Transmissible Resistance to Penicillin G, Neomycin, and Chloramphenicol in *Rhizobium japonicum*¹

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The genetic basis for resistance to a number of antibiotics was examined in *Rhizobium japonicum*. Resistance to penicillin G, neomycin, and chloramphenicol appears to be mediated by an extrachromosomal element similar to that found in the *Enterobacteriaceae*. Resistance to these antibiotics was eliminated from cells by treatment with acridine orange, and resistance to all three antibiotics could be transferred en bloc to *Agrobacterium tumefaciens* under conditions excluding transformation or transduction as possible genetic mechanisms.

Work in this laboratory earlier suggested that the resistance of *Rhizobium japonicum* to a number of antibiotics was mediated by plasmid-borne genes. This suggestion was based upon similarities in the properties of R factor-mediated resistance in enteric bacteria (1, 14) and the properties of antibiotic resistance in *R. japonicum*. These similarities were (i) the simultaneous resistance of a large number of *R. japonicum* strains to tetracycline, penicillin G (PC), neomycin (NM), and chloramphenicol (CM); (ii) the marked inoculum-dependent resistance to PC, NM, and CM, and enzymatic inactivation of these antibiotics; (iii) the elimination by acridine orange of PC, NM, and CM resistance in a high percentage of acridine-treated isolates, and (iv) the comparatively high levels of PC, NM, and CM resistance, similar to levels of resistance of enteric bacteria carrying R factors but generally lower than resistance conferred by chromosomal resistance genes. In this paper, further evidence of the plasmid nature of resistance to PC, NM, and CM, and of the transmissible nature of these resistances, is presented.

MATERIALS AND METHODS

Organisms. *R. japonicum* strains 71 and 10324 were obtained from the U.S. Department of Agricul-

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ture, Beltsville, Md., and the American Type Culture Collection (ATCC), Rockville, Md., respectively. *R. trifolii* strains C1226 and 10140 were obtained from Gösta Fåhræus, Uppsala, Sweden, and the ATCC, respectively. *Agrobacterium tumefaciens* strain P was a gift of the Department of Plant Pathology, North Carolina State University, and *A. tumefaciens* strain 23308 was obtained from the ATCC. Stock cultures were maintained on yeast extract-mannitol slants (5) stored at 4 C.

Media. A basal solution (HM) consisted of (grams per liter): Na₂HPO₄, 0.125; Na₂SO₄, 0.25; NH₄Cl, 0.32; MgSO₄·7H₂O, 0.18; FeCl₃, 0.004; CaCl₂·2H₂O, 0.013; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Calbiochem, Los Angeles, Calif.), 1.3; and 2-(N-morpholino)ethane sulfonic acid (Calbiochem), 1.1. The pH was adjusted to 6.6 with 5 N NaOH prior to autoclaving. To this solution, 0.1 ml of Tween-40 (Sigma Chemical Co., St. Louis, Mo.) was added as a diluent when single-cell suspensions were required.

Glycerol-bromothymol blue-tetrazolium agar (GBT) consisted of HM-salts supplemented with 0.5% glycerol, 0.025% yeast extract, 0.018% bromothymol blue, and 0.01% (wt/vol) triphenyl tetrazolium chloride (TTC; Sigma Chemical Co.). Sterile TTC solutions were added to presterilized media as described by Kelman (8).

Phenol red-gluconate agar (PRG) consisted of phenol red broth base (Difco) to which was added 0.5% potassium gluconate.

HM solution was supplemented with 0.5% manitol, 0.025% yeast extract, and 0.05% L-arabinose to prepare YEM-HM medium. Solid media were prepared by the addition of 1.5% Special Agar, Noble (Difco).

Culture conditions. Unless otherwise indicated, organisms were grown at 32 C in tubes (18 by 150 mm)

containing 5 ml of medium or on the surface of plates; the tubes were incubated in a controlled-temperature shaker (New Brunswick Scientific Co., New Brunswick, N.J.).

Acridine treatment. The effect of acridine treatment upon antibiotic resistance was assessed by plating approximately 100 cells of *R. japonicum* 71 on YEM-HM plates, pH 7.4, containing either 50 μ g of acridine orange/ml (Fisher Scientific Co., Fair Lawn, N.J.) or no acridine. When the colonies were about 1 mm in diameter, they were replica-plated onto YEM-HM plates containing 250 μ g of CM/ml, 150 units of PC/ml, and 100 μ g of NM/ml, or no antibiotic. These concentrations are less than or equal to one-half the minimal inhibitory concentration (MIC) for strain 71. The MIC is defined as the lowest concentration of an antibiotic which completely inhibits the growth of an organism. The replica plates were incubated until colonies appeared on antibiotic-free plates, and the colony counts of the different media were obtained.

