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Characterization of β-Lactamase-Deficient (*bla*) Mutants of the R Plasmid R1 in *Escherichia coli* K-12 and Comparison with Similar Mutants of RP1

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Thirty-eight mutants of R1, an R plasmid specifying the type IIIa (TEM) β-lactamase, were isolated; these mutants are partially or totally unable to synthesize the type IIIa β-lactamase. The loss of β-lactamase activity was associated with a reduction in the level of penicillin resistance conferred by the mutants upon their host strain. At least two of the mutants synthesized a β-lactamase with altered substrate specificity. These properties are compared with those of two β-lactamase-deficient mutants of plasmid RP1. The results suggest that, for both R plasmids, penicillin resistance is entirely attributable to the presence of β-lactamase activity. The properties of two R1 derivatives, pUB251 and pUB252, which have phenotypes similar to that of RP1, support this conclusion.

Plasmids encoding the type IIIa (TEM) β-lactamase vary widely in origin (13, 22, 31). However, they always possess a common or closely related deoxyribonucleic acid (DNA) sequence of about 3×10^6 daltons, even when no other sequences are shared (18). This striking uniformity is attributable to the fact that the β-lactamase structural gene (*bla*; previously designated *amp*) resides within a 3.2-megadalton DNA sequence, termed transposon *A* (Tn*A*) (15), which is capable of translocating at high frequency from one replicon to another (2, 14, 18).

At least two variants of Tn*A* are now recognized (33). One of these, Tn*1*, occurs on the plasmid RP4 (9, 14), which is almost certainly identical to the plasmid RP1 (4, 19). The other variant, Tn*3*, originates from the plasmid R1 (23). The differences between Tn*1* and Tn*3* are small (33): they are identical in size (17), and electron microscope heteroduplex analysis fails to disclose any obvious divergence (33).

It is generally assumed that RP1- and R1-mediated penicillin resistance is due solely to the Tn*A*-encoded β-lactamase. However Curtis et al. (7, 8) have reported the isolation of a mutant of RP1, RP1 *amp-1*, which specifies penicillin resistance in the absence of any detectable β-lactamase activity. They postulated that RP1 carries a locus, *irp*, which is responsible for this so-called intrinsic resistance to penicillins. This locus was reported to be absent from R1, since a point mutant, R1 *amp-2*, lacked both β-lactamase activity and the capacity to determine penicillin resistance (7).

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The extent to which the *irp* locus contributes to the high level of penicillin resistance mediated by wild-type RP1 is unknown. In the present study, in which we describe the properties of a newly isolated series of β-lactamase-deficient mutants of R1 and RP1, we confirm the absence of *irp* from R1 and reexamine the role of the *irp* locus on RP1. We conclude that the *irp* locus does not contribute to the penicillin resistance phenotype of wild-type RP1 and throw some doubt onto the conclusion that *irp* is responsible for the penicillin resistance mediated by RP1 *amp-1*. In the accompanying paper (6), we present a quantitative comparison of the properties of R1 and RP1 which confirms that, for both plasmids, the penicillin resistance phenotype is mediated entirely by the R-factor β-lactamase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and isolation of mutants. Bacterial strains were all *Escherichia coli* K-12 derivatives. UB1301 and UB1325, our laboratory designations for W3110 Rif^r and J6-2 Nal^r, respectively (20), were the standard host strains. JC7620, a *recB recC sbcB* strain (21), was used as a recipient for transformation. D21a12, an *ampC* derivative of D21 which lacks the chromosomal β-lactamase, was obtained from S. Normark (University of Umeå, Umeå, Sweden). The R plasmid R1 (23) carries resistance determinants for ampicillin (Amp), chloramphenicol (Chl), kanamycin (Kan), streptomycin (Str), and sulfonamides. RP1 determines resistance to ampicillin, kanamycin, and tetracycline (10). Both plasmids carry the *bla* gene encoding the type IIIa (TEM) β-lactamase. *bla* mutants were isolated by nitrosoguanidine mutagenesis, as described previously (20). R1 Kan^s, a spontaneous kanamycin-susceptible derivative of R1, was used to isolate mutants *bla-11* to *bla-25*. Mutants *bla-31* to *bla-53* were isolated from R1 Chl^s, a chlor-

amphenicol-susceptible derivative of R1 obtained by mutagenesis with ethyl methane sulfonate. RP1 *amp-1*, a β-lactamase-deficient mutant of RP1, and RP1 *amp-1 irp-1*, a mutant of RP1 *amp-1* unable to specify resistance to penicillins, were isolated by Curtis et al. (7, 8). RP1 *bla-6* (this paper) was isolated in the same way as were the R1 *bla* mutants, except that the host strain used was UB1139 (10). Only one mutant was isolated from 100,000 colonies screened, a frequency only 1% of that obtained with R1 (20). The reason for this difference is unknown.

