Underexpression of Ap from R-Plasmids in Fast-Growing *Rhizobium* Species

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The presence of the plasmid RP1 in the cells of *Rhizobium leguminosarum* strains Rld1, 300, and 248, *R. phaseoli* 1233, *R. trifolii* strains T1 and 6661, and *R. meliloti* 4013 was found to appreciably increase bacterial resistance toward kanamycin and tetracycline but not toward ampicillin. The presence of 16 other R-plasmids in *R. leguminosarum* was also found to either not increase or only marginally increase bacterial resistance toward ampicillin. It appears now that underexpression of the plasmid-specified ampicillin function is common to most fast- and slow-growing rhizobia.

It has previously been shown that the presence of plasmid pRP1, pRP4, or pR68.45 confers resistance in certain *Rhizobium leguminosarum*, *R. japonicum*, and cowpea *Rhizobium* strains to kanamycin and tetracycline, but not to penicillins such as ampicillin and carbenicillin (2, 17, 19, 20, 22). Here we investigate whether diminished expression of Ap from these R-plasmids is a property common to all fast-growing *Rhizobium* species and whether fast-growing rhizobia such as *R. leguminosarum* are impaired in the expression of Ap specified by R-plasmids of various incompatibility groups and origins. The bacterial strains employed are listed in Table 1, and the plasmids used are listed in Table 2.

present in any of the seven fast-growing strains of the *Rhizobium* species employed. As expected, the pRP1-specified Ap was expressed highly in *Escherichia coli* K-12 ED832. Also, the Km expression was observed in all of the strains of *Rhizobium* and in *E. coli* ED832. The presence of pRP1 increased the MIC of tetracycline in *E. coli* ED832 and six *Rhizobium* strains but not in *R. trifolii* T1. These results show that *R. trifolii*, *R. phaseoli*, and *R. meliloti* are similar to *R. leguminosarum* in their failure to express the Ap^r function coded by the plasmid pRP1.

Fourteen plasmids, in addition to pRP1, pRP4, and pR68.45, known to belong to seven distinct incompatibility

TABLE 1. Bacterial strains used

Designation	Properties	Source or reference
<i>Escherichia coli</i> K-12 ^a		
ED832	<i>arg his nalA thy ilv</i>	18
JC355	<i>arg his leu met</i>	18
<i>Rhizobium leguminosarum</i> ^b		
Rld1	Wild type; <i>ts</i> Tm ^r Sm ^r azide ^s ; bacteroides unbranched	21
Rld164	<i>trp</i> Sm ^s azide ^r of Rld1	23
248	Wild type; <i>ts</i> Tm ^r Sm ^r Nal ^r ; some bacteroides branched	J. E. Beringer
300	Wild type; <i>ts</i> Tm ^r Sm ^r ; bacteroides often branched	J. E. Beringer
<i>R. trifolii</i>		
T1	Wild type; <i>ts</i>	E. A. Schwinghamer
6661	Wild type; <i>ts</i> Tm ^r Sm ^r Nal ^r	J. E. Beringer
<i>R. phaseoli</i> 1233	Wild type; <i>ts</i> Tm ^r Sm ^r Nal ^r	J. E. Beringer
<i>R. meliloti</i> 4013	Wild type; Tm ^r Sm ^r Nal ^r	L. Kumari

^a *E. coli* cultures were grown on tryptone broth (tryptone [Difco Laboratories, Detroit, Mich.], 10 g; NaCl, 5 g; water, 1 liter). Minimal medium was made with Davis-Mingioli salts (9). The media were solidified by using 1.5% agar.

^b The complete media for *Rhizobium* species were GSY (K₂HPO₄, 2 g; KH₂PO₄, 2 g; yeast extract, 1 g; glucose, 1.5 g; MgSO₄, 0.1 g; CaCl₂, 0.01 g; water, 1 liter) and TY (tryptone, 5 g; yeast extract, 3 g; CaCl₂, 1.3 g; water 1 liter). The minimal medium used for growing *Rhizobium* cultures consisted of Na₂HPO₄ (2 g), (NH₄)₂SO₄ (2 g), glucose (2 g), FeCl₃ (0.002 g), MgSO₄ (0.1 g), and CaCl₂ (0.01 g) in 1 liter of water.

