type or mutant β-lactamase genes were collected during the late-exponential phase by centrifugation at 5,000 × *g* for 5 min, and β-lactamases were extracted by an osmotic shock procedure (13). The solution obtained by osmotic shock was dialyzed overnight against 2 mM sodium phosphate buffer, pH 7.0. The dialyzed proteins were

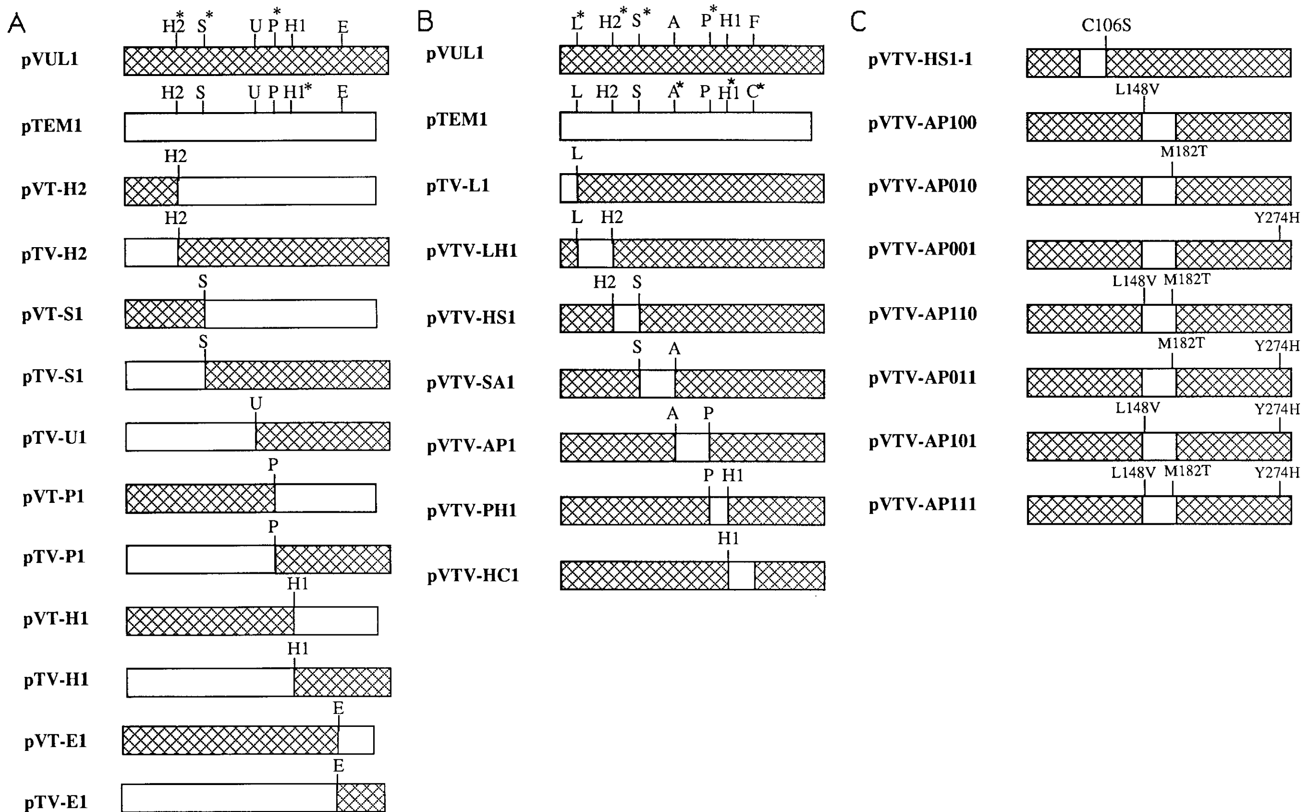

FIG. 2. Structure of hybrid genes. (A) Initially, new restriction sites were separately introduced in *P. vulgaris* and RTEM-1 β-lactamase genes (indicated by asterisk), and then the hybrid genes were constructed. Restriction endonuclease abbreviations: H1 and H2, *Hinc*II; S, *Sca*I; U, *Sau*3AI; P, *Pst*I; E, *Hae*III. (B) Following construction of hybrids represented in panel A and also introduction of the other new restriction sites (L*, A*, C*), seven hybrids containing shorter fragments from RTEM-1 β-lactamase gene were constructed. Restriction endonuclease abbreviations: L, *Apa*LI; A, *Ava*II; F, *Cfr*10I; C, *Acc*III; see also abbreviations for panel A. (C) Position of replaced residues on pVTV-HS1 and pVTV-AP1. Amino acid residue numbers are according to the numbering of Ambler (3, 4).

TABLE 2. Purification of the wild-type and hybrid β-lactamases[a]

Purification step	RTEM-1			*P. vulgaris*			pVTV-AP100[b]		
	Total activity (U)	Sp act (U/mg)	Yield (%)	Total activity (U)	Sp act (U/mg)	Yield (%)	Total activity (U)	Sp act (U/mg)	Yield (%)
Crude enzyme	5.76	143		1.77	95.5		13.3	3.77	
DE-52	6.75	358	117				6.29	10.9	47
P-11	1.64	352	28	1.02	268	58	2.65	17.0	20
Hydroxylapatite				0.36	245	20	0.9	21.9	7
DE-52	1.3	329	23						

[a] The activity of all enzymes was measured with cephaloridine as the substrate.
[b] The purification procedure for pVTV-AP010 and pVTV-AP111 was similar to that for pVTV-AP100.