Determination of heavy metal resistance. The general procedure of Novick and Roth (10) was used to determine the response of organisms to heavy metal salts. Paper disks containing 1 to 10 μ liters of heavy metal solution were placed on spread plates of YEM-HM, and the plates were incubated until growth was obvious. The MIC was defined as the smallest amount of heavy metal salt (in micrograms) which inhibited growth adjacent to the disk. The inhibitory effect of the following salts was examined: zinc nitrate, 10^{-1} M; sodium arsenite, 10^{-1} M; sodium arsenate, 10^{-1} M; lead nitrate, 10^{-1} M; cadmium nitrate, 10^{-3} M; and mercuric nitrate, 10^{-3} M.

Transfer of antibiotic resistance. Transfer of PC or CM resistance from *R. japonicum* to *A. tumefaciens* was effected by mixing approximately 10^8 organisms of donor and recipient cells per ml in 5 ml of YEM-HM. After 48 h of incubation without shaking, samples were diluted and plated on selective media. In the preparation of these mixtures, 24-h YEM-HM broth cultures of *A. tumefaciens* and 48-h YEM-HM broth cultures of *R. japonicum* were used. Plates were scored for drug-resistant recipients after 72 h of incubation.

Two media were devised which were suitable for counterselection against *R. japonicum* and *A. tumefaciens*. The first of these (GBT agar) was made counterselective against *R. japonicum* by adding 20 μ g of azetidine carboxylic acid (ACA)/ml (Calbiochem) and made counterselective against *A. tumefaciens* by adding either 100 units of PC/ml or 20 μ g of CM/ml. It was observed that *R. japonicum* was unable to grow on phenol red broth base (Difco) containing 0.5% gluconate (PRG agar), whereas *A. tumefaciens* grew well. This difference was explored as the possible basis for another selective medium. PRG was a better counterselective medium against *R. japonicum* than GBT agar plus ACA because *R. japonicum* did not grow at all on PRG, even after prolonged incubation. This MIC for *A. tumefaciens* on PRG was 50 units of PC/ml and 10 μ g of CM/ml. PRG used in transfer experiments contained 100 units of PC/ml and 20 μ g of CM/ml.

RESULTS AND DISCUSSION

To determine which resistances were lost simultaneously, cells were plated on YEM-HM containing either 50 μ g of acridine orange/ml or no acridine. Approximately 500 colonies were examined with each treatment, and the results are given in Table 1. Selection for acridine-resistant cells was ruled out, as an explanation of the results, by the nearly identical viable counts on medium with or without acridine. The MIC of the susceptible isolates, determined by the gradient plate method (13), was found to be 10 to 50 times less than that of the original strain before acridine orange treatment.

R factors may also carry genes which make the organisms resistant to the salts of heavy metals (10-12). Three strains of *R. japonicum* and two strains of *A. tumefaciens* were examined for heavy metal resistance. As shown by the results summarized in Table 2, all organisms were sensitive to 1.5 μ g of mercuric nitrate and were resistant to 3 μ g of cadmium nitrate and 165 μ g of lead nitrate. No consistent response to zinc nitrate, sodium arsenite, or sodium arsenate was measured.

R factors may or may not be transmissible, depending upon presence or absence of a discrete element, the transfer factor, which serves as the mobilizing element for the plasmid (4). A series of experiments was designed to determine whether or not PC, CM, and NM resistance was transferable under conditions which excluded transformation or transduction as the mode of genetic exchange.

To study the transfer of genetic material between live cells, it is necessary to have a

TABLE 1. Loss of antibiotic resistance after growth of *Rhizobium japonicum* strain 71 on plates containing acridine orange (AO)

Antibiotic	No. of colonies rendered susceptible ^a		
	No AO	50 μ g of AO/ml	Difference in AO
Neomycin (NM)	40	79	39
Chloramphenicol (CM)	54	109	55
Penicillin G (PC)	34	139	105
NM + CM	10	39	29
PC + NM	10	47	37
PC + CM	10	73	63
PC + NM + CM	6	31	25

^aThe number of colonies examined with each treatment was 500. Viable counts on the media were 4.2×10^7 cells/ml (with AO) and 3.5×10^7 cells/ml (without AO).