Table 3 gives the MICs of ampicillin, kanamycin, and tetracycline observed for the pRP1 carrier and noncarrier strains of the four fast-growing *Rhizobium* species. Comparison of the MICs of ampicillin for the various strains showed that little, if any, Ap expression occurred from pRP1 when

groups were transferred individually to the strains *R. leguminosarum* Rld164 and *E. coli* ED832. The MICs of ampicillin, kanamycin, and tetracycline determined for the controls and plasmid carriers are given in Table 4. A large amount of variation was observed in the ampicillin resistance among *E. coli* ED832 bacteria carrying various plasmids. The presence of plasmids in *R. leguminosarum* Rld164 did not increase or

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TABLE 2. Plasmids used and their properties

Plasmid	Properties				Bacterium from which originally isolated	Reference	<i>E. coli</i> K-12 carrier	
	In-compatibility group	Drug resistances specified	Type of β -lactamase associated with Ap	Location of Ap			Properties	Source
pR48	N	Amp Tet Smr	TEM 1	TnA	<i>Salmonella typhimurium</i>	10	J 53-1 <i>pro met nal</i>	N. Datta
pVS105	N	Amp Tet Smr			<i>E. coli</i>	4	UB 1301 <i>thi</i>	S. D. Caudry
pR390	N	Amp Tet Smr			<i>Proteus rettgeri</i>	5	J 53 <i>pro met</i>	N. Datta
pR396	N	Amp Tet Smr			<i>P. rettgeri</i>	5	J 62-1 <i>pro his trp nal</i>	N. Datta
pR412	W	Amp Tet Smr	TEM 1	TnA	<i>Klebsiella</i> sp.	6	J 53	N. Datta
pR448	N	Amp Tet Smr			<i>Proteus morgani</i>	13	J 62-1	N. Datta
pR813	F	Amp Tet Smr	TEM 1	TnA	<i>Salmonella typhi</i>	8	J 53	N. Datta
pRP1	P	Amp Tet Kan	TEM 2	TnA	<i>Pseudomonas aeruginosa</i>	24	AB 2531 <i>arg his ilv thy</i>	This laboratory
pRP4	P	Amp Kan Tet	TEM 2	TnA	<i>P. aeruginosa</i>	11	J 53	N. Datta
pR68.45	P	Amp Kan Tet	TEM 1	TnA	<i>P. aeruginosa</i>	12	AB 2531	This laboratory
pR826	S	Amp Kan Tet Smr			<i>Serratia marcescens</i>	14	J 53	G. Jacob
pR828	S	Amp Kan Tet Smr	TEM 1	TnA	<i>S. marcescens</i>	14	J 53-2 <i>pro met rif</i>	E. A. Lederberg
pR839	P	Amp Kan Tet	TEM 1	TnA	<i>S. marcescens</i>	14	J 53-1	N. Datta
pR938	P	Amp Kan Tet Smr Cm	TEM 1	TnA	<i>S. marcescens</i>	16	J 53-1	N. Datta
pIP69	M	Amp Kan Tet	TEM 1	TnA	<i>Salmonella paratyphi</i>	25	C 600 <i>thr leu thi</i>	Y. A. Chabbert
pR936	C	Amp Kan Tet	PSE 4	TnA	<i>S. marcescens</i>	7	J 53	N. Datta
pMG19		Amp Kan Tet			<i>P. aeruginosa</i>	3	E5-2 <i>trp rif</i>	E. A. Lederberg

only marginally increased the resistance of the bacteria toward ampicillin. Although a total of 17 plasmids increased MICs for ampicillin by a factor of greater than 50 and 14 of these plasmids increased MICs for ampicillin by a factor of greater than 100 in *E. coli* ED832, only 4 did so by a factor of greater than 5 in *R. leguminosarum* Rld164. In the latter organism, an approximately ninefold increase was the maximum increment obtained in the MIC of ampicillin. In both *E. coli* ED832 and *R. leguminosarum* Rld164 bacteria, a significant Km and Tc expression was observed from the plasmids known to be carrying the respective determinants. These results indicated that the fast-growing *Rhizobium* species are perhaps genetically impaired in the expression of the Ap function specified by diverse R-plasmids.

In summary, it appears that the strains of the slow-growing (17, 19) and fast-growing *Rhizobium* species that have been examined thus far are all similar in not being able to acquire a high level of resistance towards ampicillin by the presence of β -lactamase-coding (*bla*⁺) R-plasmids in them. We are unable to provide an explanation for this observation at this time. However, based on the knowledge already gained about the expression of *bla*⁺ and a variety of other genes in *E. coli*, three possibilities can be pointed out. One explanation might concern underproduction of β -lactamase enzyme in rhizobia due to the unavailability of some factor(s) required for transcription initiation from *bla*⁺ genes, *bla*⁺ transcript elongation, or *bla*⁺ transcript translation. Although no such factor has yet been identified for the synthesis of β -lactamase in *E. coli*, the involvement of such factors has not been ruled out. The second explanation might concern the stability of β -lactamase in rhizobia. It is possible that β -lactamase synthesized in rhizobia might turn over rapidly and not accumulate to the normal levels required for the expression of a high degree of ampicillin resistance. Third, the posttranslational processing and transport of β -lactamase might be impaired in rhizobia. Another topic that future works should address is the advantages to rhizobia that fail to acquire high levels of ampicillin resistance.