Characterization of mutants. Potential *bla* mutants were tested for residual β-lactamase activity and penicillin resistance phenotype (see below). All mutants were Tra^+ and expressed the same phenotype in at least two host strains (UB1301 and UB1325). Spontaneous revertants were isolated by spreading 10^9 cells onto agar containing penicillin (usually carbenicillin) and characterized in a similar manner. Those mutants which reverted to a wild-type phenotype were assumed to be point mutants.

β-Lactamase assays. Cells grown in CY medium (a minimal salts medium containing Casamino Acids and yeast extract as described by Novick [24] except that glucose was omitted and glycerol 3-phosphate was replaced by $NaH_2PO_4 \cdot 2H_2O$ [21 g/liter]) were harvested in late logarithmic phase (absorbance at 675 nm [A_{675}] = 1.0 to 1.4) and suspended in 50 mM phosphate buffer, pH 7.0. β-Lactamase activity was released by 3 min of ultrasonic disruption at 4°C, using a 100-W Dawe soniprobe 1130 at an output of 4 A. Enzyme activity was determined (in the same buffer) at 30°C by using one of two assays: the iodometric assay (28) was performed as described by Richmond et al. (30), usually with penicillin G as substrate at a concentration of 7 mM. The 87/312 assay, a spectrophotometric assay using the chromogenic cephalosporin 87/312 (27), was carried out at a substrate concentration of 0.05 mM in a Pye Unicam SP1800 spectrophotometer. Results obtained with this method were highly reproducible. Enzyme activities for both assays are expressed in the units defined by Pollock and Torriani (29): 1 U of enzyme hydrolyzes 1 μmol of substrate/h at 30°C. For the iodometric assay (with penicillin G as substrate), the term "units" is used without further qualification; in the 87/312 assay, activity is expressed as milliunits (87/312). Specific activities are in units (or milliunits) per milligram (dry weight) of cells; a culture with an A_{675} of 1.3 is assumed to contain 1 mg (dry weight) of cells per ml.

Determination of penicillin resistance. Cultures grown overnight in nutrient broth were diluted, and drops containing 50 cells at most were spotted onto plates containing twofold dilutions of the antibiotic being tested. The single cell resistance was recorded as the highest antibiotic concentration allowing the growth of colonies from single cells. Resistance values obtained by this method were reproducible to within a factor of 2.

Mapping experiments. Strains grown to an A_{675} of 0.5 were mixed in pairs (such as UB1301 [R1 Kan^s *bla-20*] and UB1301 [R1 Chl^s *bla-40*]) and incubated statically at 37°C for 7 h to permit conjugation and recombination. Portions of 0.5 ml were then inoculated into 100 ml of nutrient broth and grown overnight to permit the expression of carbenicillin resistance in cells carrying recombinant bla^+ plasmids. Such cells were selected on plates containing carbenicillin (2 mg/ml) and purified by restreaking onto fresh carbenicillin agar, to ensure the segregation and loss (through incompatibility) of the complementary bla^- recombinant plasmids. The proportion of recombinant cells that retained such a plasmid (and thus carry all the genetic information originally present on both plasmids) was probably negligible, since in some crosses less than 5% of the recombinants retained their resistance to both kanamycin and chloramphenicol (see Table 3; in other crosses the Kan^r Chl^r bla^+ cells are assumed to be genuine recombinants in which both kan^r and chl^r have become linked to bla^+). The purified recombinants were scored for resistance to kanamycin and chloramphenicol by replica plating. Control experiments ensured that the spontaneous reversion of *bla* mutants under these conditions was negligible. Segregation frequency data were analyzed as follows: if *bla* and *kan* are very closely linked, then bla^+ recombinants will be predominantly either Kan^r or Kan^s, since they will be unlikely also to have recombined between *bla* and *kan*. The order of the recombining *bla* alleles (relative to *kan*) can be deduced from the Kan phenotype that predominates among the bla^+ recombinants. If *bla* and *kan* were less closely linked, recombination between them would generate similar numbers of Kan^r bla^+ recombinants; in this case, segregation frequency data could not be used to order *bla* alleles.