TABLE 2. Levels of resistance to salts of heavy metals in *Rhizobium japonicum* and *Agrobacterium tumefaciens*

Organism	Minimal inhibitory concn ($\mu\text{g}/\text{disk}$) ^a					
	Zn(NO ₃) ₂	Hg(NO ₃) ₂	NaAsO ₂	Pb(NO ₃) ₂	Cd(NO ₃) ₂	NaAsO ₄
<i>R. japonicum</i>						
Strain 71	>300	0.6	1,300	165	>3	>310
Strain 122	30	0.6	130	165	>3	155
Strain 10324	150	1.5	130	165	3	155
<i>A. tumefaciens</i>						
Strain P	150	0.6	260	165	>3	>310
Strain 23308	60	0.6	130	165	>3	>310

^a Growth on yeast extract-mannitol-basal agar.

medium that is rigorously counterselective against both donor and recipient cells while permitting recombinants to grow. Ideally, this medium would also be differential, providing a clear contrast between donor and recipient organisms. Transfer of PC and CM resistance between strains of *R. japonicum* was attempted initially. However, no conclusive results were obtained because a medium could not be devised which was rigorously counterselective against both donor and recipient strains. All strains of *R. japonicum* exhibited similar reactions and colony size on all media tested and had similar levels of resistance to a variety of amino acid analogues. *A. tumefaciens* was chosen as an alternate recipient.

The ability of *A. tumefaciens* to produce 3-ketolactose from lactose was chosen as one unselected trait to verify that presumed recipients were indeed *A. tumefaciens* and not *R. japonicum*. The assay was performed exactly as described by Bernaerts and DeLey (3); both strains of *A. tumefaciens* gave positive tests and all strains of *R. japonicum* were uniformly negative.

Transfer of PC or CM resistance was attempted with two strains of *A. tumefaciens* and two strains of *R. trifolii* as potential recipients, and with two strains of *R. japonicum* as potential donors. The data in Table 3 demonstrate that both donor strains could transfer PC resistance to both recipient strains of *A. tumefaciens*, but no transfer was affected between the donor *R. japonicum* strains and the two *R. trifolii* strains. The apparent frequency of transfer of CM and PC resistance was influenced markedly by the composition of the counterselective medium and by the quantity of antibiotic in the medium. The frequency of transfer of CM resistance was 0.25 and 0.06%, respectively, when GBT agar plus ACA or PRG agar was used for counterselection. No drug-resistant recipient could be isolated if the amount of antibiotic in either medium exceeded twice the MIC for the recipient organism.

Watanabe (14) indicated that caution must be observed when plating mixtures of antibiotic-susceptible cells and resistant cells which produce antibiotic-inactivating enzymes, since the resistant cells inactivate enough antibiotic to permit growth of antibiotic-susceptible recipient cells. The following argument is advanced to exclude this possibility in the *R. japonicum* × *A. tumefaciens* crosses. First, the MIC for both *A. tumefaciens* and *R. trifolii* is 40 to 50 units/ml on GBT agar. If the PC concentration were reduced below these values as a result of inactivation, then both *A. tumefaciens* and *R. trifolii* colonies should have appeared on plates containing *R. japonicum* and the other organism. However, no *R. trifolii* colonies appeared

TABLE 3. Transfer of penicillin G resistance from *Rhizobium japonicum* to *Agrobacterium tumefaciens*^a

Donor strain ^b	Recipient strain	Frequency of transfer ^c (%)
71	None	0
71	<i>A. tumefaciens</i> P	0.25
71	<i>A. tumefaciens</i> 23308	— ^d
None	<i>A. tumefaciens</i> P	0
None	<i>A. tumefaciens</i> 23308	0
10324	None	0
10324	<i>A. tumefaciens</i> P	0.1
10324	<i>A. tumefaciens</i> 23308	0.1
71	<i>R. trifolii</i> C1226	0
71	<i>R. trifolii</i> 10140	0
10324	<i>R. trifolii</i> C1226	0
10324	<i>R. trifolii</i> 10140	0

^a Glycerol-bromothymol blue-tetrazolium agar + 20 μg of azetidine carboxylic acid/ml + 100 units of penicillin G/ml was used as the selective medium. Results were recorded 48 h after plating. Colonies over 1 mm in diameter which reduced tetrazolium chloride were scored as drug-resistant recipients.

^b *R. japonicum* was used as the donor in all crosses.

^c Frequency of transfer = number of drug-resistant recipients per number of recipient cells plated × 100.

^d Transfers present, but were not counted.