TABLE 3. MICs of ampicillin, kanamycin, and tetracycline for members of four *Rhizobium* species and *E. coli* ED832 and their plasmid pRP1 carriers^a

Bacterial host	Plasmid pRP1	MIC (μ g/ml) of:		
		Ampicillin	Kanamycin	Tetracycline
<i>R. leguminosarum</i> Rld1	—	20	0.5	0.1
	+	35	9.0	4.5
<i>R. leguminosarum</i> 300	—	20	1.3	0.3
	+	30	50.0	5.0
<i>R. leguminosarum</i> 248	—	13	1.3	0.3
	+	20	12.5	3.8
<i>R. phaseoli</i> 1233	—	13	2.5	0.3
	+	20	37.5	12.5
<i>R. trifolii</i> T1	—	13	2.5	3.8
	+	25	27.5	3.8
<i>R. trifolii</i> 6661	—	50	2.5	0.3
	+	50	37.5	3.0
<i>R. meliloti</i> 4013	—	50	12.5	0.3
	+	50	50.0	5.0
<i>E. coli</i> K-12 ED832	—	15	0.2	1.2
	+	5,000	20.0	39.5

^a MICs were determined by the disk diffusion method (1). Each antibiotic was serially diluted. For each dilution, 5 μ l was pipetted into a 5.5-mm disk made from Whatman filter paper. The antibiotic-containing disks were dried at room temperature. The cells in 0.1 ml of stationary bacterial culture were spread on the surface of solid complete medium in a petri dish, and nine disks were placed. The medium used for *E. coli* was TA (tryptone broth plus agar) and that for *Rhizobium* species was GSY (see Table 1, footnote b). The *Rhizobium* assay plates were incubated at 30°C for ca. 4 days, and the *E. coli* plates were incubated for 2 days at 37°C. The diameter of the zone of bacterial growth inhibition was measured in millimeters. The MICs were computed from the plots of the drug concentration in micrograms versus the diameter of inhibition zone.

TABLE 4. MICs of ampicillin, kanamycin, and tetracycline for *E. coli* ED832 and *R. leguminosarum* Rld164 and their derivatives carrying various plasmids

Plasmid transferred ^a	Ampicillin MIC (μ g/ml) for:		Kanamycin MIC (μ g/ml) for:		Tetracycline MIC (μ g/ml) for:	
	ED832	Rld164	ED832	Rld164	ED832	Rld164
None	15	9.0	0.2	0.33	1.3	0.03
pR48	100	17.5	NS ^b	NS	21.0	0.18
pIP69	360	12.5	275.0	12.5	34.0	3.25
pR396	400	22.0	NS	NS	6.0	0.29
pMG19	1,250	60.0	300.0	12.5	30.0	3.90
pR448	1,400	17.5	NS	NS	5.0	2.90
pR813	1,400	13.0	NS	NS	65.0	0.20
pVS105	1,675	11.0	NS	NS	50.0	2.00
pR390	1,825	13.5	NS	NS	5.5	0.30
pR839	2,075	17.0	30.0	12.5	67.5	4.40
pR412	2,250	9.0	NS	NS	85.0	0.26
pRP4	2,500 ^c	13.5	22.5	9.0	37.5	4.00
pR68.45	2,500 ^c	26.0	65.0	7.5	29.0	4.00
pR826	2,500 ^c	40.0	100.0	3.3	32.5	3.10
pR828	2,500 ^c	80.0	250.0	13.0	45.0	4.25
pR938	2,500 ^c	34.0	10.0	9.0	45.0	3.75
pR936	4,750	55.0	9.0	15.0	37.5	4.10
pRP1	5,000	16.0	20.0	9.0	39.5	3.25

^a Plasmid transfers were done by conjugation on solid tryptone-yeast medium (15). All plasmids were first transferred to *E. coli* ED832. These strains then served as donors of plasmids for transfer to *R. leguminosarum* Rld164 and other *Rhizobium* strains. The presence of plasmids in *E. coli* ED832 was verified by re-transfer to *E. coli* JC355, and that in *Rhizobium* carriers was verified by transfer to *E. coli* ED832 and by comparing the drug resistance patterns.

^b NS, Antibiotic resistance not specified.

^c MIC, $\geq 2,500$.

