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Multiple Antibiotic Resistance in *Rhizobium japonicum*

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A total of 48 strains of the soil bacterium *Rhizobium japonicum* were screened for their response to several widely used antibiotics. Over 60% of the strains were resistant to chloramphenicol, polymyxin B, and erythromycin, and 47% or more of the strains were resistant to neomycin and penicillin G, when tested by disk assay procedures. The most common grouping of resistances in strains was simultaneous resistance to tetracycline, penicillin G, neomycin, chloramphenicol, and streptomycin (25% of all strains tested). The occurrence of multiple drug resistance in a soil bacterium that is not a vertebrate pathogen suggests that chemotherapeutic use of antibiotics is not required for the development of multiple drug resistance.

Multiple drug resistance is a common attribute of bacterial pathogens of human and domestic animals. The presumed basis for this abundance of drug-resistant isolates is the widespread use of particular antibiotics for chemotherapy of infections and prophylaxis (1). The results of several studies with humans (5), domestic animals (14), and cultured fish (4) indicated that multiple drug resistance was common among bacterial isolates from animals or humans treated with antibiotics, but was much less common among isolates from sources that had not been subjected to antibiotic therapy. It follows from these results that, in general, multiple drug resistance should be a comparatively rare attribute among bacteria from any habitat (soil, for example) where antibiotics are not used for therapeutic purposes. The results of an extensive screening of soil bacterial isolates for antibiotic resistance (15) demonstrate that this conclusion is largely correct. The suppression of the majority of soil bacterial populations by antibiotics incorporated into media for selective isolation of specific bacteria (10, 12) also indicates that the majority of soil bacteria are susceptible to several commonly used antibiotics. In view of the generally occurring susceptibility of soil bacteria to antibiotics, we thought that it was somewhat unusual to find that *Rhizobium japonicum*—a soil bacterium that appears to be completely incapable of being a vertebrate pathogen and which was very unlikely to have been subjected to chemotherapeutic usage of antibiotics—possessed a transmissible plasmid conferring resistance to chloramphenicol, penicillin G, and neomycin (6).

In an effort to determine whether or not the strains examined previously (6) were atypical representatives of *R. japonicum* with respect to their antibiotic resistance patterns, we have surveyed strains of *R. japonicum* from several sources for antibiotic resistance. The results indicate that antibiotic resistance is the norm for *R. japonicum* and suggest that simultaneous resistance to several antibiotics can occur in bacteria in the absence of direct selective pressure.

MATERIALS AND METHODS

Organisms and medium. *R. japonicum* strains were obtained from the U.S. Department of Agriculture (USDA), Beltsville, Md.; the Department of Bacteriology, University of Wisconsin, Madison; North American Plant Breeders, Princeton, Ill.; Department of Crops and Soils, University of Iowa, Ames; Institute of Microbiology, Prague, Czechoslovakia; and Nitragin Co., Milwaukee, Wis. Stock cultures were maintained by periodic transfer on yeast extract-mannitol agar slants (9).

The medium used in this study (YEM-HM) has been described previously (6) and contains inorganic salts, D-mannitol, L-arabinose, yeast extract, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and 2(*N*-morpholino)ethanesulfonic acid. The pH was adjusted to 6.6 before autoclaving. Solid media were prepared by adding 1.5% Noble Special Agar (Difco Laboratories, Detroit, Mich.).

Antibiotics. Sensi-discs (Baltimore Biological Laboratory, Baltimore, Md.) or Dispenso-discs (Difco Laboratories, Detroit, Mich.) were used initially to test for antibiotic response. Disks were 5.5 mm in diameter. Both brands gave identical results. Representative disks were tested for potency, using YEM-HM medium and *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* as indicator organisms. The following antibiotic disks were used: chloram-

phenicol, 30 μg; streptomycin, 10 μg; neomycin, 30 μg; polymyxin B, 300 U; penicillin G, 10 U; erythromycin, 15 μg; novobiocin, 30 μg; and tetracycline, 30 μg.

Chloramphenicol, streptomycin, polymyxin B, neomycin, and penicillin G (potassium salt) powders for minimal inhibitory concentration (MIC) determinations were obtained from Calbiochem (La Jolla, Calif.); erythromycin powder was a product of Sigma Chemical Co. (St. Louis, Mo.). With the exception of penicillin G, antibiotic solutions used for incorporation into media were prepared by dissolving or suspending the antibiotic in 95% ethanol. Penicillin G was dissolved in HM-salts solution and sterilized by filtration (0.22-μm pore diameter, Millipore Corp.). All antibiotic solutions were prepared immediately before use. Sterility of the solutions was determined by incubating uninoculated tubes in parallel with tubes containing rhizobial cells.