applied onto an anion- or cation-exchange column (Whatman DE-52 or P11) and then a hydroxylapatite (Bio-Rad) column (except for RTEM-1). After elution, the β-lactamase fractions were concentrated by ammonium sulfate precipitation. The final preparation was stored at −20°C in 50 mM sodium phosphate buffer, pH 7.0, containing 50% (vol/vol) glycerol. The purity of the enzymes was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Table 2 summarizes the purification procedure.

The protein concentration was estimated by A_{280} measurements with an extinction coefficient of 26,400 M^{-1} cm^{-1} for the RTEM-1 enzyme and 30,800 M^{-1} cm^{-1} for the other pure proteins.

The N-terminal sequence of the purified *P. vulgaris* β-lactamase produced in *E. coli* was determined by Edman degradation with an Applied Biosystems gas phase sequencer at the Center for Instrumental Analysis, Hokkaido University.

Assay of β-lactamase activity. Crude β-lactamase extracts were prepared by ultrasonication (12). All cultures were adjusted to an optical density at 600 nm of 0.7, and protein concentrations were determined by the method of Bradford (6) with bovine serum albumin as the standard. Activities toward various substrates were determined spectrophotometrically on crude β-lactamase extracts with a Shimadzu (UV-160) spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of substrate for 1 min at a maximal velocity at pH 7.0 and 30°C.

β-Lactam hydrolysis of the purified enzymes was monitored at 30°C in 50 mM sodium phosphate buffer, pH 7.0. The values for the kinetic parameters were obtained by initial velocity measurements. Curve fitting, assuming the Michaelis-Menten equation, was performed with a nonlinear regression (Kaleidagraph). Usually a 10-min time course of the hydrolysis of the substrates was recorded at intervals of 30 s. The change in the extinction coefficient (Δε) for each substrate, monitored at the stated wavelength, was as follows: ampicillin (235 nm), 600 M^{-1} cm^{-1}; cephaloridine (260 nm), 14,775 M^{-1} cm^{-1}; cefuroxime (260 nm), 7,525 M^{-1} cm^{-1}; and nitrocefin (486 nm), 16,000 M^{-1} cm^{-1}.

