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A Selective Medium for the Isolation and Quantification of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* Strains from Soils and Inoculants†

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The ecological examination of members of the family *Rhizobiaceae* has been hampered by the lack of a selective medium for isolation of root nodule bacteria from soil. A novel non-antibiotic-containing medium has been developed which allows selective isolation of *Bradyrhizobium japonicum* and *B. elkanii* strains from soil and inoculants. The medium, BJSM, is based on the resistance of *B. japonicum* and *B. elkanii* strains to more than 40 μg of the metals ions Zn^{2+} and Co^{2+} per ml. BJSM does not allow growth of *Rhizobium sp.* strains. We used BJSM to isolate bacteria from a Hubbard soil and from several commercially prepared soybean inoculants. Ninety-eight percent of the isolates obtained from Hubbard soil nodulated *Glycine mar cv.* Kasota, and between 55 and 95% of the isolates from the commercial inoculants had the ability to nodulate soybeans. Numbers of bradyrhizobia obtained by using BJSM, strain-specific fluorescent antibodies, and the most-probable-number plant infection assay indicated that the three techniques were comparable in quantifying *B. japonicum* strains in soils and inoculants, although most-probable-number counts were generally 0.5 order of magnitude greater than those obtained by using BJSM. Results of our studies indicate that BJSM is useful for direct isolation and quantification of *B. japonicum* and *B. elkanii* from natural soils and inoculants. This medium may prove to be an important tool for autecological and enumeration studies of diverse populations of bradyrhizobia and as a quality control method for soybean inoculants.

Ecological studies of members of the family *Rhizobiaceae* require the quantification and isolation of indigenous and applied inoculant strains. Such studies have been hampered by the lack of a selective medium for the direct isolation and accurate enumeration of root nodule bacteria from soils. Present isolation methodology requires the use of a trap host, which biases recovered isolates to the most competitive strains which have the ability to nodulate a given legume genotype. Current enumeration methodologies include the quantitative immunofluorescence technique (22), the plant infection mostprobable-number (MPN) assay (27, 31), and direct plating on antibiotic-containing laboratory growth media (19, 27). While all three techniques provide estimates of cell numbers, they each have limitations. While the plant infection MPN assay has been a method of choice among many researchers, the technique is expensive and time consuming and is predicated on the hypothesis that a single bacterium is capable of forming a nodule. In addition, the technique is sensitive to the host species (or genotype) and growth system used (31). The immunofluorescence technique is also frequently used to quantify rhizobia in soils and inoculants. This methodology, however, tends to overestimate population sizes and is subject to problems associated with overall sensitivity, the optimal dispersal and flocculation of bacteria from different soil types, and the availability of specific, non-cross-reactive antibodies. Moreover, with the exception of the MPN assay, current enumeration methodologies are generally limited to the quantification of single strains within a species of legume-nodulating bacteria.

Some selective agents have been used for the recovery of *Rhizobium* and *Bradyrhizobium spp.* from soils and inoculants. The selective agents used have included antibiotics, dyes, and metabolic inhibitors (4, 10, 28-30). The use of antibiotics as selective agents has not proven to be particularly effective, since even strains within a given species group can have diverse resistances to many different antibiotics. Pattison and Skinner (18) reported the formulation of a selective medium for rhizobia that contained pentachloronitrobenzene (PCNB), brilliant green (BG), sodium azide, crystal violet, and penicillin. Barber (1) subsequently modified the concentrations of these inhibitors to make the medium more selective for *R. meliloti*.

Metal tolerance among soil microorganisms has been extensively studied (5, 7-9, 11, 25). High-level resistance to zinc, cobalt, and cadmium has been well studied in *Klebsiella spp. (2)* and *Alcaligenes eutrophus* (6, 14-17, 29). Despite the presence of metal-contaminated soils, there have been few reports on metal and metalloid resistance in rhizobia. Previously, Kinkle et al. (13) examined two genera of soybean nodulating rhizobia for levels of resistance to eight different metals. Marked differences in levels of resistance to several heavy metals were found, even for different rhizobial strains within a single species group. Moreover, *B. japonicum* strains were found to be more resistant to several heavy metals than were the several *Rhizobium sp.* strains examined.

The objectives of the current study were to examine the heavy metal resistance levels of various groups of bradyrhizobia and to develop a heavy-metal-based, non-antibiotic-containing, selective medium for the direct isolation and quantification of *B. japonicum* strains from agricultural soils.

