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Beta-Lactamase-Directed Barrier for Penicillins of *Escherichia coli* Carrying R Plasmids

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Strains of *Escherichia coli* and *Salmonella typhimurium* carrying R plasmids, which were obtained from ampicillin-resistant clinical isolates of *E. coli* and *Klebsiella* spp. and specified either the type IIIa (TEM-type) or type Va (oxacillin-hydrolyzing) β-lactamase, are resistant not only to ampicillin but also to carbenicillin and sulbenicillin. The latter two derivatives, however, are poorly hydrolyzed in vitro by the β-lactamases. Although values of K_m of the enzymes are lower for sulbenicillin and carbenicillin than for ampicillin, the ratios of V_{max} to K_m for sulbenicillin and carbenicillin are not high enough to explain the high resistance in *E. coli* bearing the R plasmid. Two mutants of the plasmids conferring a temperature-sensitive ampicillin resistance were induced by nitrosoguanidine treatment. It was confirmed that *E. coli* CSH2, harboring the mutant plasmid, produces a temperature-sensitive β-lactamase and is resistant only at low temperatures (below 33°C), but not at 42°C, to ampicillin, sulbenicillin, and carbenicillin simultaneously. It is thus concluded that β-lactamase itself is responsible for the mechanism of resistance not only to ampicillin but also to sulbenicillin and carbenicillin, even though the enzyme as determined in cell-free extracts hydrolyzes the latter two drugs poorly. An unknown barrier for sulbenicillin and carbenicillin directed by β-lactamase in *E. coli* strains carrying R (*bla*) plasmids is postulated.

Beta-lactamases, which open the β-lactam ring of the penicillins and cephalosporins, have been understood as a major mechanism of resistance to these drugs in both gram-positive and gram-negative bacteria (11). The characteristics of these enzymes, however, are different between gram-positive and gram-negative bacteria. The β-lactamases of gram-negative rods are mainly localized in the periplasmic space between the outer and cytoplasmic membranes and are produced constitutively (or rarely inducibly) in rather small amounts (12). They are specified by either the chromosome or plasmids. In contrast, β-lactamases of gram-positive cocci are predominantly exoenzymes mediated by nontransferable mini-plasmids and are produced in relatively large amounts. They are typical inducible enzymes.

Substrate affinities for both groups of β-lactamases also vary. In general, β-lactamases of gram-positive bacteria often display a high affinity for the "natural penicillins," whereas gram-negative bacteria produce enzymes with a variety of substrate specificities (3, 12).

Carbenicillin and sulbenicillin are broad-spectrum penicillins developed for infections of *Pseudomonas aeruginosa* (1, 8). They are more stable than ampicillin against various types of β-lactamases. This paper deals with the role of β-lactamase in resistance to ampicillin, sulbenicillin, and carbenicillin in *Escherichia coli* strains carrying R (*bla*) plasmids.

MATERIALS AND METHODS

R plasmids. Plasmids of RK1 (*amp cml tet str kan sul*) and RE45 (*amp cml tet str sul*) were employed as representative plasmids specifying the type IIIa (TEM-type) and type Va (oxacillin-hydrolyzing) β-lactamases, respectively (6, 12). They were selected from among 61 R (*amp* ---) plasmids obtained from 92 clinical isolates of *E. coli* and *Klebsiella* spp. in Juntendo Hospital, Tokyo, Japan, resistant to ampicillin.

Bacteria. *E. coli* CSH2 (F^-) *metB* (2), *Salmonella typhimurium* LT2, and *Proteus mirabilis* Pm1 *lac thy nia nal* were employed as host bacteria for the R plasmids.

Chemicals. Ampicillin, sulbenicillin, and cephaloridine were provided by Takeda Chemical Industry Co. Ltd. and Shionogi Pharmaceutical Co. Ltd., Osaka, Japan, respectively. Commercially available carbenicillin (Fujisawa Chemical Industry Co. Ltd., Osaka) was also used.

Media. Heart infusion agar (Eiken Chemical Co. Ltd., Tokyo, Japan) and MacConkey agar containing 1% lactose (Eiken) were used as plating media. L broth (4) was employed as the liquid medium.