RESULTS

Site-directed mutagenesis. In order to substitute the various coding regions of the RTEM-1 gene with the equivalent portion from the *P. vulgaris* β-lactamase gene, we first introduced an *Apa*LI, a *Hin*cII, a *Sca*I, and a *Pst*I restriction site in the *P. vulgaris*, and an *Ava*II, a *Hin*cII, and an *Acc*III site in the RTEM-1 β-lactamase genes (Fig. 1). The MIC for *E. coli* cells harboring the *P. vulgaris* β-lactamase gene was >2,000 μg/ml for ampicillin. The creation of an *Apa*LI site in the *P. vulgaris* β-lactamase gene resulted in the substitutions of two amino acids, Q41G and G42A, and gave a low level of ampicillin resistance to the cells harboring them. Although residues 41 and 42 of the *P. vulgaris* β-lactamase are different from the consensus sequences (4), they did affect the activity. Alteration of S84V and E85D by introduction of a *Hin*cII-2 site in the *P. vulgaris* β-lactamase gene had no effect on ampicillin resistance of the mutant cells. Substitution of Ala-104 and Ser-106 by Glu and Cys, respectively, by introduction of a *Sca*I site in the *P. vulgaris* β-lactamase gene resulted in a low ampicillin MIC (200 μg/ml). Therefore these residues, especially Ser-106, which is a conserved amino acid residue among class A β-lactamases (4), should play an important role on activity of the mutant cells. Amino acid Ile-184 was changed into Ala by creation of a *Pst*I site in the *P. vulgaris* β-lactamase gene, and this substitution had no effect on resistance of the cells harboring it. Note that except for the S106C, the substituted amino acid residues in the *P. vulgaris* β-lactamase by site-directed mutagenesis were identical with the amino acid residues of the RTEM-1 β-lactamase. The creation of restriction sites *Ava*II, *Hin*cII, and *Acc*III in the RTEM-1 gene introduced no amino acid alteration. The effect of mutations on activity is also summarized in Table 1.

Hybrid proteins. *E. coli* cells containing all the hybrid β-lactamase genes represented in Fig. 2A were completely susceptible to the antibiotics ampicillin, cefuroxime, and cephaloridine. Details of hybrids represented in Fig. 2B are shown in Table 3. Among these hybrids, *E. coli* cells harboring pTV-L1, pVTV-LH1, pVTV-HS1 pVTV-PH1, or pVTV-HC1 plasmid(s) were susceptible to the antibiotics. *E. coli* cells harboring pVTV-SA1 or pVTV-AP1 showed resistance toward the antibiotics (Table 4). From all of the hybrids represented in Fig. 2A and B, except pVTV-HS1 and pVTV-AP1, we did not obtain any active hybrid gene by spontaneous mutation in a *dnaQ* mutant of *E. coli*. Although we isolated and sequenced five independent mutants (MIC of ampicillin, 200 μg/ml) from pVTV-SA1 with an ampicillin MIC of 600 μg/ml, no change in amino acid residues was found. Thus, because the product of pVTV-SA1 has a partial activity against ampicillin (MIC, 800 μg/ml), antibiotic susceptibility is decreased probably by another mutation within the plasmid resulting in high protein expression.

Isolation of mutants from pVTV-HS1. Cells carrying the hybrid pVTV-HS1 were completely susceptible to ampicillin. By spontaneous mutation, four independent hybrid mutants were obtained (MIC, 200 μg/ml) and complete structural genes were sequenced. In three of four hybrid mutants, Cys-106 was

TABLE 3. Replaced regions in the hybrid proteins between *P. vulgaris* and RTEM-1 β-lactamases

Plasmid	Residues replaced[a]	No. of residues in replaced region	Identical residues in replaced region		Residue(s) interacting directly with substrates[b]
			No.	%	
pTV-L1	25–42	17[c]	1	6	
pVTV-LH1	41–85	45[d]	15	33	S70, K73
pVTV-HS1-1	84–104	21	5	24	
pVTV-SA1	104–142	39	22	56	S130, N132
pVTV-AP1	146–184	39	20	51	E166
pVTV-PH1	184–203	20	7	35	
pVTV-HC1	208–235	28	6	21	K234

[a] Amino acid residue numbers are according to the numbering of Ambler (3, 4).
[b] These residues are estimated to interact with substrates by X-ray crystallography (36).
[c] 18 residues from *P. vulgaris* were replaced with 17 residues from RTEM-1.
[d] 44 residues from *P. vulgaris* were replaced with 45 residues from RTEM-1.