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TABLE 1. Bacteria used in this study and their sources

Strain(s)	Source[a]
B. japonicum USDA 4, USDA 38, USDA 62, USDA 110, USDA 122, USDA 123, USDA 127, USDA 129, USDA 135	1
B. elkanii USDA 46, USDA 61, USDA 76, USDA 94, USDA 130	1
R. etli Tal 182, Tal 1383	2
R. fredii USDA 191, USDA 205, USDA 257	1
HH 103	3
R. leguminosarum bv. trifolii Tal 1820, Tal 1824	2
R. leguminosarum bv. viciae Tal 634, Tal 640	2
R. meliloti Tal 380, Tal 1372	2
102F34	4
R. tropici CIAT 899	5

[a] Sources: 1, U.S. Department of Agriculture, Beltsville, Md.; 2, The NifTAL Project, Paia, Hawaii; 3, B. Ben Bohlool, University of Hawaii, Honolulu; 4, L. T. Smith, University of California, Davis; 5, P. H. Graham, University of Minnesota, St. Paul.

MATERIALS AND METHODS

Strains and media used. The *Rhizobium* and *Bradvrhizobium* strains used in this studv and their sources are listed in Table 1. Strains were routinely grown and maintained on AG medium (21) at 28°C.

Soils and inoculants. Commercially prepared soybean inoculant I was a peat-based inoculant obtained from Lipha Tech Inc. (Milwaukee, Wis.), inoculant II was a peat-based soybean inoculant from Urbana Laboratories (St. Joseph, Mo.), and inoculant III was a liquid soybean inoculant from Lipha Tech Inc. Monmouth fine sandy loam soil (Typic Hapludults) was obtained in Maryland and contained fewer than 10 *B. japonicum* organisms per g of soil as determined by the plant infection MPN method (27, 31). Hubbard loamy sand soil (Udorthentic Haploboroll) was obtained from the University of Minnesota Agricultural Experiment Station in Becker. The soil has been used for soybean cultivation.

Determination of resistance to heavy metals and inhibitors of fungi and gram-positive bacteria. *Rhizobium* and *Brad yrhizobhun* strains were grown in AG broth medium at 28°C to the late log phase and serially diluted in 0.85% NaCl, and dilutions were spread plated or streaked onto the surface of metal-amended or unamended AG agar medium. The metals used were added as $ZnCl_2$, $CoCl_2 \cdot 6H_2O$, $CuCl_2$, $CdCl_2 \cdot 2H_2O$, $PbCl_2$, $HgCl_2$, MoO_3, and NiCI- Stock solutions of each metal ion were prepared at 10 g/liter in distilled water, except for Pb^{2+}, which was prepared as a 5-g/liter stock solution. All metal solutions were filter sterilized before addition to sterile and molten media. Plates were amended with either one or two of the metals. Each strain was tested on different concentrations of all eight metals. All plates were replicated at least three times and incubated at 28°C. Plates were routinely scored after 10 to 12 days of growth. MICs are presented as micrograms of metal ions per milliliter. Percent relative growth efficiency of *B. japonicum* and *B. elkanii* strains was determined in liquid AG medium supplemented with various concentrations of metals. Growth was quantified after 5 days of incubation at 28°C by measuring optical density at 590 rim. Resistance to PCNB, cycloheximide, and BG was tested by spread plating dilutions of AG-grown B. *japonicum* cultures onto AG agar medium amended with several concentrations of each inhibitor. Plates were incubated at 28°C, and viable cell numbers on duplicate plates were determined after 10 days of growth.

Soil inoculations. Mid-log-phase, AG-grown cultures of strains of rhizobia were harvested by centrifugation at 10,000 x g for 10 min at 4°C, washed in 0.85% NaCl solution, and centrifuged at 10,000 x g for 10 min at 4°C. Cell pellets were suspended in 10-fold-diluted HM salts solution (21) and added to soils (10 g) to achieve initial cell numbers of 10^5 to 10^7 rhizobia per g of soil. The final moisture content of the soils was adjusted to approximately field capacity. Soils were incubated in sterile 150-ml broad-mouth bottles at 28°C for 7 days (13).

Extraction of rhizobia from soils and inoculants. Rhizobia were extracted from soils and inoculants by a modification of the gelatin-ammonium phosphate method of Kingsley and Bohlool (12). Ten-gram aliquots of soils and inoculants were extracted in 95 ml of gelatin-ammonium phosphate solution containing 0.5 ml of Tween 80 and 0.1 ml of silicone antifoam AF72 (General Electric Co., Waterford, N.J.). Soil and inoculant suspensions were shaken on a wrist action shaker for 30 min and allowed to settle for an additional 30 min. The upper aqueous phase was removed to a sterile test tube, and serial dilutions were used for plant infection MPN assays (27, 31), direct quantitative fluorescent-antibody (FA) enumeration (12, 22) and plating onto the various selective media.