Measurement of drug resistance levels. Minimal

growth inhibitory concentrations of β-lactam drugs were measured by the plate dilution method. One-loopful portions of a 100-fold-diluted overnight culture of test bacteria in L broth were streaked onto heart infusion agar plates containing serially increasing amounts of drugs and incubated at 37°C overnight. Fifty percent inhibitory concentrations of the drugs were measured by the liquid dilution method. Cells of test microbes reaching midlogarithmic growth phase at 30°C were inoculated into fresh L broth containing serially increasing amounts of drugs to make a final density of 1×10^5 cells/ml and incubated for 5 h at temperatures ranging from 33 to 45°C. The growth rate in the tubes was measured turbidimetrically with a Klett-Summerson colorimeter with a no. 66 filter.

β-Lactamase assay. Crude extracts of *E. coli* CSH2 carrying the RK1 or RE45 plasmids were prepared by growing the cells in 100 ml of L broth at 37°C with shaking, washing with and suspending in 3 ml of 0.01 M phosphate buffer (pH 7.0), and disrupting by four 15-s bursts of sonic treatment at 10 kc. The sonic extracts were centrifuged at 4°C for 15 min at 40,000 × *g*, and the resulting supernatant fluids were used as the crude enzymes (14). The β-lactamase assay was carried out at 30°C in 0.1 M phosphate buffer (pH 7.0) by either the macroiodometric, microiodometric, or biological method (13). For the biological assay, *Bacillus subtilis* PCI 219 and *E. coli* CSH2 were employed instead of *Staphylococcus aureus* and *Sarcina lutea*. The specific activity of β-lactamase is expressed as micromoles of substrate hydrolyzed per 60 min per milligram of protein. Protein concentrations in the crude extracts were measured by either the biuret method or the method of Lowry et al. (5).

Temperature-sensitive mutants of plasmids. The overnight culture of *E. coli* CSH2 carrying plasmid RK1 in L broth was treated with 150 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml by the method of Mise and Suzuki (7). The mutagenized bacteria were spread on MacConkey agar plates free from drugs, and more than 1,000 subclones were tested for resistance to ampicillin at 30 and 42°C by replica plating onto heart infusion agar plates containing 100 μg of ampicillin per ml. Mutant plasmids that confer temperature-sensitive ampicillin resistance were confirmed by successive transfer of them from *E. coli* CSH2 (R^+) to R^- strains of *E. coli* AB1157 and then *E. coli* CSH2 by conjugation. A new clone of *E. coli* CSH2 carrying the temperature-sensitive plasmids was used for the experiments.

Assay of temperature sensitivity. Fifty percent inhibitory concentrations for ampicillin and sulbenicillin in *E. coli* CSH2 carrying the mutant plasmids RK117 and RK1051 were measured by the method described previously at various temperatures. For testing temperature sensitivity of the β-lactamases specified by the mutant plasmids, the enzyme was first purified 50-fold by gel filtration with Sephadex G-75 (10). The partially purified enzymes were incubated at 42°C at protein concentrations of 0.02 and 0.4 μg/ml in 0.01 M phosphate buffer (pH 7.0) for 2.5 to 20 min, and residual activities of β-lactamases were measured by either the macroiodometric or the microiodometric method (13), with ampicillin as the substrate both before and after incubation.

RESULTS

Resistance levels of gram-negative bacteria carrying the RK1 or RE45 plasmid to β-lactam drugs. Table 1 shows the resistance levels of *E. coli*, *S. typhimurium*, and *P. mirabilis* bearing plasmids RK1 or RE45 to ampicillin, sulbenicillin, carbenicillin, and cephaloridine. In both *E. coli* and *S. typhimurium*, resistance levels conferred by either plasmid were much higher to sulbenicillin and carbenicillin than to ampicillin, although in *P. mirabilis* resistance levels to the former two drugs were lower than that to ampicillin. This evidence indicates that the same R (*bla*) plasmids specify different resistance levels to various β-lactam drugs in different bacterial species.