(Table 3), indicating that the PC concentration did not fall below the MIC. Second, if cells of the recipient culture survived, it is more probable that *R. trifolii* rather than *A. tumefaciens* would survive. Bacterial cells are more susceptible to PC during log phase. Under conditions where the PC concentration decreases with time because of the presence of penicillinase-producing cells, a PC-susceptible organism which begins growth sooner on PC-containing medium would be confronted with a higher PC concentration than one that initiates growth at a later time. On this basis, one would expect *R. trifolii* rather than *A. tumefaciens* to survive, because *A. tumefaciens*, as indicated by the time of colony appearance, initiates growth much sooner than does *R. trifolii* and thus would be subjected to a higher PC concentration. The fact that the colonies which appeared were *A. tumefaciens* and not *R. trifolii* indicates that PC inactivation does not explain the results obtained.

CM inactivation in mixtures is excluded by the fact that only cell-free extracts, but not whole cells, of *R. japonicum* are capable of inactivating CM (Cole, unpublished data). In addition, the CM concentration of medium containing *R. japonicum* remained constant up to 96 h of incubation at 32 C.

Direct evidence that inactivation could not explain the observed results was obtained by diluting separately the donor and recipient cells and plating 10^4 cells of each organism on the same plate. When this procedure was used, no plasmid transfers were found, demonstrating that mixed cultivation in broth was required to obtain transfer, and also that mixed cultivation on plates cannot explain the results obtained.

There was no increase in the number of plasmid transfers when donor cells were irradiated with ultraviolet light to 10% survivors, which further suggests that the determinants of PC and CM resistance are plasmids. If the PC and CM determinants were integrated into the bacterial chromosome, an increase in the frequency of transfer should have been observed when irradiated donor cells were used (15).

Unlike transformation, plasmid transfer usually results in the simultaneous transfer of several antibiotic resistances. Co-transfer of NM, CM, and PC resistance from *R. japonicum* 71 to *A. tumefaciens* P was demonstrated by selecting initially for CM-resistant recombinants on phenol red-gluconate agar plates containing 20 μ g of CM/ml. The identity of the recipients was verified by use of the tests in Table 4; in all cases, the results for the transfers were identical to those of *A. tumefaciens*. The

TABLE 4. Characteristics of donor and recipient strains

Characteristic	<i>R. japonicum</i>	<i>A. tumefaciens</i>
Genetic role	Donor	Recipient
Reactions on glycerol-bromo thymol blue-tetrazolium agar		
Tetrazolium chloride reduction	—	+
Acid reduction	—	+
Growth on phenol red-gluconate agar	—	+
Ketolactose production	—	+
Minimal inhibitory concentration ^a		
Neomycin	300	<25
Penicillin G	>500	50
Chloramphenicol	>300	12
Tetracycline	100	<10
Azetidone carboxylic acid	<10	>40
Time required for appearance of 1-mm diameter colony (days; GBT agar) . . .	6	1

^a Determination was made on GBT agar. Results are expressed in micrograms per milliliter, except for penicillin G, which is expressed in units per milliliter.

isolates were grown in YEM-HM broth to stationary phase, and the MIC of NM, CM, and PC for each isolate was determined by streaking a loopful of cells on a series of YEM-HM plates containing various concentrations of a single antibiotic (Table 5).

The conditions under which transfer was demonstrable, the frequency of transfer, and the simultaneous transfer of several antibiotic resistances strongly suggested that plasmid transfer was the mode of genetic exchange. However, it was necessary to eliminate both transformation and transduction as an explanation of the results. Two key criteria for distinguishing between plasmid transfer and transformation are the requirement for live donor cells for plasmid transfer and the complete inhibition of transformation by treatment of the donor material with deoxyribonuclease.

Formation of PC- or CM-resistant recipients required live donor cells, since a mixture of *R. japonicum* cells, killed by heating at 60 C for 30 min, and live *A. tumefaciens* cells did not yield plasmid transfers. Incubation of mixtures of donor and recipient cells with 10 μ g of deoxyribonuclease/ml did not inhibit transfer.