Composition of *Bradyrhizobium* selective medium (BJSM). The *B. japonicum* selective medium used consisted of AG medium supplemented with 1.0 μg of BG per ml, 500 μg of PCNB per ml, 83 μg of ZnCl. per ml, and 88 μg of $CoCl_2$ per ml. PCNB was prepared and added to the medium as previously described (1, 18). The BG, heavy metals, and cycloheximide were added to the sterile and molten medium as filter-sterilized stocks.

Plant infection assays. Plant infection assays were done in plastic growth pouches as described by Somasegaran and Hoben (27). Each pouch contained two plants. Bacteria isolated on BJSM from inoculated and uninoculated field soils and inoculants were tested for the ability to nodulate soybean plants. Plant nodulation tests were done with surface-sterilized (27) soybean seeds *(Glycine max* cv. Kasota). Plants were watered with N-free plant nutrient solution (3). Individual small, white colonies were picked from BJSM and grown for 3 days in AG medium at 28°C, and 1-ml aliquots were inoculated onto pre-germinated seedlings. Positive (inoculated with *B. japonicum* USDA 110) and negative (inoculated with blank AG broth) controls were included. MPN analyses were done with four replicates as previously described (27). Inoculated seedlings were incubated in a growth chamber at 25°C with a 16-h photoperiod for 4 weeks.

Direct quantitative immunofluorescence assay. Bradyrhizobia in extracts of soils and inoculants were quantified by direct immunofluorescence microscopy as previously described (12, 22). Strain-specific FAs were prepared as previously described (22).

RESULTS AND DISCUSSION

Resistance of bradyrhizobia and rhizobia to heavy metals. We examined whether heavy metals could be used as selective agents for the direct isolation of *B. japonicum* and B. *elkanii* strains from soils and inoculants. Initially, five strains of bradyrhizobia (representing type members of five

TABLE 2. Tolerance of *B. japonicum* strains to heavy metals

Strain	MIC (μg/ml) of:							
	Zn	Co	Cu	Cd	Pb	Hg	Mo	Ni
USDA 61	480	160	80	80	480	1	3,200	80
USDA 110	320	80	80	40	640	1	1,600	40
USDA 122	320	80	20	40	640	<1	1,600	80
USDA 123	80	80	40	<20	480	<1	1,600	20
USDA 127	80	80	80	<20	480	1	1,600	40

and eight strains of rhizobia were tested for intrinsic resistance to eight heavy metals. With few exceptions, the *B. japonicum* and *B. elkanii* strains tested were resistant to relatively high concentrations of most of the metals (Table 2). Our results are in general agreement with those reported by Kinkle *et al.* (13). USDA 123 and USDA 127 proved to be the least tolerant *B. japonicum* strains tested. The MICs of Mo^{2+} were the highest (1,600 to 3,200 μg/ml) for the bradyrhizobia tested. For *B. elkanii* USDA 61, the MICs of seven of the eight tested metals were the highest. Tolerance to Zn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} varied significantly among strains USDA 61, USDA 110, USDA 122, USDA 123, and USDA 127. Tolerance to Cd^{2+} also varied greatly among the bradyrhizobia tested, with MICs ranging from <20 to 80 μg/ml. All of the bradyrhizobia tested were sensitive to Hg^{2+}, with MICs of 1 μg/ml or less.

To be effective selective agents for the bradyrhizobia, heavy metals must inhibit the growth of the phylogenetically related *Rhizobium* strains. Consequently, intrinsic resistance to heavy metals was also examined among several diverse *Rhizobium* species: *R. leguminosarum* bv. viciae Tal 634 and Tal 640, *R. meliloti* Ta1380 and Tal 1372, *R. etli* Tal 182 and Tal 1383, and *R. leguminosarum* bv. trifolii Tal 1820 and Tal 1824. For most of the *Rhizobium* sp. strains examined, the MICs of all of the tested metals were nearly identical, with the exception of those of Mo (Table 3). In contrast to the bradyrhizobia, the *Rhizobium* strains had lower MICs of Zn^{2+}, Co^{2+}, and Cd^{2+} (Table 3). Low MICs of Cu, Al, Ni, Zn, and Cd have also been previously reported for 11 *R. fredii* strains (13). Since *Bradyrhizobium* strains were uniformly more resistant to Zn^{2+} and Co^{2+} than were *Rhizobium* strains, our results suggested that these heavy metals might be useful as selective agents for the direct isolation of bradyrhizobia from soils.