Determination of antibiotic susceptibility. The response of organisms to antibiotic disks was determined by spreading ~1 × 10^6 cells per plate on YEM-HM plates before depositing the disk, unless otherwise indicated. The plates were incubated at 28°C until there was a moderate amount of growth on the plates; 1 to 5 days of incubation was required, depending upon the strain. The diameter of the inhibitory zone (if any) was measured to the nearest millimeter. The data presented are the means at least three separate determinations for each strain.

The MIC was determined by preparing plates with a series of antibiotic conentrations, spotting 10 μl of cell suspension (10^7 to 10^8 cells/ml) on a sector of the plate (6), and incubating as described.

It was not possible to perform regression analysis (11) to establish the relationship between zone diameter and the MIC for the antibiotics used because of the extreme clustering of the data. As an alternative, strains were scored as resistant to an antibiotic by the following method: an arbitrarily chosen MIC was selected as indicating resistance to a specific antibiotic, and the average zone diameter of all strains whose MIC equaled or exceeded the selected value was calculated. This zone diameter was then used as the upper limit of zone diameter indicating resistance. For example, all strains whose MIC for streptomycin was ≥100 μg/ml had zone diameters ≤12 mm surrounding 10-μg streptomycin disks (Table 1). This method could not be used for novobiocin because the antibiotic powder was not available. In this case, Anderson's criteria (3) were employed, since the zone diameters of *R. japonicum* strains were similar to those reported for other bacteria.

Grouping of antibiotic resistances was determined by computer analysis, employing a program which generated all combinations of the five antibiotics given in Table 4 and which listed strains possessing a given combination of resistances. The full data (Table 2) were used as inputs for the program and entered in a coded form (1 = resistant; 0 = susceptible).

RESULTS

Since YEM-HM medium is not a standard medium for antibiotic susceptibility testing, it was deemed necessary to test organisms other than the rhizobia on this medium to insure that the medium components did not prevent migration of the antibiotics or antagonize the antibiotics' effects upon the cells. This determination was made for all antibiotics listed above by plating portions of 24-h broth cultures of the organisms on YEM-HM agar and on nutrient agar (Difco), at which time antibiotic disks were placed on the surface of the plates. The following bacteria were examined: *Escherichia coli*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Staphylococcus aureus*, and *Erwinia carotovora*. Results were scored after 24 and 72 h of incubation at 32°C. Zone diameters ranged from 25 mm (polymyxin B) to 65 mm (tetracycline) when a susceptible indicator organism was utilized. There were no differences in the antibiotic responses of an organism, regardless of which medium was used. The results indicated that YEM-HM was a suitable medium for assessing the antibiotic response of *R. japonicum*. Mannitol was used as the carbon source for all antibiotic work, because the pH of the medium remains constant (pH = 6.6), whereas the pH rises to about 8 when gluconate is the carbon source. The pH rise was undesirable, since the activity of a number of antibiotics is markedly pH dependent (8).

The responses of 48 strains of *R. japonicum*

TABLE 1. *Criteria used in scoring antibiotic resistance*

Antibiotic	Zone diam (mm)	Corresponding MIC (μg/ml)
Polymyxin B	6	≥400
Chloramphenicol	6	≥150
Streptomycin	≤12	≥100
Neomycin	≤10	≥200
Tetracycline	≤18	≥50
Penicillin G	≤12	≥60
Erythromycin	6	≥100
Novobiocin	≤17	ND[a]

[a] ND, Not determined.

TABLE 2. *Distribution of resistance to eight antibiotics among 48 R. japonicum strains*

Antibiotic	No. of strains resistant[a]	% of strains resistant[a]
Polymyxin B	41	86
Chloramphenicol	32	67
Streptomycin	32	67
Neomycin	41	86
Tetracycline	20	41
Penicillin G	23	47
Erythromycin	34	71
Novobiocin	8	16

[a] A strain was scored as resistant by the disk diffusion criteria given in the text.

to eight antibiotics are presented in Table 2. The majority of strains were susceptible to tetracycline and novobiocin, whereas zone diameters for erythromycin, chloramphenicol, polymyxin B, and neomycin were less than 10 mm for 70% or more of the strains tested. Of the strains tested, 86% showed no zone of inhibition around polymyxin B or neomycin disks, and the greatest zone diameter observed was 8 mm.

The resistance of many strains to the antibiotic concentration in most of the disks made it necessary to use higher concentrations of these antibiotics. The MIC for selected strains was determined by the spot-test method (Table 3). Regardless of the overall pattern of resistances exhibited by the isolates, the level of resistance to chloramphenicol, neomycin, and tetracycline was quite uniform among all of the strains tested (Table 3). In contrast, the range of resistance to penicillin G was broader, with half of the strains examined showing resistance to at least 200 U of penicillin G per ml. This variation is not due to differences in the levels of β-lactamase produced, since several strains that produce large amounts of β-lactamase were highly susceptible to β-lactams (I. K. Stovall and M. A. Cole, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, A45, p. 8).

