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Tellurium and Selenium Resistance in Rhizobia and Its Potential Use for Direct Isolation of *Rhizobium meliloti* from Soil†

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Forty-eight *Rhizobium* and *Bradyrhizobium* strains were screened for resistance to tellurite, selenite, and selenate. High levels of resistance to the metals were observed only in *Rhizobium meliloti* and *Rhizobium fredii* strains; the MICs were 2 to 8 mM for Te(IV), >200 mM for Se(VI), and 50 to 100 mM for Se(IV). Incorporation of Se and Te into growth media permitted us to directly isolate *R. meliloti* strains from soil. Mutant strains of rhizobia having decreased levels of Se and Te resistance were constructed by Tn*5* mutagenesis and were found to have transposon insertions in DNA fragments of different sizes. Genomic DNAs from Te^r rhizobium strains failed to hybridize with Te^r determinants from plasmids RP4, pHH1508a, and pMER610.

Heavy metals and metalloids are a concern when they are found in high concentrations in agricultural soils, surface waters, and groundwater (13). Microorganisms are actively involved in transformations of many metals and metalloids, including the metalloids tellurium (Te) and selenium (Se) (2). While the geochemical cycling of selenium has received much attention (6, 8), less is known about the biotransformation reactions involved in the cycling of tellurium. Recently, however, there has been growing concern about tellurium toxicity and about sources of Te in natural environments (21). Plasmid-encoded tellurium resistance has been reported in strains of *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, and *Alcaligenes* sp. (9, 28).

Despite the presence of metal-impacted agricultural soils, there have been few studies of metal and metalloid resistance in rhizobia. Previously, Kinkle et al. (10) examined two genera of soybean-nodulating rhizobia to determine levels of resistance to eight different metals. Marked differences in the levels of resistance were found with several heavy metals, even for different rhizobial strains belonging to the same species group. More recently, Tong and Sadowsky (26) reported that bradyrhizobia are more resistant to several heavy metals than *Rhizobium* strains are.

There are currently no adequate direct selection media for isolating rhizobia directly from soil. Current isolation methods rely on using a suitable trap host and plant nodulation, which is specific for certain species or even individual strains of rhizobia (17, 24, 26). In recent reports, however, workers have described direct isolation from soil of strains of rhizobia which lack the ability to nodulate the appropriate host plants, suggesting the possibility that there are large and diverse soil populations of uncharacterized rhizobia (20, 23).

In this paper we describe the levels of resistance to Te and Se in various groups of rhizobia, describe the use of Te and Se in a selective medium which was used for direct isolation of *Rhizobium meliloti* from soil, and describe the isolation and preliminary characterization of Te^s and Se^s *Rhizobium fredii* strains.

Rhizobium strains were obtained from the USDA Culture Collection, Beltsville, Md., and from the NifTAL Project, Paia, Hawaii, and were maintained on slants of AG medium (17). *Escherichia coli* WA803(pGS9) was maintained on Luria-Bertani medium (18, 22). Levels of resistance were determined on solid AG medium amended with filter-sterilized solutions of Te(IV), Se(IV), and Se(VI). Plates were streaked with cells grown in AG medium, incubated at 28°C, and scored for visible growth after 7 days. The media used for direct isolation of *R. meliloti* from soil included (i) TY medium (3); (ii) TY medium containing Te (400 μg of potassium tellurite per ml); (iii) *R. meliloti* selective medium (RMSM) (TY medium amended with 1.5 μg of brilliant green per ml, 500 μg of pentachloronitrobenzene per ml, and 400 μg of potassium tellurite per ml); and (iv) RMSM containing Se (5,000 μg of sodium selenite per ml). The pentachloronitrobenzene and brilliant green were prepared and added to RMSM medium as previously described (1, 15).