However, since Zn^{2+} MICs varied widely among strains of bradyrhizobia in plate assays, we more precisely evaluated the

TABLE 3. Tolerance of *Rhizobium* strains for heavy metals

Strain	Host	MIC (μg/ml) of:							
		Zn	Co	Cu	Cd	Pb	Hg	Mo	Ni
Tal 634	LH[a]	40	40	80	20	640	1	1,600	80
Tal 640	LW[b]	20	40	80	20	640	<1	6,400	80
Tal 380	MS[c]	40	40	80	40	640	1	6,400	80
Tal 1372	MS	40	40	80	20	640	1	4,800	80
Tal 182	PV[d]	40	20	80	10	640	<1	4,800	80
Tal 1383	PV	40	20	80	10	640	<1	4,800	80
Tal 1820	TR[e]	40	40	80	10	640	<1	3,200	80
Tal 1824	TP[f]	20	40	80	20	640	<1	1,200	80

[a] LH, *Lathyrus hirsutush.*
[b] LW, *Lens culinaris.*
[c] MS, *Medicago sativa.*
[d] PV, *Phaseolus vulgaris.*
[e] TR, *Trifolium repens.*
[f] TP, *Trifolium pratense.*

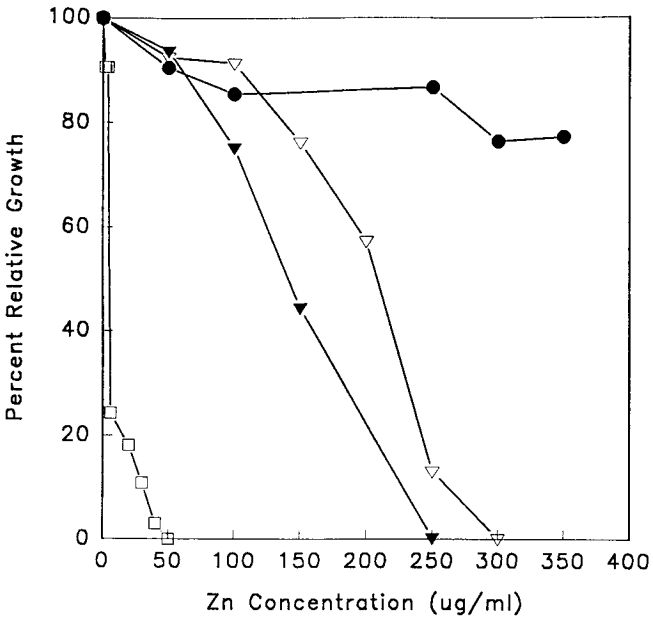

FIG. 1. Growth of *Rhizobium* and *Bradyrhizobium* strains in AG liquid medium containing various concentrations of Zn^{2+}. Symbols: ●, USDA 61; ▽, USDA 110; ▼, USDA 122; □, Tal 380.

impact of Zn^{2+} concentration on the growth of *B. elkanii* USDA 61, *B. japonicum* USDA 110 and USDA 122, and *R. meliloti* Tal 380 growing in liquid culture (Fig. 1). *R. meliloti* Tal 380 had little growth when the Zn^{2+} concentration was greater than 40 μg/ml and less than 25% of the growth of the metal-unamended control medium when the Zn^{2+} concentration reached 10 μg/ml. In contrast, the three *Bradyrhizobium* strains tested (USDA 122, USDA 110, and USDA 61) had between 78 and 90% of the growth of the control culture when the Zn^{2+} concentration reached 100 μg/ml. *B. japonicum* USDA 122 and USDA 110 failed to grow adequately at medium Zn^{2+} concentrations higher than 250 μg/ml. The *B. elkanii* strain tested, USDA 61, proved to be very tolerant to high Zn^{2+} concentrations and had relatively good growth even when Zn^{2+} reached 350 μg/ml in liquid medium (Fig. 1).

Development of the B. japonicum selective medium (BJSM). We inoculated *B. japonicum* and *B. elkanii* strains into Monmouth soil and evaluated their recovery on AG medium amended with 40 μg of Zn^{2+} per ml and 40 μg of Co^{2+} per ml (Table 4). Monmouth soil has previously been shown to contain very low numbers of *B. japonicum* and *B. elkanii* strains (20). The identity of the bacteria recovered was verified by using strain-specific FAs. The metal-amended medium was highly effective in recovering all of the tested bradyrhizobia from soils and did not appreciably reduce viable cell counts relative to AG medium. This medium allowed for the recovery of the more metal-sensitive strains *B. japonicum* USDA 123 and USDA 127 and totally inhibited the growth of the indigenous bacteria present in Monmouth soil. Several grampositive, bacillus-like bacterial colonies; small, compact, colored colonies; and several fungal colonies, however, grew on medium containing 40 μg of Zn^{2+} per ml and 40 μg of Co^{2+} per ml at low soil dilutions (about 10 colonies from the 10^{-1} soil dilution).