Conjugation and transformation. Conjugation was carried out by using standard procedures (20). Transformation (5) was performed as described by Bennett et al. (1).

Chromogenic cephalosporin. The chromogenic cephalosporin 87/312 was the gift of Glaxo Research Ltd. (27).

RESULTS

Classification of mutants. The data presented in Table 1 are representative of the properties of 40 β-lactamase-deficient mutants (38 mutants of R1 and 2 of RP1) that we have characterized. Three phenotypes were found: class I mutants were unable to confer penicillin resistance above the R^- level and specified negligible levels of type IIIa β-lactamase activity (less than 10 mU [87/312]/mg [dry weight] when assayed either in the host strain D21a12, which lacks the chromosomal type Ib β-lactamase, or in the presence of carbenicillin, an inhibitor of the chromosomal enzyme [31]). Class II mutants determined low levels of penicillin resistance and β-lactamase activity (10 to 100 mU [87/312]/mg [dry weight], i.e., less than 1% of the wild-type level), and class III mutants specified relatively high levels of resistance and enzyme activity (greater than 250 mU [87/312]/mg [dry weight]). Of the 38 R1 mutants, 15 were class I, 14 were class II, and 9 were class III. Significantly, no mutants were able to determine pen-

TABLE 1. *Properties of bla mutants of R1 and RP1*

R factor[a]	Revertibility[b]	Single cell resistance[c] (μg/ml)			β-Lactamase activity (mU [87/312]/mg [dry wt])	
		PenG	Amp	Carb	Total	Without Ib activity[d]
None		16	2	4	87	7*
R1		250	250	2,000	11,500	11,500*
RP1[e]		>1,000	>1,000	≥10,000	136,000	136,000*
Class I R1 mutants						
bla-11	+	16	2	4	73	<1**
bla-13	+	16	2	8	85	9*
bla-15	+	16	2	4	87	<1*
bla-20	+	16	2	4	78	<1**
Class II R1 mutants						
bla-14	+	32	16	125	94	26*, **
bla-40	+	ND	32	64	88	25*
bla-45	+	16	16	32	87	18*, **
bla-50	−	16	16	125	85	23*
Class III R1 mutants						
bla-18	−	64	32	250	1,300	—[f]
bla-34	+	ND	32	250	430	350*
bla-38	−	ND	125	1,000	1,800	—[f]
bla-39	−	ND	64	500	975	—[f]
bla-44	+	32	16	250	625	—[f]
bla-46	−	ND	64	500	370	300*
RP1 mutants						
amp-1	−	125	250	500	230	—[g]
bla-6	−	16	4	64	245	—[g]

[a] Host strain was UB1301. R1 Kan[s] and R1 Chl[s] have a penicillin phenotype identical to that of R1.

[b] Spontaneous revertants to a wild-type phenotype were isolated (+) or not isolated (−). *bla-50* also reverted, but not to a wild-type phenotype (see Table 4).

[c] Determined as described in Materials and Methods. PenG, penicillin G; Amp, ampicillin; Carb, carbenicillin. ND, Not determined.

[d] The 87/312 assay was performed either in the presence of carbenicillin (0.5 μM) to inhibit the chromosomal type Ib β-lactamase (*) or in strain D21a12, a derivative of *E. coli* K-12 that lacks the Ib enzyme (**).

[e] Data from references 7 and 31 indicate that RP1 specifies single resistances to PenG, Amp, and Carb of 5,000 to 10,000 μg/ml, 2,500 to 5,000 μg/ml, and 10,000 to 20,000 μg/ml, respectively (note that minimal inhibitory concentration values are in general double resistance values).

[f] Assays using UB1325 as host strain gave activities in the presence of 0.5 μM carbenicillin that were 85 to 100 mU/mg (dry weight) less than in its absence, although the absolute levels of β-lactamase activity were up to 50% less than in UB1301.

[g] Not determined. Instead, iodometric assays using D21a12 as host strain were carried out (see Table 2).

icillin resistance in the absence of a detectable level of type IIIa β-lactamase activity.