Total genomic DNAs were extracted from *Rhizobium* strains as described previously (16). Genomic DNAs from *R. fredii* USDA 201 and HH303 and *R. meliloti* 1024 were digested with *Eco*RI, separated by horizontal electrophoresis, and transferred to Nytran membranes (Schleicher and Schuell, Keene, N.H.) as described previously (19). ^{32}P-labeled gene probes were prepared and hybridized to membranes under stringent conditions as described previously (19). The gene probes used were a 7-kb *Sal*I fragment from IncHII Te^r plasmid pHH1508a cloned into pUC8, a 3.1-kb *Bam*HI-*Hin*dIII fragment from IncP Te^r plasmid RP4 cloned into pUC8, and a 5-kb *Bgl*II-*Pst*I fragment from IncHI-2 Te^r plasmid pMER610 cloned into pLV59. Positive and negative control DNAs were used in the hybridization experiments.

Transposon Tn*5* mutants of *R. fredii* USDA 201 were isolated following mutagenesis with plasmid pGS9 as previously described (22). The Te^s and Se^s *R. fredii* mutants were identified by replica plating randomly selected Tn*5* mutants onto unamended TY medium and TY medium amended with Te or Se. Genomic DNAs of Te^s mutants were digested with *Eco*RI or *Hin*dIII, separated by horizontal electrophoresis, and transferred to MagnaCharge membranes (Micro Separation,

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TABLE 1. Te and Se resistance in *Rhizobium* and *Bradyrhizobium* strains

Rhizobial strain	Host plant	Metalloid MIC (mM)		
		Te(IV)	Se(IV)	Se(VI)
R. fredii USDA 201	*Glycine max*	8	6	400
B. japonicum USDA 138	*Glycine max*	2	6	50
B. japonicum USDA 162	*Glycine max*	2	12	50
R. meliloti TAL 380	*Medicago sativa*	16	100	400
R. meliloti TAL 1372	*Medicago sativa*	16	100	400
Rhizobium etli TAL 182	*Phaseolus vulgaris*	2	6	100
R. etli TAL 1383	*Phaseolus vulgaris*	1	6	50
R. leguminosarum bv. viceae TAL 1399	*Vicia faba*	2	200	400
R. leguminosarum bv. trifolii TAL 1820	*Trifolium praetense*	2	12	200
R. leguminosarum bv. trifolii TAL 1824	*Trifolium praetense*	2	12	200
R. leguminosarum bv. viceae TAL 634	*Lathyrus hirsutus*	2	50	200
R. leguminosarum bv. viceae TAL 640	*Lens culinaris*	2	25	100
Bradyrhizobium sp. strain TAL 169	*Vigna unguiculata*	2	6	50
Bradyrhizobium sp. strain TAL 310	*Macrotyloma uniflorum*	8	6	100
Rhizobium sp. strain TAL 1145	*Leucaena leucocephala*	2	12	100

Inc., Westboro, Mass.) as described previously (18). Purified pGS9 was prepared by using a Quiagen Maxi Plasmid kit (Quiagen, Inc., Chatsworth, Calif.) according to the manufacturer's directions. Biotin-labeled pGS9 probe was prepared, hybridized under stringent conditions, and detected on membranes by following the directions of the manufacturer (New England Biolabs, Beverly, Mass.).

The soil used for inoculation studies was a Waukegan silt loam prepared as previously described (11). Sterile and nonsterile soil samples were inoculated with a mid-log-phase culture of *R. meliloti* RCR 2011 to a concentration of 10^9 cells per g of soil. *R. meliloti* was grown in TY medium and washed in 0.85% saline prior to inoculation. The moisture contents of the inoculated soil samples were adjusted to field capacity, and the preparations were incubated at 28°C for 3 days. The uninoculated soil used was a Waukegan silt loam obtained from a field planted with alfalfa. Rhizobia were extracted from soils as previously described (11), and soil dilutions were plated onto different selective media and incubated as described above.