To suppress the growth of contaminating gram-positive organisms and fungi, we amended the AG medium containing 40 μg each of Co^{2+} + and Zn^{2+} per ml with 1.0 μg of BG per ml

TABLE 4. Enumeration of *B. japonicum* and *B. elkanii* strains from soils and inoculants with selective media and the MPN plant infection assay

Source	No. of bacteria on following medium[a]: AG	Zn^{2+} + Co^{2+}	BJSM	No. of bacteria determined by plant infection MPN assay
Strains added to Monmouth soil				
USDA 61[b]	9.0×10^7[c]	4.0×10^7	2.4×10^7	1.6×10^8
USDA 110	2.2×10^7	1.9×10^7	1.8×10^7	2.9×10^7
USDA 122	2.1×10^7	7.2×10^6	2.0×10^6	1.6×10^7
USDA 123	6.0×10^7	1.3×10^7	0	ND[d]
USDA 127	1.6×10^7	8.7×10^7	0	ND
Inoculants[e]				
I	4.9×10^8	9.0×10^6	9.0×10^5	1.6×10^8
II	2.1×10^8	4.0×10^6	1.8×10^5	2.9×10^6
III	7.0×10^7	ND	5.5×10^6	1.7×10^7
Soils[f]				
Hubbard	9.0×10^6	8×10^4	1.1×10^4	3.5×10^5
Monmouth	1.0×10^5	0	0	29

[a] The media used were AG medium and AG medium amended with 40 μg of Zn^{2+} per ml and 40 μg of Co^{2+} per ml. BJSM is AG medium amended with 40 μg of Zn^{2+} per ml, 40 μg of Co^{2+} per ml, 1.0 μg of BG per ml, and 500 μg of PCNB per ml.

[b] The *B. japonicum* strains were inoculated into Monmouth soil and incubated for 7 days at 28°C.

[c] Values are means of replicated plates ($n = 2$) or growth pouches ($n = 4$) and are expressed as numbers of bacterial cells per g of soil.

[d] ND, not determined.

[e] Commercial inoculants were used. Inoculant I was Lipha Tech peat based. Inoculant II was Urbana Laboratories/Research Seeds peat based. Inoculant III was Lipha Tech liquid inoculant.

[f] Soils were uninoculated.

and 500 μg of PCNB per ml. We named this medium BJSM, for *B. japonicum* selective medium. These inhibitors have been previously used in selective media for other strains of rhizobia (1, 18). The selective agents effectively inhibited the growth of contaminants (the average number of contaminants ranged from 2 to 5 colonies per plate at the 10^{-1} soil dilution for Monmouth soil). Following recovery from Monmouth soil, viable cell counts of strains USDA 61 and USDA 110 were seemingly unaffected by the inhibitors (Table 4), while viable counts of *B. japonicum* USDA 122 were slightly reduced relative to those in the same medium without the inhibitors. Since BJSM was completely inhibitory for the growth of *B. japonicum* USDA 123 and USDA 127, the results in Table 4 suggested that BJSM selectively recovered bacteria in different serological groups.

BJSM was also tested for the ability to recover bacteria from commercially prepared soybean inoculants and Hubbard soil (Table 4). While the Hubbard soil contained 9.0 x 10^6 total bacteria per g of soil (on AG medium), BJSM recovered about 10^4 bacteria per g of soil (these were tentatively called bradyrhizobia Isee below]). BJSM also allowed for the isolation of 9.0 x 10^5 and 1.8 x 10^5 bacteria per g of peat from the Lipha Tech (formally Nitragin Co.) and Urbana Laboratories peat based inoculants, respectively.