TABLE 4. Hydrolytic activity of the hybrid and mutant β-lactamases at 30°C

Plasmid	MIC (μg/ml)			Activity (10^2 U/mg)[a]			
	Amp	Cxm	Cer	Amp	Nit	Cxm	Cer
pHSG-397	<2	<2	<2	ND	ND	ND	ND
pVUL1	>2,000	>1,500	>800	440	2,000	1,200	1,400
pTEM1	>2,000	5	200	19,000	3,100	3.0	5,800
pTV-L1	5	5	5	ND	ND	ND	ND
pVTV-LH1	5	5	5	ND	ND	ND	ND
pVTV-HS1	5	5	5	ND	ND	ND	ND
pVTV-HS1-1	200	100	50	160	530	220	530
pVTV-SA1	800	100	50	130	770	60	1,700
pVTV-AP1	100	50	10	7.0	60	2.0	11
pVTV-PH1	5	10	5	ND	ND	ND	ND
pVTV-HC1	10	10	10	ND	ND	ND	ND
pVTV-AP100	1,000	100	50	38	150	18	30
pVTV-AP010	1,000	400	50	100	500	160	100
pVTV-AP001	1,000	400	50	47	410	130	41
pVTV-AP110	1,500	800	400	340	1,200	260	400
pVTV-AP011	1,500	800	400	280	1,800	280	180
pVTV-AP101	1,500	800	400	240	1,800	290	170
pVTV-AP111	>2,000	1,000	400	340	2,200	280	300
pVUL001	>2,000	>1,500	800	760	5,000	2,700	1,500

[a] One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of antibiotics for 1 min at a maximal velocity at 30°C. ND, not determined because of low activity.

replaced with Ser by altering TGC to AGC, and in the other one by altering TGC to TCC, since both AGC and TCC encode serine. Ser-106 in the wild-type *P. vulgaris* β-lactamase is a consensus amino acid, and substitution with Cys during site-directed mutagenesis resulted in a notable increase of ampicillin susceptibility of the mutant cells (Table 1). In fact, we obtained a genuine hybrid named pVTV-HS1-1 for which the cells harboring it exhibited resistance to antibiotics (MIC of ampicillin, 200 μg/ml) (Table 4). Note that whereas there were equal chances of altering other amino acids, selection ability was only at amino acid residue 106. These results suggest that Ser-106 is an important amino acid residue even in the hybrid protein and also that the *dnaQ* mutant of *E. coli* is a very effective strain to isolate mutants.

Isolation of mutants from pVTV-AP1. The 117-bp replacement of the *P. vulgaris* β-lactamase gene in pVTV-AP1 resulted in a remarkable decrease of ampicillin MICs (100 μg/ml). Ten independent hybrid mutants conferring resistance to ampicillin (MIC, 1,000 μg/ml) were obtained from this plasmid. On the basis of the amino acid alteration, these 10 mutants were divided into three groups. The first group contained only one member, pVTV-AP100. In this mutant Leu-148 was replaced with Val (CTA→GTA), which is the amino acid residue of the *P. vulgaris* β-lactamase. In the second group, with three members, pVTV-AP010, Met-182 was replaced with Thr (ATG→ACG). Both Val-148 and Thr-182 are the consensus amino acid residues of class A β-lactamases (4). In the third group, with six members, pVTV-AP001, Tyr-274 was replaced with His (TAC→CAC) (Fig. 2C). All these selected mutation sites were confirmed by DNA sequencing of the complete structural genes.