Bacteria isolated on selective media from the uninoculated alfalfa field soil were tested for their ability to nodulate alfalfa. A total of 200 single-colony isolates were obtained from the RMSM cultures, and 46 isolates were isolated from the cultures on RMSM containing Se. Plant nodulation tests were performed by using surface-sterilized seeds of alfalfa (*Medicago sativa* cv. Sarnac 7061) as described previously (24). Individual isolates were picked from selective media and grown overnight in TY medium, and 1-ml aliquots were inoculated onto seedlings. Positive controls (seeds inoculated with *R. meliloti* RCR 2011) and uninoculated controls were included in triplicate.

A total of 47 randomly selected colonies were picked from RMSM and RMSM-Se plates that had been inoculated with extracts from nonsterile soil amended with *R. meliloti* RCR 2011. These 47 isolates were tested for their reactions with fluorescent antibodies specific for strain RCR 2011. Strain-specific fluorescent antibodies were prepared as described by Schmidt et al. (19). In addition, 46 purified colonies that were used in the alfalfa nodulation assays described above were also tested for their reactions with an *R. meliloti*-specific monoclonal antibody (14).

The MICs of Te(IV), Se(IV), and Se(VI) for a diverse group of rhizobial strains that nodulate 10 different legumes are shown in Table 1. A total of 15 strains were examined, and the highest overall MICs of the three metalloid species were observed with the *R. meliloti* strains. The high Se MICs for the rhizobium strain isolated from *Vicia faba*, strain TAL 1399, and for *R. fredii* USDA 201 were also noteworthy. High levels of resistance to Te were found with the two *R. meliloti* strains, the *R. fredii* strain, and the strain capable of nodulating *Macrotyloma* sp., strain TAL 310. The Te and Se MICs for an additional 14 strains of *R. fredii* and 20 strains of *R. meliloti* are shown in Table 2. With few exceptions, strains of these two species of rhizobia exhibited uniformly high levels of resistance to Te and Se. Most of the *R. fredii* strains examined were resistant to at least 8 mM Te(IV), 12 mM Se(IV), and 400 mM Se(VI). Similarly, the 20 *R. meliloti* strains were uniformly resistant to the metalloids. While all of the *R. meliloti* strains were resistant to at least 50 mM Se(IV) and 400 mM Se(VI), the Te(IV) MICs varied from 2 to 16 mM. There was an apparent relationship among resistance to Se(IV), resistance to Se(VI), and resistance to Te(IV) for the *Rhizobium* strains tested (Tables 1 and 2). This finding is in contrast to the findings of Burton et al. (5), who reported a lack of correlation among levels of resistance to various metalloids for a diverse group of bacteria isolated from aquatic and sediment samples from a Se-polluted reservoir and a relatively pristine reservoir.

The *R. meliloti* and *R. fredii* strains formed small (1- to 4-mm), black, nonmucoid, raised colonies with smooth margins when they were grown on Te-containing media. Strains of *R. meliloti* and *R. fredii* grown on Se-containing media produced small red colonies. The formation of red and black colonies was presumably due to reduction of the added Se and Te compounds, respectively, to their elemental forms.

Since the *R. meliloti* strains were resistant to fairly high levels of the metalloids, we examined the use of the metalloids as selective agents for recovery of *R. meliloti* strains from media and soils. The following three selective media were used: TY medium containing Te, RMSM, and RMSM containing Se. While *R. meliloti* RCR 2011 grew on all three selective media, we observed differential suppression of bacterial growth depending on the medium used and the source of the inoculum (Table 3). After *R. meliloti* RCR 2011, grown on TY medium, was plated onto the different selective media, the viable cell counts were lower on the Te- and Se-containing media than on unamended TY medium. The presence of additional antifungal and anti-gram-positive organism inhibitors (pentachloronitrobenzene and brilliant green, respectively) further reduced the plating efficiency of *R. meliloti* on RMSM and RMSM