To determine whether other bradyrhizobia were capable of growing on the selective medium, we streak inoculated BJSM with AG-grown cultures of nine additional serologically dis tinct *B. japonicum* and *B. elkanii* strains. Although BJSM allowed growth of *B. japonicum* USDA 4, USDA 38, and USDA 62, it inhibited the growth of strains USDA 129 and USDA 135. With the exception of strain USDA 135, all of the

TABLE 5. Effects of inhibitors on growth of *B. japonicum* USDA 123 and USDA 127[a]

Inhibitor	Concn (μg/ml)	CFU of *B. japonicum* USDA 123	CFU of *B. japonicum* USDA 127
None	0	2.5×10^7	2.9×10^7
BG	0.5	7.4×10^6	2.4×10^7
	1.0	0	4.6×10^6
	1.5	0	0
PCNB	250	3.2×10^6	1.4×10^7
	500	3.0×10^6	4.9×10^6

[a] The values shown are means of duplicate plates after 10 days of growth. *B. japonicum* strains were grown in AG medium supplemented with the different inhibitors at the concentrations indicated.

inhibited strains belong to serocluster 123 (23). All of the *B. elkanii* strains tested (USDA 46, USDA 76, USDA 94, and USDA 130) grew on BJSM. BJSM failed to support the growth of the fast-growing, soybean-nodulating *R. fredii* strains USDA 191, USDA 205, USDA 257, and HH 103, indicating that the ability to nodulate soybeans is not directly correlated with the ability to grow on the selective medium. Other *Rhizobium* species, *R. tropici* CIAT 899 and *R. mehloti* 1021734, also failed to grow on BJSM. Our results indicated that BJSM is capable of supporting the growth of a majority of *Bradyrhizobium* serogroup strains found in soils within the United States.

Selective inhibition of the growth of *B. japonicum* USDA 123 and USDA 127 on BJSM appears to be due to the sensitivity of these strains to the relatively high concentrations of the inhibitors used (Table 5). The AG medium amended with BG at a concentration greater than or equal to 1.0 μg/ml completely inhibited the growth of strain USDA 123 and reduced viable counts of strain USDA 127 by about 1 order of magnitude relative to unamended AG medium. Similarly, PCNB at 500 μg/ml (the concentration used in BJSM) reduced the viable plate counts of USDA 123 and USDA 127 by about 1 order of magnitude relative to unamended AG medium (Table 5). Maximum cell counts for both strains were obtained at BG and PCNB concentrations of 0.5 and 250 μg/ml, respectively. Cycloheximide, at 200 μg/ml, was a useful antifungal substitute for PCNB and allowed isolation of about 100-fold more USDA 123 colonies than did BJSM (data not presented). Results of these studies indicate that BJSM could be modified to include lower concentrations of BG (0.5 μg/ml) and PCNB or substitution of the fungal inhibitor cycloheximide (100 to 150 μg/ml) if greater numbers of serocluster 123 strains were to be isolated.

Comparison of enumeration methodologies. Table 4 also shows the comparison of bacterial counts (expressed as numbers of bacteria per gram of soil) recovered from Monmouth soil individually spike inoculated with *B. japonicum* USDA 61, USDA 110, and USDA 122; uninoculated Hubbard soil; and three commercially prepared soybean inoculants by using the MPN plant infection assay and plate counts on BJSM. In most cases, the MPN counts of *B. japonicum* were about 3 to 10 times greater, but usually about 0.5 order of magnitude higher, than those obtained with BJSM. For USDA 110 and the Urbana Laboratories peat inoculant, MPN values were nearly identical to those determined with BJSM. In most instances, as expected, total bacterial counts on AG medium were greater than MPN or BJSM counts. Nevertheless, MPN count< were generally in close agreement with the bacterial counts obtained with BJSM, with the exception of those obtained wit.

Lipha Tech peat-based inoculant.

TABLE 6. Comparison of BJSM, the FA technique, and the MPN plant infection method for enumeration of *B. japonicum* USDA 110 recovered from Monmouth soil[a]

Incubation time (days)	No. of cells/g determined by:		
	FA count	MPN plant infection assay	BJSM plate count
0	1.5×10^5	2.3×10^4	1.8×10^4
7	7.9×10^4	1.3×10^4	1.0×10^4
Monmouth soil[b]	0	2.9×10^1	0

[a] The values shown are means of duplicate samples.
[b] Uninoculated soil.

A comparison of bacterial counts of strain USDA 110 from spiked Monmouth soil obtained with the FA method, the MPN plant infection assay, and BJSM plate counts is shown in Table 6. While the numbers of strain USDA 110 obtained with the FA technique are generally a little higher than those obtained with the other two methods, the plant infection MPN assay and BJSM plate counting results were virtually identical at both time points. The differences between plate and FA counts have been noted previously *(12)* and could be due to problems associated with plating efficiencies on the selective medium or the FA counting of dead or dying bacteria.