Residue 274 is not conserved at all in class A enzymes, but the effect of this substitution in the hybrid gene is conspicuous. To understand the role of this residue, we replaced it with His in the wild-type *P. vulgaris* β-lactamase by recombinant DNA technique (pVUL001), and its susceptibility to antibiotics was tested. As shown in Table 4, the wild-type and the mutant pVUL001 transformants showed the same MICs of ampicillin, cephaloridine, and cefuroxime.

We tried to obtain second mutations from the hybrid mutants which are resistant to higher levels of ampicillin. Plasmids containing hybrid mutant genes, pVTV-AP100 and pVTV-AP010, were transformed into the *dnaQ* mutant of *E. coli* again and tested to isolate highly resistant colonies (see materials and methods). The MIC of ampicillin against *E. coli* AS2261 cells harboring isolated plasmids was 1,500 μg/ml, and the hybrid β-lactamase genes were then sequenced. From pVTV-AP100 we obtained three independent mutants. In its first mutation Leu-148 was replaced with Val, and in the three second mutations, Met-182 was replaced with Thr (pVTV-AP110), which is the same as the mutation site of pVTV-AP010. In the three independent mutants of pVTV-AP010, the second mutations were the replacements of Tyr-274 with His (pVTV-AP011) which are identical with the mutation site of pVTV-AP001 (Fig. 2C). These results suggest that the recovery of active hybrid enzyme is restricted to these three mutation sites.

We introduced codons for mutant L148V into the β-lactamase gene of pVTV-AP001, resulting in plasmid pVTV-AP101 conferring the same level of resistance as pVTV-AP110 and pVTV-AP011 (Table 4). As each of three mutations, L148V, M182T, and Y274H, resulted in improved hydrolysis of ampicillin relative to hybrid pVTV-AP1, we also introduced all three codons into one hybrid gene (pVTV-AP111).

Susceptibility of *E. coli* carrying plasmids to antibiotics and β-lactamase activities. The susceptibility profiles of *E. coli* transformed with each of the plasmids are summarized in Table 4.

MICs for *E. coli* cells containing the wild-type *P. vulgaris* β-lactamase were >2,000 μg/ml (ampicillin), >1,500 μg/ml (cefuroxime), and 800 μg/ml (cephaloridine). In contrast, strains producing the RTEM-1 β-lactamase were resistant to cephaloridine (MIC, 200 μg/ml) but completely susceptible to cefuroxime. *E. coli* cells harboring all hybrids in Fig. 2A and B, except pVTV-HS1-1, pVTV-SA1, and pVTV-AP1, were susceptible to these antibiotics as cells harboring the vector plasmid, but the strains with pVTV-HS1-1 and pVTV-SA1 were resistant to cefuroxime (MIC, 100 μg/ml) and cephaloridine (MIC, 50 μg/ml). pVTV-AP1 hybrid was resistant to cefuroxime (MIC, 50 μg/ml), but it was susceptible to cephaloridine. pVTV-AP100, pVTV-AP010, and pVTV-AP001 showed increased levels of resistance to cefuroxime and cephaloridine as well as ampicillin. Hybrid mutants pVTV-AP110, pVTV-AP011, and pVTV101 plasmids with double mutations were resistant to cefuroxime (MIC, 800 μg/ml) and to cephaloridine (MIC, 400 μg/ml). For the hybrid mutant with three mutation sites, pVTV-AP111, MICs were 1,000 μg/ml (cefuroxime) and 400 μg/ml (cephaloridine). The behavior of the mutant pVUL001 toward cephaloridine and cefuroxime was identical to that of the wild type, pVUL1. These results suggest that antibiotic resistance is conferred with synergistic effect by the substitution of 1, 2, or 3 amino acids in the inactive hybrid protein.