TABLE 3. *Levels of resistance to chloramphenicol, streptomycin, neomycin, tetracycline, penicillin G, and polymyxin B in 24 randomly selected strains of R. japonicum*

Antibiotic	Concn (μg/ml)	No. of strains surviving
Chloramphenicol	300	17
	200	17
	150	22
	19	22
	2	24
Streptomycin	125	1
	100	9
	50	15
	25	19
	12	21
Neomycin	300	4
	200	23
	150	23
	125	23
	12	24
Tetracycline	125	0
	100	1
	50	15
	25	19
	12	20
	1	21
Penicillin G	135	5
	120	12
	60	18
	30	18
	13	21
	1	24
Polymyxin B	400	19
	320	19
	240	19
	160	19
	80	19
	40	21

The patterns of resistance to tetracycline, penicillin G, neomycin, chloramphenicol, and streptomycin were analyzed by a computer. These were selected since they are the major antibiotics to which plasmid-associated resistances are found in gram-negative bacteria. Table 4 summarizes the results of the computer analysis. The antibiotic resistances were grouped in a nonrandom fashion. Of the 31 possible combinations of 5, 4, 3, 2, or 1 antibiotic resistances, 9 of these combinations included 71% of the 48 strains examined. The other 29% generally comprise single strains resistant to combinations of antibiotics other than those listed in Table 4. Based on the frequency of individual resistances (Table 2) and assuming that all resistances behave in an independent manner, 7.4% of all strains would be expected to be resistant to five antibiotics; the actual frequency was 25% (Table 4). The nonrandom distribution of resistances among the strains was quite similar to those reported for enteric bacteria carrying R-factors (2, 16). When groups of antibiotic resistances were considered, rather than resistance to a single antibiotic, a high degree of source variability was observed. Strains obtained from the USDA comprised 75% of the strains exhibiting simultaneous resistance to five antibiotics, whereas

TABLE 4. *Commonly observed groups of resistances in 48 R. japonicum strains*

Resistant to[a]	% Occurrence
Tc, Pc, Nm, Cm, Sm	25
Tc, Nm, Cm, Sm	4
Pc, Nm, Cm, Sm	4
Tc, Nm, Cm	8
Nm, Cm, Sm	4
Nm, Cm	8
Nm, Sm	6
Nm only	8
Pc only	4

[a] Strains were scored as resistant based on the disk diffusion criteria stated in the text. Cm, Chloramphenicol; Pc, penicillin G; Nm, neomycin; Tc, tetracycline; Sm, streptomycin.

65% of the Nitragin strains were resistant to at least four antibiotics. Multiple resistance was also seen in strains from other sources, but the frequency of multiple-resistant isolates was much lower than that observed with Nitragin or USDA isolates.

DISCUSSION

The *R. japonicum* strains examined in this work were isolated from different soils and different host legume varieties, using different media. A number of them have been in culture for many years, particularly the USDA strains. In spite of many differences in the biochemical properties of the isolates (9), most isolates were similar in their resistance to polymyxin B and chloramphenicol, and many were also resistant to tetracycline, neomycin, and streptomycin. The variety of resistance patterns seen in these strains is probably not due to loss of some of the resistance characters as a result of a long period of culture in the laboratory, because strains recently isolated from soy beans in North Carolina and Illinois showed similar patterns of resistance. Our results indicate that streptomycin and penicillin resistances are relatively variable characters, unlike resistance to chloramphenicol, neomycin, and polymyxin B. *R. japonicum* is not unique among the rhizobia in exhibiting high-level antibiotic resistance, since Schwinghamer (13) has described several parent strains of *R. leguminosarum* and *R. trifolii* whose growth was not inhibited by polymyxin B, chloramphenicol, or oxacillin at 150, 250, and 120 μg/ml, respectively.

We have shown previously that some *R. japonicum* strains possess a plasmid conferring resistance to chloramphenicol, neomycin, and penicillin G (6). The similarities in the antibiotic resistance patterns of those strains with a demonstrable plasmid and the strains examined in this study suggest that most *R. japonicum* strains possess this plasmid. Curing experiments are underway to determine the role of this plasmid in conferring antibiotic resistance.

Unlike pathogenic bacteria such as the enteric group, *R. japonicum* has not been subjected to rigorous selection for antibiotic resistance by the widespread use of antibiotics. Consequently, the fact that many *R. japonicum* strains are multiple drug resistant when isolated suggests that this resistance has a survival value in natural habitats, but the exact value to the organism is unknown.

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