Nodulation ability of soil and inoculant bacteria recovered on BJSM. Small, whitish colonies obtained from soils and inoculants on BJSM were tested for the ability to nodulate soybeans (Table 7). All of the bacteria recovered on BJSM from B. *japonicum*-spiked Monmouth soil were capable of nodulating soybean plants. Moreover, since 98% of the randomly selected bacterial colonies *(*49 of 50) recovered from Hubbard soil on BJSM were also able to nodulate soybean plants, our results indicated that BJSM was capable of effectively isolating about 10^4 soybean-nodulating bacteria per g of soil among the total of 9×10^6 bacteria per g of soil. Moreover, our results indicated that contrary to what was reported for R. *leguminosarum* soil isolates (24, 26), *B. japoni*cum strains isolated from soils are relatively stable for nodulation ability.

High percentages of soybean-nodulating bacteria were also recovered from the Lipha Tech Company peat- and liquid based inoculants (Table 7), suggesting that BJSM is useful for quantifying bradyrhizobia in commercial and laboratory inoculants. However, only 55% of the BJSM-recovered colonies from the Urbana Laboratories peat-based inoculant had the ability to nodulate soybean plants. The exact reason for the low level of nodulation ability among the bacteria recovered from the Urbana Laboratories inoculant is unknown. One plausible explanation is that the peat carrier used contains bacteria (or a bacterium) that are highly resistant to the selective agents used and morphologically resemble *B. japonicum* colonies growing on BJSM.

TABLE 7. Nodulation of soybean cv. Kasota by *B. japonicum* strains recovered from soils and inoculants with BJSM medium

Source	No. tested	No. nodulating	% Nodulation
USDA 61 from Monmouth soil[a]	20	20	100
USDA 110 from Monmouth soil	20	20	100
USDA 122 from Monmouth soil	20	20	100
Lipha Tech peat-based inoculant	20	19	95
Urbana Laboratories peat-based inoculant	20	11	55
Lipha Tech liquid inoculant	60	60	100
Hubbard soil[b]	50	49	98

[a] *B. japonicum* strains were inoculated into nonsterile Monmouth soil to obtain about 10^7 cells per g of soil. Soils were incubated for 7 days at 28°C, and were recovered on BJSM.
[b] Indigenous strains.

Taken together, the results of our studies indicate that non-antibiotic-containing BJSM is useful for the direct isolation of *B. japonicum* and *B. elkanii* from natural soils and legume inoculants and that this medium may be an important tool for autecological studies. In addition, since BJSM can be used to isolate and quantify bradyrhizobia irrespectively of serological group or resistance to antibiotics or phage, the medium will allow ecological examination of diverse populations of bradyrhizobia at the genus level. Moreover, since plant infection MPN assays are very expensive and time consuming and often delay acquisition of results when used in large-scale studies and inoculant quality control programs, the newly described *Bradyrhizobium* selective medium could prove to be the method of choice for cheap and relatively rapid enumeration of bradyrhizobia in soils and inoculants.

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ADDENDUM IN PROOF

After this paper was accepted, we learned of another reported medium for the selective isolation of B. *japonicum* from soils (R. R. Gault and E. A. Schwinghamer, Soil Biol. Biochem. *25:1161-1166, 1993).*

REFERENCES

1. **Barber, L. E.** 1979. Use of selective agent for recovery of *Rhizobium meliloti* from soil. Soil Sci. Soc. Am. J. 43:1145-1147.
2. **Brynhildsen, L., and T. Rosswall.** 1989. Effects of cadmium, copper, magnesium, and zinc on the decomposition of citrate by a *Klebsiella sp.* Appl. Environ. Microbiol. 55:1375-1379.
3. **Cregan, P. B., and H. H. Keyser.** 1986. Host restriction of nodulation by *Bradyrhizobium japonicum* strain USDA 123. Crop Sci. 26:911-916.
4. **Danso, S. K. A., M. Habte, and M. Alexander.** 1973. Estimating the density of individual bacterial populations introduced into natural ecosystems. Can. J. Microbiol. 19:1145-1451.
5. **Dean-Ross, D., and A. L. Mills.** 1989. Bacterial community structure and function along a heavy metal gradient. Appl. Environ. Microbiol. 55:2002-2009.
6. **Diels, L., and M. Mergeay.** 1990. DNA probe-mediated detection of resistant bacteria from soils highly polluted by

dissolution of transition and heavy metal oxides. Appl. Environ. Microbiol. 54:1009-1014.

