APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2001, p. 65–74 Vol. 67, No. 1
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.1.65–74.2001

Novel Tellurite-Amended Media and Specific Chromosomal and Ti Plasmid Probes for Direct Analysis of Soil Populations of *Agrobacterium* Biovars 1 and 2

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Received 22 May 2000/Accepted 16 October 2000

Ecology and biodiversity studies of *Agrobacterium* spp. require tools such as selective media and DNA probes. Tellurite was tested as a selective agent and a supplement of previously described media for agrobacteria. The known biodiversity within the genus was taken into account when the selectivity of K_2TeO_3 was analyzed and its potential for isolating *Agrobacterium* spp. directly from soil was evaluated. A K_2TeO_3 concentration of 60 ppm was found to favor the growth of agrobacteria and restrict the development of other bacteria. Morphotypic analyses were used to define agrobacterial colony types, which were readily distinguished from other colonies. The typical agrobacterial morphotype allowed direct determination of the densities of agrobacterial populations from various environments on K_2TeO_3-amended medium. The bona fide agrobacterium colonies growing on media amended with K_2TeO_3 were confirmed to be *Agrobacterium* colonies by using 16S ribosomal DNA (rDNA) probes. Specific 16S rDNA probes were designed for *Agrobacterium* biovar 1 and related species (*Agrobacterium rubi* and *Agrobacterium fici*) and for *Agrobacterium* biovar 2. Specific pathogenic probes from different Ti plasmid regions were used to determine the pathogenic status of agrobacterial colonies. Various morphotype colonies from bulk soil suspensions were characterized by colony blot hybridization with 16S rDNA and pathogenic probes. All the *Agrobacterium*-like colonies obtained from soil suspensions on amended media were found to be bona fide agrobacteria. Direct colony counting of agrobacterial populations could be done. We found 10^3 to 10^4 agrobacteria · g of dry soil^{-1} in a silt loam bulk soil cultivated with maize. All of the strains isolated were nonpathogenic bona fide *Agrobacterium* biovar 1 strains.

The ecology and biodiversity of *Agrobacterium* have been studied mainly by using collections of isolates from crown gall tumors. However, soil agrobacteria are usually nonpathogenic, and a better understanding of agrobacteria in soil habitats is necessary. Media suitable for studying low concentrations of agrobacteria in soil are still needed in spite of earlier attempts to produce them (for a review see reference 18). The percentage of cells recovered with some media depends upon the agrobacterial genotype (15), and such media should not be used to study the biodiversity of agrobacteria. Other media, such as those described by Brisbane and Kerr (6), do not result in significant differences in the percentage of cells recovered and can be used for biodiversity studies. Most of these media have been developed for isolating *Agrobacterium* from rich soils or tumors, and they are not selective enough to inhibit the growth of undesired microorganisms from biotopes containing relatively low concentrations of agrobacteria. Agrobacterium-like colonies selected by visual inspection also require additional tests to ensure that they are bona fide *Agrobacterium* colonies. As a result, agrobacterial density cannot be determined by direct counting.

Kinkle et al. (14) showed that several *Rhizobium* species were resistant to selenite and tellurite. Incorporation of selenite and tellurite into growth media has allowed direct isolation of *Rhizobium meliloti* from soil (14). The genera *Agrobacterium* and Rhizobium are close relatives. Thus, incorporation of selenite, which is present at a low concentration in the media of Brisbane and Kerr, or tellurite might improve the selectivity of media used to isolate *Agrobacterium* spp. However, such media could be used to study biodiversity only if the added oxidative metalloids did not significantly alter recovery of any of the agrobacterial genotypes.

Several bona fide species of the genus *Agrobacterium* and some putative new species not completely described yet have been identified by conventional morphological and biochemical analysis and by DNA-DNA hybridization studies. A relationship has been established between the classic assignments of agrobacteria in biovars (12) and the species designations, as follows: *Agrobacterium vitis* for biovar 3 (25), *Agrobacterium rhizogenes* for biovar 2, and *Agrobacterium radiobacter* for biovar 1 (35). This latter name was contested by Bouzar (3), who proposed *Agrobacterium tumefaciens* instead. Notwithstanding this, exhaustive studies have shown that there are at least nine genomic species within biovar 1 alone (31). Thus, the general term biovar 1 as defined by Keane et al. (12) is used in this paper to designate a cluster of closely related genomic species that includes, but is not restricted to, *A. tumefaciens* sensu Bouzar (3). Two other putative species of *Agrobacterium* still remain to be completely described. One putative species includes agrobacteria related to strain NCPPB1650 (13, 35). The other consists of agrobacteria isolated from weeping fig trees (5), which have been named *Agrobacterium fici* in the Biolog

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catalog. The species and biovar designations have been corroborated by 16S rRNA (*rrs*) analysis (5, 35, 41). All bona fide and putative *Agrobacterium* species contain specific *rrs* sequences. As a result, the *rrs* gene is now routinely used to identify the main species or biovars of newly isolated *Agrobacterium* strains, for instance, restriction fragment length polymorphism analysis by PCR (24, 30). Specific oligonucleotide probes based on variable parts of the *rrs* gene can thus be designed for rapid, accurate species identification on colony blots.

Pathogenic agrobacteria also occur in soils (3). As pathogenicity requires a large plasmid designated the Ti plasmid, some of the regions of this plasmid are routinely targeted by PCR amplification in order to identify pathogenic strains (29, 30). DNA probes based on the same Ti plasmid regions used in these PCR screening analyses can also be used to detect Ti plasmids on colony blots.