There was no inhibition or lysis of *R. japonicum* or *A. tumefaciens* when up to 10^8

TABLE 5. Levels and patterns of resistance to penicillin G, chloramphenicol, neomycin, and tetracycline in *A. tumefaciens* recombinants

Isolate	Genotype	Minimal inhibitory concn ^a			
		Penicillin G	Chloramphenicol	Neomycin	Tetracycline
Parent strain	R-	>40, <75	>10, <20	<25	<10
1104A, 1104C, 1104D	R (NM)	>40, <75	>10, <20	>80	<10
1104B	R (PC, NM)	>75, <150	>10, 20	>80	<10
1104E, 1104F	R (PC, CM, NM)	>150	>80	>80	<10

^a Minimal inhibitory concentrations are expressed in micrograms per milliliter, except for PC, which is expressed in units per milliliter.

cells of each organism were plated together. The absence of lysis and inhibition strongly suggests that neither organism harbors a prophage that is capable of parasitizing both *R. japonicum* and *A. tumefaciens*.

Consistent with points i through iv given in the introduction, a very strong circumstantial case for resistance of *R. japonicum* to PC, CM, and NM being mediated by a plasmid similar to that reported in the *Enterobacteriaceae* (1, 14) is presented by these data. The simultaneous transfer of PC, CM, and NM resistance to *A. tumefaciens* shows that the resistance determinants for these antibiotics are closely associated and are readily transmissible to susceptible recipients. The requirement for live donor cells and the lack of effect of deoxyribonuclease treatment indicate that transformation is not the mechanism of genetic exchange in the transfer of PC and CM resistance.

It is not likely that transduction is the mode of genetic exchange for three reasons. First, there was no indication of lysis of *A. tumefaciens* when plated or when incubated in liquid medium with *R. japonicum*. Second, the intergeneric nature of the cross renders it unlikely that a phage exists which has both *R. japonicum* and *A. tumefaciens* as hosts. Roslycky and co-workers (12) examined 19 different *A. radiobacter* phages for their ability to lyse 40 strains (representing eight species) of *Rhizobium*. Only one of the phages lysed any of the rhizobia, and only two strains of *R. meliloti* and three strains of *R. trifolii* were attacked. Third, the observed frequency of transfer in these experiments is approximately three orders of magnitude higher than is observed with transduction.

The resistance plasmid (or plasmids) found in *R. japonicum* differs in several ways from R factors found in *E. coli* but resembles the mechanism in *Salmonella* isolates. The R (TC, CM, SM, SU) R factor in *E. coli* is generally lost as a unit when cells are treated with acridine

dyes, with a low percentage of cells retaining R (TC) only (16), and the frequency of loss is relatively low. In contrast, resistance to PC, CM, and NM in *R. japonicum* is lost primarily as the individual determinants, and the frequency of loss is relatively high. The loss of individual resistance determinants resembles the mechanism observed by Anderson (1) in *Salmonella*. The frequency of transfer of R factors in *E. coli* is greatly increased by ultraviolet irradiation of donor cells (15). This phenomenon was not observed with *R. japonicum*, which suggests that the resistance determinants exist primarily or exclusively in the autonomous state. The high rate of loss of resistance following acridine orange treatment is also explicable on this basis.

All rhizobia tested were more sensitive than plasmid-carrying strains of *Staphylococcus aureus* to mercury, arsenite, arsenate, and lead. Strain 71 was resistant to zinc and strain 10324 was resistant to cadmium. There was no correlation in the zinc- or cadmium-resistant strains between heavy metal resistance and the antibiotic resistance patterns. This lack of correlation suggests that *R. japonicum* does not carry a plasmid similar to that found in *S. aureus* which confers resistance to erythromycin, penicillin, lead, arsenate, and cadmium (10). The differences in the levels of heavy metal resistance that exist between the rhizobia tested and *S. aureus* do not exclude the possibility of plasmid-mediated heavy metal resistance in the rhizobia, since the level of resistance conferred by plasmid genes can vary widely from organism to organism.

The marked sensitivity of the rhizobia to mercury suggests that seed or nodule sterilization with mercuric chloride is undesirable because low quantities of residual mercuric chloride would probably inhibit the rhizobia. The use of an alternate seed and nodule sterilant such as ethanol or formaldehyde is suggested.

The report of Lindberg and co-workers (9)

that staphylococcal antibiotic plasmids can be transferred to susceptible recipients by transformation suggests that a reappraisal of transformation of antibiotic resistance in rhizobia may be necessary. Transformation of antibiotic resistance in rhizobia has been reported by several workers (2, 6, 7). A survey of rhizobia other than *R. japonicum* for plasmid-mediated resistance would be desirable to determine whether or not other rhizobia possess transmissible antibiotic resistance.

Current work is oriented toward physical characterization of the plasmid(s) in *R. japonicum* and toward determining the kinetics of plasmid transfer between *R. japonicum* and *A. tumefaciens*.

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