Enzymological properties of β-lactamases specified by RK1 and RE45 plasmids. Kinetic parameters of β-lactamases specified by RK1 and RE45 are shown in Table 2. Although the plasmids conferred high resistance levels to sul-

TABLE 1. *Resistance levels to β-lactam antibiotics of E. coli CSH2, S. typhimurium LT2, and P. mirabilis Pm1 carrying the RK1 or RE45 plasmid*

Bacteria	Plasmid present	Minimal inhibitory concentration (μg/ml)			
		Ampicillin	Sulbenicillin	Carbenicillin	Cephaloridine
E. coli CSH2		1.6	1.6	1.6	3.2
	RK1	3,200	25,600	25,600	25
	RE45	400	400	400	3.1
S. typhimurium LT2		0.4	1.6	1.6	1.6
	RK1	6,400	25,600	25,600	200
	RE45	200	400	400	3.1
P. mirabilis Pm1		0.2	0.4	0.4	3.1
	RK1	400	100	100	25
	RE45	50	12.5	25	6.3

TABLE 2. *Kinetic properties of β-lactamases specified by the RK1 and RE45 plasmids in E. coli CSH2*

Plasmid	Substrate	V_{max}[a]	K_m (μM)	V_{max}/K_m[a]
RK1	Ampicillin	100	37	100
	Sulbenicillin	5.2	7.1	27
	Carbenicillin	9.4	14	25
	Cephaloridine	120	714	6.3
RE45	Ampicillin	100	20	100
	Sulbenicillin	7.7	34	4.6
	Carbenicillin	16	59	5.4
	Cephalorodine	28	200	2.8

[a] Expressed as values relative to those for ampicillin taken as 100.

benicillin and carbenicillin on *E. coli* CSH2, V_{max} values of β-lactamases specified by those plasmids for sulbenicillin and carbenicillin were less than 16% of that for ampicillin. The V_{max} for cephaloridine was high in the RK1 β-lactamase, but rather low in the enzyme specified by RE45.

K_m values of β-lactamases for the drugs are also shown in Table 2. Since Pollock (9) reported that values of ratios of V_{max} to K_m might be a better indicator for the resistance levels, those for ampicillin, sulbenicillin, carbenicillin, and cephaloridine have been calculated. The value of V_{max} to K_m for the RK1 enzyme for cephaloridine was low enough to account for the low resistance level for that drug, although those for sulbenicillin and carbenicillin were insufficiently high to explain the high resistance levels.

Competitive inhibition of β-lactamase by sulbenicillin. Figure 1 shows a Lineweaver-Burk plot of the RK1 β-lactamase with ampicillin as the substrate, in the presence or absence of various amounts of sulbenicillin. Sulbenicillin inhibited the enzyme activity competitively, with a K_i value of 6.5 μM. Sulbenicillin, therefore, appears to have a common active site on the enzyme with ampicillin.

Accuracy of the iodometric assay. Since activities of β-lactamase were measured iodometrically, it appeared necessary to establish that the methods were accurate enough not only for ampicillin but also for sulbenicillin and carbenicillin. Values of V_{max} for the RK1 β-lactamase were identical regardless of whether determined by macroiodometry or by the bioassay (Table 3).

Hydrolysis of ampicillin, sulbenicillin, and cephaloridine by intact whole cells of *E. coli* CSH2 carrying RK1 was examined (Table 4). Sulbenicillin was hydrolyzed to a lesser extent than ampicillin, even by whole cells, although the efficiency of the enzyme was lower than with the cell-free extracts.

Temperature-sensitive mutants of plasmid RK1. Two mutants of plasmid RK1, RK117 and RK1051, which confer temperature-sensitive ampicillin resistance on the hosts, were obtained after nitrosoguanidine treatment. New subclones of *E. coli* CSH2 carrying the mutant