RP1 *amp-1* and RP1 *bla-6* had properties intermediate between classes II and III (Table 1). To confirm that, for these mutants, the increase in β-lactamase activity over the R⁻ level was attributable to the R-factor enzyme, both R factors were assayed with D21a12 as host strain (Table 2). The β-lactamase activity of RP1 *amp-1* was comparable to that in Table 1 (about 0.1% of the wild-type activity), but the enzyme had an altered substrate profile. RP1 *bla-6* also specified a significant level of β-lactamase activity, but the results in Tables 1 and 2 are not directly comparable since control experiments demonstrated that, at the time the iodometric assays were carried out (Table 2), RP1 *bla-6* was no longer able to confer resistance to carbenicillin above the R⁻ level. It is of interest that RP1 *amp-1 irp-1* (alias pUB306 [32]), a mutant of RP1 *amp-1* that had lost the ability to specify penicillin resistance, was not quite β-lactamase negative, although it retained less than 15% of the β-lactamase activity of RP1 *amp-1* (Table 2).

R1 mutants with altered substrate specificities. Hall and Knowles have reported that the R_{TEM} β-lactamase, which is indistinguishable from the R1 enzyme (22), can be modified by mutation so that its substrate specificity is altered (12). To test the β-lactamase substrate specificities of our mutants, we reassayed our enzyme preparations by the iodometric method, with penicillin G as substrate. This assay is less sensitive than the 87/312 assay, so that reproducible results were in general only obtained for

TABLE 2. *β-Lactamase activity of mutants with altered substrate specificities*

Host strain	R factor	β-Lactamase activity[a] (U/mg [dry wt])		Substrate profile[b]				
		Iodometric	Microiodometric	Amp	Cer	Carb	Meth	Clox
UB1301	RP1	550	—	113	113	11	—	—
	R1	81	75	125	126	9	0	2
	R1 *bla-40*	8	2.9	40	<50	—	—	—
	R1 *bla-45*	30	2.7	225	35	11	1	1
D21a12	None	<0.01	—					
	RP1 *amp-1*	0.24	—	50	—	—	—	—
	RP1 *bla-6*	0.07	—	125	—	—	—	—
	RP1 *amp-1 irp-1*	0.03	—	100	—	—	—	—

[a] Substrate (penicillin G) concentrations were 7 and 0.1 mM for the iodometric and microiodometric assays, respectively. The microiodometric assay was a modification of the method described by Novick (24). —, Not tested.

[b] Enzyme activities determined by the iodometric assay (substrate concentration, 6 to 7 mM) were expressed as a percentage of the activity when penicillin G was used as substrate. Amp, Ampicillin; Cer, cephaloridine; Carb, carbenicillin; Meth, methicillin; Clox, cloxacillin; —, not tested.

class III mutants. The results obtained iodometrically and by the 87/312 assay are compared graphically in Fig. 1. For several mutants (in particular *bla-36*, *-38*, *-39*, *-47*, and *-51*) the β-lactamase substrate specificity was indistinguishable from that of the R1 enzyme. However, in a number of cases the ratio of activities for the two substrates differed slightly from that of the R1 β-lactamase (*bla-11*, *-18*, *-34*, *-44*, and *-46*), and in two cases the altered substrate specificity was unambiguous (*bla-40* and *-45*). To confirm that the β-lactamases encoded by these two mutants had altered substrate profiles, conventional iodometric assays were carried out with a range of substrates. The substrate profiles obtained were strikingly different both from that of the wild-type R1 β-lactamase and from each other (Table 2). Furthermore, both enzymes had a reduced affinity for penicillin G (Table 2; compare results obtained with the iodometric and microiodometric assays; see also the accompanying paper (6)). Since both *bla-40* and *bla-45* reverted spontaneously (Table 1) and are presumably point mutations, it is apparent that at least two amino acid alterations within the β-lactamase molecule can cause dramatic changes in the kinetic characteristics of the enzyme.