By using the crude extract of *E. coli* cells, the enzyme activity of the hybrids and hybrid mutants was examined (Table 4). The activity of the crude enzyme of the hybrid pVTV-SA1 for ampicillin and nitrocefin was lower than for the wild type, pVUL1. The hybrid pVTV-AP1 with substitution of 39 amino acid residues from RTEM-1 (Table 3) produced an enzyme with very low activity for ampicillin, cephaloridine, and cefuroxime. For hybrid mutants with single replacements, pVTV-AP100, pVTV-AP010, and pVTV-AP001, a substitution at Leu-148 with Val, Met-182 with Thr, and Tyr-274 with His,

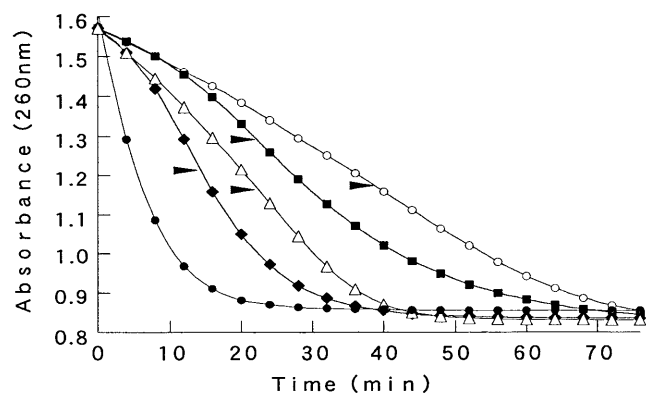

FIG. 3. Time course of the hydrolysis of cefuroxime by the different enzymes. To 3 ml of 0.1 mM cefuroxime in 50 mM sodium phosphate buffer, pH 7.0, 4 μl of crude extracts (0.71 mg of protein per ml) was added. Because the product of the pVTV-AP1 exhibited very low activity, 50 μl of the crude extract was used. Hybrids pVTV-AP100 and pVTV-AP001 exhibited the same features as pVTV-AP010, and pVTV-AP011 and pVTV-AP110 exhibited the same features as pVTV-AP101. Arrows indicate the maximum velocity of the time course. ●, pVUL1; ◆, pVTV-AP111; △, pVTV-AP1; ■, pVTV-101; ○, pVTV-AP010.

respectively, yielded enzymes with clearly higher activity than that of the pVTV-AP1 toward ampicillin, nitrocefin, and cephaloridine. For pVTV-AP110, pVTV-AP101, and pVTV-AP011, substitution of two amino acids resulted in higher activity of the crude extracts compared with the hybrid mutants with a single mutation. The mutant pVTV-AP111, with substitution of three amino acids, produced an enzyme with high activity toward ampicillin and nitrocefin at the same level as the wild type, pVUL1, while for cefuroxime and cephaloridine, activity was still lower than that of the wild-type enzyme. pVTV-HS1 did not produce an enzyme with detectable activity, while a single amino acid substitution (C106S) resulted in the production of an enzyme with activity toward ampicillin, nitrocefin, and cephaloridine.

The extract from pVUL1 showed clear hydrolytic activity against cefuroxime. The RTEM-1 extract shows little activity against cefuroxime, while pVTV-HS1-1 and pVTV-SA1 exhibit activity against cefuroximase.

Progress curves for cefuroxime hydrolysis by crude extracts from pVTV-AP1 and derivatives were abnormal. A lag, characteristic of substrate-induced activation, was observed upon hydrolysis of cefuroxime (Fig. 3). A similar result has previously been reported for class A β-lactamases (7, 26). This feature was not observed with the wild-type enzyme nor with the other hybrids studied here. These observations suggest that the initially inactive or weakly active mutant proteins undergo a slow conformational change to an active enzyme upon binding the substrate. After the reaction was allowed to proceed to complete conversion (40 min), newly added substrates were hydrolyzed with the same feature (data not shown), suggesting that this phenomenon is reversible. The purified enzymes also showed a pattern similar to that for the crude extracts, and the lags did not allow us to measure the kinetic parameters accurately for cefuroxime.

Kinetic parameters of purified enzymes. To obtain further information about the effect of the mutations described above on the catalytic activity, the hybrid mutants L148V and M182T and the triple mutant were purified and the kinetic parameters for ampicillin, nitrocefin, and cephaloridine hydrolysis were determined (Table 5).