LITERATURE CITED

- Bauer, A. W., W. M. Kirby, J. C. Sherris, and M. Turk. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol.* **45**:493-496.
- Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **84**:188-198.
- Bryan, L. E., M. S. Shahrabadi, and H. M. van den Elzen. 1974. Gentamicin resistance in *Pseudomonas aeruginosa*: R-factor-mediated resistance. *Antimicrob. Agents Chemother.* **6**:191-199.
- Caudry, S. D., and V. A. Stanisich. 1979. Incidence of antibiotic-resistant *Escherichia coli* associated with frozen chicken carcasses and characterization of conjugative R plasmids derived from such strains. *Antimicrob. Agents Chemother.* **16**:701-709.
- Coetzee, J. N., N. Datta, and R. W. Hedges. 1972. R factors from *Proteus rettgeri*. *J. Gen. Microbiol.* **72**:543-552.
- Datta, N., and R. W. Hedges. 1972. R factors identified in Paris. Some conferring gentamicin resistance constitute a new compatibility group. *Ann. Inst. Pasteur* **123**:849-852.
- Datta, N., R. W. Hedges, D. Becker, and J. Davies. 1974. Plasmid determined fusidic acid resistance in the Enterobacteriaceae. *J. Gen. Microbiol.* **83**:191-196.
- Datta, N., and J. Olarte. 1974. R factors in strains of *Salmonella typhi* and *Shigella dysenteriae* 1 isolated during epidemics in Mexico: classification by compatibility. *Antimicrob. Agents Chemother.* **5**:310-317.
- Davis, B. D., and E. S. Mingioli. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B₁₂. *J. Bacteriol.* **60**:17-28.
- Drabble, W. T., and B. A. D. Stocker. 1968. R factors in *Salmonella typhimurium*: pattern of transduction by phage P22 and UV protection effect. *J. Gen. Microbiol.* **53**:109-123.
- Falcow, S., and L. S. Baron. 1962. Episomic element in a strain of *Salmonella typhosa*. *J. Bacteriol.* **84**:581-589.
- Hass, D., and B. W. Holloway. 1976. R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. *Mol. Gen. Genet.* **144**:234-251.
- Hedges, R. W., N. Datta, J. N. Coetzee, and S. Dennison. 1973. R factors from *Proteus morganii*. *J. Gen. Microbiol.* **77**:249-259.
- Hedges, R. W., A. E. Jacob, N. Datta, and J. N. Coetzee. 1975. Properties of plasmids produced by recombination between R factors of group J and F II. *Mol. Gen. Genet.* **140**:289-302.
- Hopwood, D. A., R. J. Harold, A. Vivian, and H. M. Ferguson. 1969. A new kind of fertility variant in *Streptomyces coelicolor*. *Genetics* **62**:461-477.
- Jacob, A. E., J. A. Shapiro, D. I. Smith, S. N. Cohen, and D. Berg. 1977. Plasmids studied in *Escherichia coli* and other enteric bacteria, p. 607-704. In A. I. Bukhari, J. A. Shapiro and S. L. Adhya (ed.), *DNA insertion elements, plasmids and episomes*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Kennedy, C., B. Dreyfus, and J. Brockwell. 1981. Transfer, maintenance and expression of P plasmids in strains of cowpea *Rhizobia*. *J. Gen. Microbiol.* **125**:233-240.
- Kumar, S., and S. Srivastava. 1983. Cyclic AMP and its receptor protein are required for expression of transfer genes of conjugative plasmid F in *Escherichia coli*. *Mol. Gen. Genet.* **190**:27-34.
- Kuykendall, L. D. 1979. Transfer of R factors to and between genetically marked sublines of *Rhizobium japonicum*. *Appl. Environ. Microbiol.* **37**:862-866.
- Nagahari, K., T. Koshikawa, and K. Sakaguchi. 1979. Expression of *Escherichia coli* tryptophan operon in *Rhizobium leguminosarum*. *Mol. Gen. Genet.* **171**:115-119.
- Ram, J., R. P. Grover, R. B. Riwar, and S. Kumar. 1978. Improvement in the nitrogen fixing effectiveness of *Rhizobium leguminosarum* by incorporating genetic resistance to azide. *Indian J. Exp. Biol.* **16**:1321-1322.
- Ram, J., and S. Kumar. 1979. Host dependence of RP1-specified resistance to ampicillin: differential expression in *Escherichia coli* and *Rhizobium leguminosarum*. *Gene* **7**:349-353.
- Singh, A., J. Ram, V. K. Sikka, and S. Kumar. 1984. Derivation of multiply marked strains in *Rhizobium leguminosarum* Rld 1 by nitrosoguanidine and transposon mutagenesis. *Indian J. Exp. Biol.* **22**:239-247.
- Stanisich, V. A., and B. W. Holloway. 1971. Chromosome transfer in *Pseudomonas aeruginosa* mediated by R factors. *Genet. Res.* **17**:169-172.
- Witchitz, J. L., and G. R. Gerbaud. 1972. Classification des plasmides conferant la resistance a la gentamicine. *Ann. Inst. Pasteur* **123**:333-337.