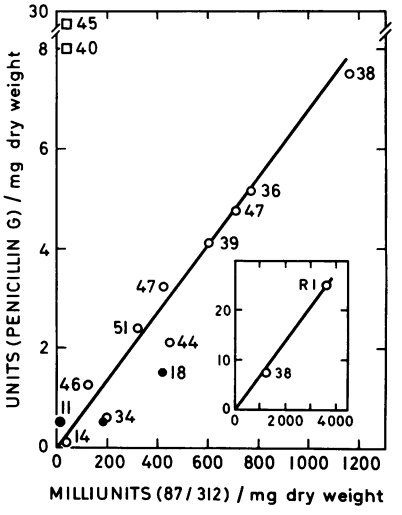

FIG. 1. *Comparison of β-lactamase activities of R⁺ strains by the iodometric and 87/312 assays. Enzyme activities are corrected for the activity of the chromosomal type Ib β-lactamase, where applicable, by subtraction of 0.2 U (for the iodometric assay) or 80 mU (for the 87/312 assay) per mg (dry weight). Note that enzyme activities determined with UB1325 as host strain are lower than those obtained with UB1301 (compare with Table 1). Host strain: ○, UB1325; □, UB1301; ●, D21a12. R1 mutants are designated by their bla allele number. Inset: One-tenth scale to show the wild-type R1 β-lactamase.*

Mapping experiments. Mapping experiments were performed to demonstrate that the class I phenotype can arise from a lesion within *bla*, the β-lactamase structural gene. (*bla* mutations could conceivably lie within a promoter or control region, and thus control the expression of more than one gene.) *bla*⁺ recombinants were formed between four point mutants of R1 Kan^s and either R1 *bla-40* or R1 *bla-45* (both of which are derived from R1 Chl^s) and scored for resistance to kanamycin and chloramphenicol (Table 3). The results indicate that, although *chl* is not sufficiently closely linked to *bla* to permit a segregation frequency analysis, such an analysis is possible for *kan* (see Materials and Methods). The allele order derived from the data using *bla-40* as the reference allele is *bla-20–40–(13,14,15)–kan*, whereas the order using *bla-45* is *(bla-20,13,14)–45–15–kan*. The position of *bla-15* between *bla-45* and *kan* is less certain than

TABLE 3. *Segregation patterns for Chl and Kan among bla⁺ recombinants*[a]

Cross	No. of bla⁺ recombinants tested	Segregation pattern (%)				Total (%)	
		Chl^r Kan^r	Chl^r Kan^s	Chl^s Kan^r	Chl^s Kan^s	Chl^r	Kan^r
bla-13 × *bla-40*	60	43	12	33	12	55	76
× *bla-45*	60	3	30	13	54	33	16
bla-14 × *bla-40*	114	38	7	53	2	45	91
× *bla-45*	39	5	49	18	28	54	23
bla-15 × *bla-40*	116	31	17	52	0	48	83
× *bla-45*	39	18	33	44	5	51	62
bla-20 × *bla-40*	47	2	62	11	25	64	13
× *bla-45*	60	0	45	13	42	45	13

[a] Analysis of these data was carried out as described in Materials and Methods.

the positions of the other alleles since only 62% of the *bla*⁺ recombinants were Kan^r (Table 3). However, an analysis of the actual segregation frequency data for the least common class of recombinants (in this case, Kan^s Chl^s *bla*⁺) and comparison with the equivalent data for the other crosses support the allocation made. The relative order of the various alleles is therefore *bla-20–40–(13,14)–45–15–kan*. *bla-13* and *-14* must therefore be point mutations within the β-lactamase structural gene, which is defined by *bla-40* and *bla-45* (see above; note that this analysis says nothing about *bla-15* and *-20*, though the likelihood is that these are also structural gene mutations). It should be noted that these conclusions are not invalidated by the known circularity of the R1 genetic map; the tight linkage between *bla* and *kan* refers only to the shortest distance between them.

Revertants of R1 *bla* mutants conferring very high levels of penicillin resistance. Several R1 *bla* mutants reverted spontaneously to derivatives with a penicillin resistance phenotype different from that of R1. One such revertant, isolated from the class I mutant R1 *bla-22*, was unstable. Plasmid DNA extracted from a strain carrying this revertant was used to transform JC7620. When the transformed plasmid, designated pUB251, was transferred by conjugation to UB1301, 4% of the exconjugants were found to have lost the Chl^r Str^r markers characteristic of pUB251 (Kan^r is absent since R1 *bla-22* was isolated from R1 Kan^s). One such Chl^s Str^s segregant was pUB252. Both pUB251 and pUB252 determined high levels of penicillin resistance and β-lactamase activity that were more characteristic of RP1 than R1 (Table 4). The high β-lactamase activity may be a gene dosage effect, since pUB251 also specifies high levels of chloramphenicol acetyltransferase and streptomycin adenylylsynthetase, the enzymes encoded by *chl* and *str* respectively (W. V. Shaw, unpublished data). Gene dosage effects for *bla*, *chl*, and *str* have previously been reported for other multicopy mutants derived from R1 (36).