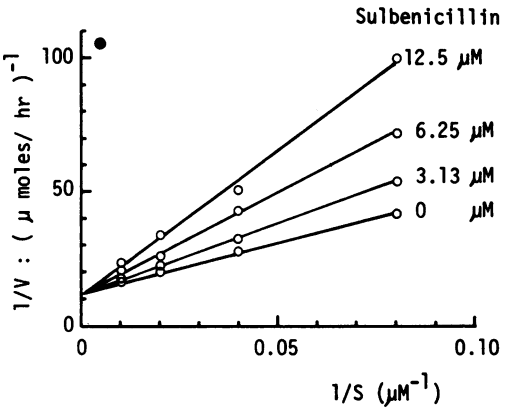

FIG. 1. *Lineweaver-Burk plots of β-lactamase specified by the RK1 plasmid in E. coli CSH2. The reaction was carried out in 2 ml of 0.1 M phosphate buffer, pH 7.0, containing 0.15% gelatin at 30°C with ampicillin as the substrate in the presence of various amounts of sulbenicillin. After 15 min of incubation, the reaction was terminated by chilling and subsequently adding 1 ml of 0.15 M sodium tungstate in 2 M acetate buffer, pH 4.0. Precipitates were removed by centrifugation, and 2 ml of the resulting supernatant was mixed with an equal volume of an aqueous solution containing 0.2% starch, 80 μM I_2, and 1.6 mM KI. The mixtures were kept at room temperature for 15 min and measured spectrophotometrically at 620 nm. The observed values at each point were subtracted by those of blanks processed in the same manner without enzyme during incubation at 30°C.*

TABLE 3. *Comparison of activities of the RK1 β-lactamase[a] measured by different methods*

Substrate	V_{max} values[b] measured by:		
	Macroiodometry	Bioassay with:	
		B. subtilis PCI 219	*E. coli* CSH2
Ampicillin	1,100	1,181	1,100
Sulbenicillin	77	69	61
Carbenicillin	102	134	94

[a] The crude enzyme of *E. coli* CSH2 carrying RK1 was employed.

[b] Expressed as micromoles of substrate hydrolyzed per 60 min per milligram of protein.

TABLE 4. *Hydrolysis of β-lactam antibiotics by whole cells and a cell-free extract of E. coli CSH2 carrying plasmid RK1.*

Enzyme source	μmol of substrate hydrolyzed per 60 min		
	Ampicillin	Sulbenicillin	Cephaloridine
Whole cells[a]	36	9.6	3,228
Extracts of cells[a]	28,800	2,400	30,360

[a] A total of 10^{10} cells.

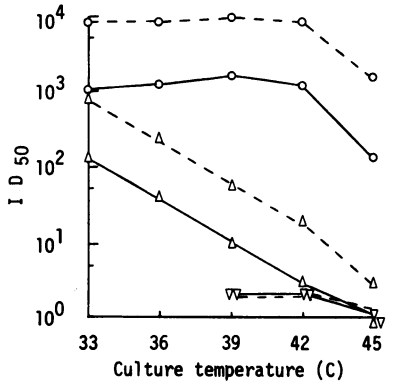

FIG. 2. *Influence of temperature shift on resistance levels to ampicillin and sulbenicillin of E. coli CSH2 harboring or not harboring plasmid RK1 and its temperature-sensitive mutant plasmid RK1051. Bacterial cells grown at 30°C in L broth were diluted with fresh L broth containing serially increasing amounts of ampicillin or sulbenicillin to 1 × 10^5 cells/ml. Portions (2 ml) were incubated at given temperatures for 5 h. Fifty percent inhibitory concentrations (ID_{50}) of antibiotics were determined turbidimetrically and expressed as micrograms per milliliter. Symbols: ——, ampicillin; -----, sulbenicillin; ○, RK1⁺; △, RK1051⁺; ▽, R⁻.*

plasmids were highly resistant to ampicillin only at low temperatures (below 33°C), but the resistance to chloramphenicol, streptomycin, tetracycline, and kanamycin remained unaffected by the mutations. The resistance level to sulbenicillin of cells carrying either RK117 or RK1051 decreased with an increase of culture temperatures, identical to that found for ampicillin (Fig. 2). This fact indicates that the same temperature-sensitive mechanism is responsible for resistance to both ampicillin and sulbenicillin.

Figure 3 shows the influence of incubation at 42°C on the enzymatic activity of the β-lactamases that were partially purified about 50-fold from cell-free extracts of *E. coli* CSH2 harboring RK1 or its mutants, RK117 and RK1051. As shown, more than 90% of the activity of the RK1 β-lactamase remained after 20 min of incubation at 42°C at a protein concentration of 0.4 μg/ml, whereas only 55 and 13% of the activities in the RK117 and RK1051 enzymes were retained, respectively, under the same conditions. When enzyme preparations with lower protein concentration, i.e., 0.02 μg/ml, were incubated at 42°C, loss of enzyme activity was more prominent. Under this condition, more than 95% of the activity of the RK1051 β-lactamase was lost after only 10 min of incubation at 42°C.