TABLE 2. Te and Se resistance in *R. fredii* and *R. meliloti* strains

Rhizobial strain(s)	Metalloid MIC (mM)		
	Te(IV)	Se(IV)	Se(VI)
R. fredii strains			
USDA 191, USDA 194, USDA 201, USDA 206	8	6–12	400
USDA 192, USDA 193, USDA 205, USDA 208, USDA 214	6–8	25	400
USDA 257	8	25	25
HH103	8	50	400
USDA 217, HH003	16	25–50	400
HH303	16	200	400
R. meliloti strains			
USDA 1150, USDA 1179, USDA 1148, USDA 1149, USDA 1031	2–4	50–100	400
USDA 1002, USDA 1093, USDA 1098, USDA 1107, USDA 1163	8	50–100	400
USDA 1005, USDA 1021a, USDA 1027, USDA 1035, USDA 1045, USDA 1146, USDA 1170, USDA 1171, USDA 1180	16	50–100	400
USDA 1024	16	200	400

containing Se. Similar reductions in CFU were also observed when the same strain was recovered from sterile soil and nonsterile soil that had been inoculated with *R. meliloti* RCR 2011 (Table 3). Moreover, the presence of Se in the selective medium greatly reduced the number of rhizobia recovered. Similar reductions in the plating efficiencies of selective *Bradyrhizobium japonicum* strains on metal- or selective agent-amended media have also been reported (1, 15, 26).

Colonies recovered from sterile and nonsterile soils (which were inoculated with *R. meliloti* RCR 2011) on RMSM and RMSM containing Se were examined by using a fluorescent antibody specific for *R. meliloti* RCR 2011; 87 and 100% of the colonies recovered from the nonsterile and sterile soils, respectively, reacted with the fluorescent antibody.

We also used the same selective media in an attempt to directly isolate rhizobia from uninoculated field soil. While the nonselective TY medium plates were overgrown with both fungi and bacteria (confluent growth), the plates containing the two selective media, RMSM and RMSM supplemented with Se, predominantly contained colonies whose appearance was similar to the appearance of laboratory strains of *R. meliloti*. On plates containing TY medium supplemented with TE, RMSM, and RMSM supplemented with SE, we observed 4×10^3, 5.2×10^3, and 1.1×10^2 CFU, respectively (for the determinations on TE-containing medium we included only the small [1- to 4-mm], black, dry colonies; the small number of large, mucoid colonies present were not counted). The selective plates, however, did contain a small number (less than 1% of the total count) of gram-positive bacteria that formed large mucoid colonies and fungi. To determine if bacteria isolated from uninoculated field soil by using the selective media were capable of nodulating alfalfa, pure cultures were obtained and used to inoculate *M. sativa* seedlings. Of 200 strains isolated from the RMSM cultures, only 20 (10%) nodulated the alfalfa plants. In contrast, 10 of 46 colonies (22%) isolated from cultures on RMSM containing Se effectively nodulated alfalfa, and 1 isolate nodulated ineffectively. The positive control plants inoculated with a known *R. meliloti* strain were well nodulated, and the uninoculated negative controls remained free of nodules.

TABLE 3. Recovery of *R. meliloti* RCR 2011 on selective and nonselective media

Source of *R. meliloti*	CFU of *R. meliloti* RCR 2011 on[a]:			
	TY medium	TY medium containing Te	RMSM	RMSM containing Se
TY broth	1.8×10^9	1.3×10^9	1.1×10^9	8.7×10^8
Seeded sterile soil	7.5×10^6	2.2×10^5	7.5×10^4	4.2×10^2
Seeded nonsterile soil	4.5×10^6	1.8×10^5	6.3×10^5	6.6×10^3

[a] For CFU determinations on Te-containing media we included only small (1- to 4-mm), black, dry colonies. The small number of large, mucoid colonies present were not counted.