TABLE 4. *Properties of superresistant mutants of R1*

R factor[a]	β-Lactamase activity[b] (U/mg [dry wt])	Single cell resistance (μg/ml)[c]	
		PenG	Carb
R1	81	250	2,000
RP1	830	>1,000	≥10,000
pUB251	510	>1,000	10,000
pUB252	1,390	>1,000	≥10,000
pUB254	8.6	700	4,000

[a] Host strain was UB1301.

[b] Iodometric assay with penicillin G (PenG) as substrate.

[c] See Table 1, footnote *c*.

A totally different phenotype was found for pUB254, a derivative of R1 *bla-50* isolated by two steps of selection on penicillin agar. This plasmid specified a high level of penicillin resistance (twice as high as R1) but less than 10% the β-lactamase activity of R1 (Table 4). This unusual resistance phenotype is entirely dependent upon the activity of the β-lactamase, since cells growing in the presence of 0.4 mg of methicillin per ml (an inhibitor of the type IIIa β-lactamase [26]) were no more resistant to penicillin G than R$^-$ cells (data not shown).

DISCUSSION

The structural gene for the most widespread R plasmid-specified β-lactamase, the type IIIa (TEM) enzyme, is located on a transposon, Tn*A*, that is shared by many otherwise unrelated R plasmids (18, 22). The ability of these R plasmids to confer penicillin resistance upon their host bacteria is generally considered to be due to the action of this β-lactamase. The generality of this view has, however, been challenged (7): it has been proposed that the R plasmid RP1 carries an *irp* locus specifying so-called intrinsic (i.e., non-β-lactamase) resistance to penicillins, in addition to the *bla* gene that encodes the type IIIa β-lactamase. The functional interaction of *irp* and *bla* is unclear; however, it has been pointed

out that the existence of *irp* could account for the very high level of carbenicillin resistance specified by RP1 (considerably greater than the level of resistance to penicillin G), which is otherwise difficult to reconcile with the poor rate of carbenicillin hydrolysis (10% that of penicillin G) afforded by the type IIIa β-lactamase (7).

The same authors have reported that another Tn*A*-carrying R plasmid, R1, does not have an *irp* locus (7). Yet R1 also specifies a high level of resistance to carbenicillin, relative to penicillin G (though the absolute levels are lower than for RP1; Table 1). Could R1, then, have an *irp* gene after all?

The evidence in this paper clearly rules out this possibility. We have isolated several R1 point mutants that neither determine penicillin resistance nor specify any detectable β-lactamase activity (class I mutants; Table 1). These mutants are identical to R1 *amp-2*, the mutant isolated by Curtis and Richmond (7). It is important to show that the class I phenotype is not due to, for example, a promoter mutation that prevents the expression of both *bla* and *irp* (promoters shared by two operons have been described for the *argECBH* region [3]). Our data exclude this possibility, since at least one class I mutant, *bla-13*, maps within the β-lactamase structural gene (Table 3). Since it is known from DNA sequencing studies that this gene terminates adjacent to one of the terminal inverted repeats of Tn*A* (35), *bla-13* cannot be having a polar effect on a hypothetical *irp* gene located on the same operon and distal to *bla*.

The conclusion that R1-mediated penicillin resistance is entirely attributable to the type IIIa β-lactamase is supported by the fact that in no case did any R1 mutant specify penicillin resistance in the absence of β-lactamase activity (Table 1). On the contrary, there was a general correlation between the level of penicillin resistance and the specific activity of the β-lactamase. In cases where the correlation was not exact (for example, compare R1 *bla-46* and *bla-44* in Table 1), it is likely that the kinetic characteristics of at least one of the β-lactamases are altered. By comparing two different assays, we found firm evidence for an altered substrate specificity in two cases (*bla-40* and *bla-45*; Fig. 1) and suggestive evidence in several other cases (for example, the *bla-34* enzyme is over three times as active against cephalosporin 87/312 as the *bla-46* β-lactamase, relative to the activities of these enzymes against penicillin G). Some mutants determined the same resistance to one penicillin but conferred very different levels of resistance against another penicillin (Table 1; for example, *bla-44* and *bla-45*). Our interpretation in these cases is the same: one or both mutants encode an altered β-lactamase.