The k_{cat} and the k_{cat}/K_m values of the L148V and M182T mutant enzymes for ampicillin were about half of the values for the *P. vulgaris* enzyme. For the triple mutant the k_{cat}/K_m value was 72% of that for the *P. vulgaris* enzyme. The increase in the k_{cat}/K_m value of the triple mutant compared with those of the single mutants is due to the alteration in the both k_{cat} and K_m values. On the other hand, the L148V and M182T mutants and the triple mutant exhibited reduction in the k_{cat} and K_m values for cephaloridine and nitrocefin compared with that of the wild-type *P. vulgaris* β-lactamase. However, the decrease in k_{cat} was balanced by a reduction in K_m so that the overall k_{cat}/K_m was relatively near to that of the wild-type *P. vulgaris* β-lactamase. In the case of nitrocefin, the k_{cat}/K_m value for L148V substitution was higher than that of the wild type. The change was due to the dramatic decrease in K_m value. For nitrocefin, the k_{cat} value of the triple mutant was about twice those of the single mutants but a lower k_{cat}/K_m was due to the increase in K_m value. The kinetic values of the purified enzymes did not directly reflect the susceptibility determinations. A similar result has already been reported (42).

DISCUSSION

There is no report of three-dimensional analysis of the *P. vulgaris* β-lactamase. Because the class A β-lactamases have similar structures, we will discuss the structures of hybrid and mutant proteins on the basis of the RTEM-1 β-lactamase conformation (18, 36).

The *P. vulgaris* and RTEM-1 β-lactamases have evolved from the same ancestor protein. If the essential amino acid residues for the enzyme activity are conserved in common, all hybrid proteins between them should be active. This is not the case. The hybrid genes shown in Fig. 2A produced mature size proteins (data not shown) but no active enzymes at all. The hybrids represented in Fig. 2B containing a rather narrow and a highly similar region of the heterogeneous gene could produce partially active enzymes directly or after single amino acid substitution. In general, residues that are either structurally or functionally important are conserved. However, hybrid proteins at either the amino or carboxy terminus are inactive while

TABLE 5. Kinetic parameters (mean ± SD) of β-lactamases

β-Lactamase	Ampicillin			Cephaloridine			Nitrocefin		
	K_m (μM)	k_{cat} (s^{-1})	$10^6 k_{cat}/K_m$ ($M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	$10^6 k_{cat}/K_m$ ($M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	$10^6 k_{cat}/K_m$ ($M^{-1} s^{-1}$)
RTEM-1	51 ± 6	1,470 ± 210	29	390 ± 50	790 ± 95	2.0	25 ± 4	194 ± 40	7.8
P. vulgaris	53 ± 5	98 ± 11	1.8	95 ± 3	140 ± 9	1.5	24 ± 2	94 ± 17	3.9
L148V[a]	69 ± 7	54 ± 4	0.8	18 ± 1	17 ± 0.5	0.9	4 ± 0.5	22 ± 2	5.5
M182T[a]	53 ± 3	48 ± 3	0.9	14 ± 1	18 ± 1	1.3	7 ± 0.5	27 ± 2	3.9
Triple mutant[a]	47 ± 3	60 ± 3	1.3	9 ± 0.5	15 ± 1	1.7	17 ± 3	51 ± 8	3.0

[a] pVTV-AP1 derivatives, pVTV-AP100, pVTV-AP010, and pVTV-AP111 (Fig. 2C).

the sequence similarities of both terminal regions are very low in class A β-lactamases. Both N- and C-terminal regions constitute α-helices and interact each other. Although these α-helices are far away from the active site serine or enzyme-substrate interaction sites, they could affect the enzyme activity. The loss of enzyme activity should result from changes in a particular interaction between enzyme and substrate or from general conformational alterations, but, at present, we cannot identify precisely the particular residues in the hybrid proteins responsible for the altered catalytic activity.