Because of the possible existence of nonnodulating *Rhizobium* populations in soil (20, 22), the same 46 strains isolated from the cultures on selective medium were then tested with a monoclonal antibody that has been shown to react with 63 *R. meliloti* strains (14). Despite their distinctive colony morphology, resistance to Te and Se, and color on selective media, only 5 of the 46 strains isolated on RMSM containing Se reacted positively with the monoclonal antibody. It is possible, however, that since the monoclonal antibody has been tested previously only with strains of rhizobia isolated from alfalfa nodules, there may be soil populations of *R. meliloti* that do not have the specific surface antigen. This possibility is supported by the fact that several of the bacterial strains which were isolated from soil on our selective media and were shown to nodulate alfalfa did not react with the monoclonal antibody. The other possibility is that these strains of soil bacteria are not *R. meliloti* strains and merely have the ability to grow in the presence of, and to reduce, Te and Se. Two ways to distinguish between these two possibilities would be to use an rRNA probe specific for, and characteristic of, all *R. meliloti* strains and to test the metalloid-resistant soil bacteria for the ability to nodulate alfalfa following acquisition of the Sym plasmid from a known *R. meliloti* strain.

Four different Te^s mutants of *R. fredii* USDA 201 were obtained by random Tn*5* mutagenesis. The levels of resistance to Se and Te of the mutants are shown in Table 4. Since the Te resistance and Se resistance exhibited by strain USDA 201 appeared to be uncoupled in most of the mutants, our results indicated that different genes may control resistance to the metalloids. These genes, however, may interact in some manner, since in some mutants both Te resistance and Se resistance were simultaneously affected. In addition, since Tn*5* was located in *Eco*RI fragments of different sizes in the genomic DNAs of the Te^s mutants (Table 4), our results suggest that more than one gene may be involved in Te resistance.

No hybridization was observed between total genomic DNAs isolated from Te^r rhizobium strains and the Te^r deter-

TABLE 4. Characterization of wild-type and Te^s *R. fredii* USDA 201 mutants

R. fredii strain	Size of *Eco*RI fragment containing Tn*5* (kb)	Metalloid MIC (mM)		
		Te(IV)	Se(IV)	Se(VI)
USDA 201		8	6	400
201-1b	12.6	1	6	200
201-2a	7.1	0.5	1	50
201-13a	9.2	0.5	4	200
201-14a	16.0	2	6	200

minants from IncHII plasmid pHH1508a (from *K. aerogenes*), IncP plasmid RP4 (from *P. aeruginosa*), and IncHI-2 plasmid pMER610 (from an *Alcaligenes* sp. strain). This suggests that the rhizobia may have evolved novel Te resistance determinants. While the mechanism(s) involved in Te and Se resistance in rhizobia and most bacteria is not known (25), it has been shown recently that an arsenical ATPase efflux pump mediates Te resistance in *E. coli* (27), that Te and Se resistance in *Rhodobacter sphaeroides* is due to membrane-associated enzymatic reduction (12), and that Se may be an essential element for symbiotic growth of some strains of rhizobia (4).

In conclusion, while previously described selective media for rhizobia have included antibiotics and other selective agents (1, 7, 15, 26), none of the media have been shown to simultaneously allow the growth of all strains of a certain *Rhizobium* species and prevent the growth of all other soil bacteria. An antibiotic-containing medium that is selective for *R. meliloti* has been described previously (1). This medium, however, was restricted in its use to soils containing relatively high numbers of *R. meliloti*. In this study, two non-antibiotic-containing media, RMSM and RMSM supplemented with Se, were selective enough for *R. meliloti* to be used at low soil dilutions, without extensive overgrowth by other soil bacteria. Thus, these media may be very useful as quality control media for nonsterile alfalfa inoculants. It should be noted, however, that the media slightly suppressed the growth of *R. meliloti* recovered from soils, suggesting that bacteria recovered from soil may be physiologically compromised in some manner. Consequently, the media described above may need to be reformulated by reducing the toxicity of constituents (26) in order to adequately isolate rhizobia from different soils.

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