The two RP1 mutants, RP1 *amp-1* and RP1 *bla-6*, have phenotypes that are intermediate between those of the class II and III R1 mutants (Table 1). Specifically, both mutants specify the synthesis of a significant level of β-lactamase activity (well above that characteristic of R^- cells). This result contrasts with the original report that RP1 *amp-1* was β-lactamase negative (8). Our data were obtained with the same assay and the same host strain as were originally used, and the resistance phenotype of RP1 *amp-1* (Table 1) was not significantly different from that originally reported (7, 8). We believe that the low β-lactamase activity of RP1 *amp-1* was overlooked in this original description; in our experience, reproducible results with such low levels of activity are difficult to obtain without a stable, sensitive spectrophotometer.

Since RP1 *amp-1* is not β-lactamase negative, it is no longer certain that its penicillin resistance phenotype is due to the *irp* locus. An equally acceptable interpretation would be that, by analogy with the class II and III mutants of R1 discussed above, this phenotype is due to an altered β-lactamase present in cells at low specific activity, and the substrate specificity of the RP1 *amp-1* β-lactamase is indeed altered (Table 2). The properties of pUB254, an R1 derivative able to confer higher levels of penicillin resistance than R1, but with less than 10% of the enzyme activity (Table 4), support the idea that mutants of the type IIIa (TEM) β-lactamase can arise in which enzymes of low specific activity confer relatively high levels of penicillin resistance. (A quantitative analysis of the relationship between penicillin resistance and the properties of the R plasmid β-lactamase is described in the accompanying paper [6]). An intermediate situation is also possible, in which both *bla* and *irp* contribute to the penicillin resistance phenotype of RP1 *amp-1*.

The present data do not distinguish between these alternatives. The strongest evidence that *irp* is responsible for the penicillin resistance mediated by RP1 *amp-1* is that the *irp-1* mutation brings about the loss of this resistance, together with the loss of the other *irp* properties (low level resistance to rifampin, lysozyme, and certain other non-β-lactam antibiotics) characteristic of RP1 and RP1 *amp-1* (7). However, the *irp-1* mutation also leads to the loss of the residual β-lactamase activity of RP1 *amp-1* (Table 2). It may therefore be a multisite mutation simultaneously affecting *bla* and *irp*, which would be consistent with the fact that it was isolated by using nitrosoguanidine, a mutagen known to cause closely linked multiple mu-

tations (11). Although *irp* has not been mapped, there is evidence suggesting that it may be closely linked to Tn*A*. This comes from the properties of an RP1 deletion mutant, pUB307, which is entirely unable to confer penicillin resistance, and in which only Tn*A* DNA (including about half of the *bla* gene) and DNA adjacent to Tn*A* (at the opposite end from that at which *bla* is located) have been deleted (1). Assuming that the inability of pUB307 to confer penicillin resistance is due to mutations in both *bla* and *irp*, these properties suggest that *irp* maps adjacent to Tn*A*, in the region deleted from pUB307. It cannot lie within Tn*A*, which has recently been sequenced and which encodes no proteins other than the type IIIa β-lactamase that are not involved in transposition (16). It is of interest that the aeruginocin resistance determinant (Aer) of RP1 is also absent from pUB307 (1, 34). This suggests that *aer* is also located adjacent to Tn*A* and that it could be identical with *irp*.

Regardless of whether *irp* contributes to the penicillin resistance phenotype of RP1 *amp-1*, its contribution to the penicillin resistance mediated by RP1 is probably negligible. This conclusion is drawn from a comparison of the properties of pUB251, pUB252, and RP1: the former two plasmids are derivatives of R1 whose penicillin resistance phenotypes are indistinguishable from that of RP1 (Table 4). Further support is provided by the model presented in the accompanying paper (6), in which the differences in penicillin resistance mediated by R1 and RP1 are quantitatively accounted for solely in terms of the properties of the R-factor β-lactamase.

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