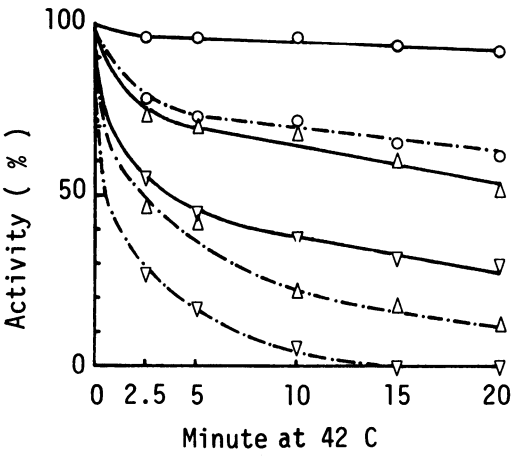

FIG. 3. *Heat stability of β-lactamases from E. coli CSH2 carrying the RK1 plasmid or its temperature-sensitive mutants RK117 and RK1051. Symbols: ○——○, RK1 β-lactamase at a protein concentration of 0.4 μg/ml; ○-·-·-·-·○, RK1 β-lactamase at a protein concentration of 0.02 μg/ml; △——△, RK117 β-lactamase at a protein concentration of 0.4 μg/ml; △-·-·-·-△, RK117 β-lactamase at a protein concentration of 0.02 μg/ml; ▽——▽, RK1051 β-lactamase at a protein concentration of 0.4 μg/ml; ▽-·-·-·-▽, RK1051 β-lactamase at a protein concentration of 0.02 μg/ml.*

Since temperature-sensitive ampicillin resistance of *E. coli* CSH2 carrying the mutant plasmid was confirmed to be based upon a temperature-sensitive β-lactamase, and the sulbenicillin resistance became temperature sensitive simultaneously, the mechanism of resistance to sulbenicillin and ampicillin is considered to be the β-lactamase itself, even though sulbenicillin is poorly hydrolyzed by the cell-free enzyme activity.

DISCUSSION

It appears that *E. coli* and *S. typhimurium* harboring R plasmids that specify the type IIIa

(TEM-type) or type Va (oxacillin-hydrolyzing) β-lactamase are resistant not only to ampicillin but also to sulbenicillin and carbenicillin, even though crude or partially purified enzymes from these strains hydrolyze poorly the latter two drugs.

As pointed out by Pollock (9), the enzyme is never likely to operate under optimum conditions in vivo so that the physiological efficiency of β-lactamase depends upon the values of both K_m and V_{max}. The value of the ratio of V_{max} to K_m is, therefore, a good indicator for the resistance levels to β-lactam drugs. The results obtained have indicated that K_m values of β-lactamases specified by plasmids RK1 and RE45 are lower for sulbenicillin and carbenicillin than for ampicillin, although values of V_{max} to K_m of the enzymes are not high enough for an explanation of the high resistance levels to sulbenicillin and carbenicillin.

Richmond and Curtis (11) have emphasized the importance of the interplay of β-lactamase and intrinsic factors by the recent discovery showing that R plasmid RP1, which was obtained from a strain of *P. aeruginosa*, carries two genetic regions that affect resistance to penicillins. The first is a determinant for the β-lactamase, but the second is a new gene(s) that imparts enhanced intrinsic resistance to bacteria. In contrast, the present results show that, even without the second gene(s), *E. coli* strains carrying R (*bla*) plasmids are resistant not only to ampicillin but also to sulbenicillin and carbenicillin, which are poorly hydrolyzed by the β-lactamases in vitro. This fact was confirmed by isolation of mutant plasmids that specify temperature-sensitive β-lactamases and confer simultaneously temperature-sensitive resistance to three antibiotics. Since the spontaneous reversion frequencies of the mutant plasmids were between 1×10^{-8} and 1×10^{-9}, the mutation in these plasmids was considered to be a point mutation.

It is therefore postulated that resistance to sulbenicillin and carbenicillin in *E. coli* strains carrying R (*bla*) plasmids is based on an unknown barrier directed by β-lactamase itself, which does not effectively hydrolyze these antibiotics, but binds them and prevents their passage through the periplasmic space to the penicillin-sensitive site.

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