9. **Giller, K. E., S. P. McGrath, and P. R. Hirsch.** 1989. Absence of nitrogen fixation in clover grown on soil subject to long-term contamination with heavy metals is due to survival of only ineffective *Rhizobium*. Soil Biol. Biochem. 21:841-848.
10. **Graham, P. H**. 1969. Selective medium for growth of *Rhizobium*. Appl. Microbiol. 17:769-770.
11. **Hermansson, M., G. W. Jones, and S. Kjelleberg.** 1987. Frequency of antibiotic and heavy metal resistance, pigmentation, and plasmids in bacteria of the marine air-water interface. Appl. Environ. Microbiol. 53:2338-2342.
12. **Kingsley, M. T., and B. B. Bohlool.** 1981. Release of *Rhizobium* spp. from tropical soils and recovery for immunofluorescence enumeration. Appl. Environ. Microbiol. 42:241-248.
13. **Kinkle, B. K., J. S. Angle, and H. H. Keyser.** 1987. Long-term effects of metal-rich sewage sludge application on soil populations of *Brad yrhizobium japonicum*. Appl. Environ. Microbiol. 53:315-319.
14. **Nies, D., M. Mergeay, B. Friedrich, and H. G. Schlegel.** 1987. Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus* CH34. J. Bacteriol. 169:48654868.
15. **Nies, D. H., A. Nies, L. Chu, and S. Silver.** 1989. Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. *Proc. Natl. Acad. Sci. USA* 86:7351-7355.
16. **Nies, D. H., and S. Silver**. 1989. Plasmid-determined inducible efflux is responsible for resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus*. J. Bacteriol. 171:896-900.
17. **Nies, D. H., and S. Silver**. 1989. Metal ion uptake by a plasmid-free metal-sensitive *Alcaligenes eutrophus* strain. J. Bacteriol. 171:4073-1075.
18. **Pattison, A. C., and F. A. Skinner.** 1974. The effect of antimicrobial substances on *Rhizobium* spp. and their use in selective media. J. Appl. Bacteriol. 37:230-250.
19. **Postma, J., J. D. van Elsas, J. M. Govaert, and J. A. van Veen.** 1988. The dynamics of *Rhizobium leguminosarum* biovar *trijblii* introduced into soil as determined by immunofluorescence and selective plating techniques. FEMS Microbiol. Ecol. 53:251-260. 20. Sadowsky, M. J., P. B. Cregan, and H. H. Keyser. 1990. A DNA hybridization probe for use in determining restricted nodulation among *Bradyrhizobium japonicum* serocluster 123 field isolates. Appl. Environ. Microbiol. 56:1468-1474.
21. **Sadowsky, M. J., R. E. Tully, P. B. Cregan,** and H. H. Keyser. 1987. Genetic diversity in *Bradyrltizobitun japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybeans. Appl. Environ. Microbiol. 53:2624-2630.
22. **Schmidt, E. L., R. O. Bankole, and B. B. Bohlool.** 1968. Fluores-cent-antibody approach to study of rhizobia in soil. J. Bacteriol. 95:1987-1992.
23. **Schmidt, E. L., M. J. Zidwick, and H. H. Abebe.** 1986. Bradyrhi-zobium *japonicum* serocluster 123 and diversity among member isolates. Appl. Environ. Microbiol. 51:1212-1215.
24. **Segovia, L., D. Pinero, R. Palacios, and E. Martinez-Romero**. 1991. Genetic structure of a soil population of nonsymbiotic *Rhizobium leguminosarum*. Appl. Environ. Microbiol. 57:426-333. 25. Silver, S., and T. K. Misra. 1988. Plasmid-mediated heavy metal resistances. Annu. Rev. Microbiol. 42:717-743.
26. **Soberon-Chavez, G., and R. Najera.** 1989. Isolation from soil of *Rhizobium leguminosarum* lacking symbiotic information. Can. J. Microbiol. 35:464-468.
27. **Somasegaran, P., and H. J. Hoben.** 1985. Methods in legume-*Rhizobium* technology. NifTAL Project, Paia, Hawaii.
28. **Thompson, J. A., and J. M. Vincent.** 1967. Methods of detection and estimation of *Rhizobia* in soil. Plant Soil. 26:72-84.
29. **Top, E., M. Mergeay, D. Springael, and W. Vestrate.** 1990. Gene escape model: transfer of heavy metal resistance genes from *Escherichia* coli to *Alcaligenes eutrophus* on agar plates and in soil samples. Appl. Environ. Microbiol. 56:2471-2479.
30. **Van Schreven, D. A.** 1970. Some factors affecting growth and survival of *Rhizobium spp. in* soil-peat cultures. Plant Soil 32:113130.
31. **Woomer, P. L., P. W. Singleton, and B. B. Bohlool**. 1988. Reliabil-ity of the most-probable-number technique for enumerating rhizobia in tropical soils. Appl. Environ. Microbiol. 54:1494-1497.