The degree of resistance of *E. coli* cells harboring different hybrids does not depend on the quantity of the homology existing between native and substituted regions. As shown in Table 2, the amino acid homology of replaced region in the pVTV-AP1 hybrid is more than 50% while the *E. coli* cells harboring it are susceptible to antibiotics. In contrast, the replaced region in pVTV-HS1-1 shows low homology (24%), while the cells harboring it are more resistant to antibiotics.

The substitution of one amino acid, L148V, M182T, or Y274H, in the pVTV-AP1 hybrid gene by spontaneous mutation surprisingly increased the MICs for the cells harboring them (1,000 μg/ml for ampicillin). It is worth noting that the mutation at position 274, which is not in the hybrid region, occurred frequently in the independent conditions although this amino acid is quite variable among the class A β-lactamases and no special role has been found for it in hydrolysis.

To understand the role of substituted residues in the hydrolysis of β-lactam antibiotics, we evaluated kinetic parameters of some mutant enzymes (pVTV-AP100, pVTV-AP010, and pVTV-AP111) toward ampicillin, cephaloridine, and nitrocefin. As shown in Table 5, the replacement of one amino acid, Leu-148 with Val or Met-182 with Thr in the pVTV-AP1 hybrid, increased the enzyme activity toward all three substrates. Both Val-148 and Thr-182 are consensus amino acid residues of the class A β-lactamases. Val-148 is located near Lys-73 and turns its side chain toward the inside of the enzyme. The side chain of Val-148 might affect the enzyme activity through the conformational change of Lys-73. Table 3 also shows that the replaced regions contain several residues which are conserved all through the class A β-lactamases and would interact directly with substrates (36).

Approximately 5- to 10-fold reductions in the K_m values of the pVTV-AP1 derivatives compared with that of the wild-type enzyme suggest that the replaced region with or without the mutations led to structural change which decreased the K_m values for a substrate.

For all three substrates, ampicillin, cephaloridine, and nitrocefin, the k_{cat} value of the purified enzymes did not follow the same patterns as activity of the crude extracts. This variation may be due to the different stability of the proteins in the periplasm. The triple mutant enzyme seems to be more stable than the single mutants since the amount of its periplasmic β-lactamase is higher than that of single mutants (data not shown).

The efficiency of substitution of one amino acid residue in the *dnaQ* strain of *E. coli* was about 10^{-5} to 10^{-6} which was enough to introduce one base substitution into all the nucleotides of a hybrid gene at nearly equal frequencies. However, there were several restrictions on amino acid substitution. For instance, of the 19 possible replacements of amino acids at residue Met-182 in pVTV-AP1, only six amino acids (Val, Leu, Ile, Lys, Arg, and Thr) can be substituted by one nucleotide alteration. The Thr substitution could confer activity to the protein. This restriction also existed for all residues of the protein. Only the substitutions L148V, M182T, and Y274H of the possible replacements in the hybrid gene of pVTV-AP1 could produce active enzymes. We could not isolate any active mutant from 16 hybrid plasmids with MKS148 except pVTV-AP1 and pVTV-HS1. This probably means that any single nucleotide substitution in these hybrid genes does not produce active enzymes at all. Note that, under these conditions, it is experimentally impossible to obtain a double mutant in one step. Nonetheless, we believe that the *dnaQ* strain of *E. coli* is a very efficient strain for mutagenesis.

We are now constructing a series of reverse hybrids which contain a small segment from the *P. vulgaris* β-lactamase gene in the RTEM-1 gene. It will be interesting to compare these two series of hybrids, and we are expecting to get more precise information about the relationship between amino acid residues and activity of enzymes.

ACKNOWLEDGMENTS

We thank N. Nakajima for his helpful discussion, H. Maki and M. Sekiguchi for *E. coli* MK148, F. de la Cruz and H. Adachi for their plasmids, and M. N. G. James for coordinates of the RTEM-1 crystal structure.

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