Here, we investigated whether media amended with selenite or tellurite are suitable for both direct counting and isolation of bona fide agrobacteria from soil. Most of the presently known biodiversity of *Agrobacterium* spp. was considered in order to evaluate the resistance of individual strains to selenite and tellurite and the effects of the two additives on cell recovery. Chromosome and Ti plasmid probes were used to establish the agrobacterium and pathogenicity status of the agrobacterium-like colonies isolated from soil by using amended or unamended media.

MATERIALS AND METHODS

Bacterial strains and media. The strains of *Agrobacterium* spp. listed in Table 1 include representatives of all bona fide species plus representatives of putative new species and members of heterogeneous biovar 1, as described by Popoff et al. (31). Most strains used in this study that were isolated from the same host were confirmed to be genotypically different by using molecular methods described by Ponsonnet and Nesme (30), and these strains reflected the wide diversity of pathogenic populations involved in crown gall outbreaks (4, 29). Pathogenicity was tested by inoculating standard host plants as previously described (30). Bacteria were grown at 28°C for 48 h on nonselective MG agar medium and for 5 days on 1A medium (selective for all biovar 1 strains) and 2E medium (selective for *A. rhizogenes*) (6, 12). Strains were also grown overnight in LPG broth (30).

MICs of potassium selenite and potassium tellurite. The MICs of tellurite for pure cultures were determined on MG medium, as well as 1A and 2E media (selective for *Agrobacterium* biovars 1 and 2, respectively). Twofold serial dilutions were prepared with 0.9% (wt/vol) NaCl, and dilutions were plated onto MG, 1A, and 2E media with or without K_2TeO_3. A stock solution (100 μg ml^{-1}) of K_2TeO_3 was prepared in ultrapure water and sterilized by filtration. Each strain was tested with different concentrations of metal. The experiment was performed by using three plates per dilution, and the plates were incubated for 7 days.

Soil. The 10- to 30-cm superficial layer of a standard silt loam soil from a maize field close to Lyon (La-Côte-Saint-André, France) was used as a source of soil. Two soil samples, one collected in May 1998 and the other collected in July 1998, were sieved through a <2-mm mesh. The soil properties were as follows: 17% clay, 35.3% loam, 47.7% sand, and 2% organic matter; pH (water) 7; and water-holding capacity, 25.8 g of H_2O 100 g (dry weight)$^{-1}$ (32).

Counting the *Agrobacterium* population in soil. The microorganisms were extracted from 5-g portions of soil by blending samples with 50 ml of sterile distilled water for 90 s in a blender (Waring Commercial, New Hartford, Conn.). The resulting soil suspensions were serially diluted in sterile distilled water, and 100-μl aliquots of appropriate dilutions were spread on agar plates. Three plates were inoculated per dilution.

Total viable heterotrophic bacteria were counted on Trypticase soy agar (TSA) (Gibco BRL, Rockville, Md.) diluted 1/10. Agrobacteria were counted on 1A and 2E media with or without 80 μg of K_2TeO_3 per ml. Cycloheximide (200 mg · liter^{-1}) was used as an antifungal agent. All counting was done after incubation for 3 and 5 days at 28°C. Data were expressed as means and standard errors of the means based on three independent replicate determinations. *Agrobacterium*-like colonies were purified by suspending individual colonies for at least 30 min in sterile distilled water and then streaking them on LPG agar. The process was repeated until all colonies appeared to be homogeneous. Production of 3-ketolactose (2) and production of acid from erythritol were used to separate the strains into biovars 1 and 2 (11).

DNA probes, PCR, and hybridization conditions. DNA oligonucleotide probes were designed by comparing nine 16S rRNA sequences (from four biovar 1 strains, one strain of *Agrobacterium rubi*, one strain isolated from *Ficus benjamina* [*A. fici*], and three biovar 2 strains). The sequences were compared by using the multiple-alignment ClustalW algorithm (40). Consensus probes F639*rrs*AT41 (AAACCCCGAATGTCAAGAGC) and F640*rrs*AT42 (ATACCC CGAATGTCAAGAGC) were designed to detect the cluster containing *A. tumefaciens*, *A. rubi*, and *Agrobacterium* isolated from *F. benjamina*. F641*rrs*AR5 (CCATATCTCTACGGGTAACA) was designed to detect *A. rhizogenes*. DNA probes were defined by using OLIGO software (33), and their specificities for the targets were confirmed by a BLASTn analysis performed with the GenBank database (1). The specificities of the DNA probes were tested with collection strains by using a slot blot technique with 16S rDNA PCR products obtained with primers FGPS6 and FGPS1509′, exactly as described by Ponsonnet and Nesme (30). The oligonucleotide probes were synthesized by Eurogentec (Seraing, Belgium). Synthetic DNA oligonucleotide probes were 3′ end labelled by using a DNA tailing kit (Boehringer Mannheim, Meylan, France) with [α-^{32}P]dATP (NEN Life Science Products, Boston, Mass.) at a specific activity of 6,000 Ci/mmol according to the manufacturer's recommendations. Unincorporated nucleotides were removed with a Qiaquick column, as recommended by the manufacturer (Qiagen S.A., Courtaboeuf, France).

The PCR DNA pathogenicity probes consisted of three regions of the Ti plasmid. These probes were amplified from genomic DNA of strain C58 by using primers FGP*tmr*530 and FGP*tmr*701′ (for the tumor morphology root [*tmr*] probe) and primers FGP*nos*1236′ and FGP*nos*975 (for the nopaline synthase [*nos*] probe, specific for nopaline pTi20). These two DNA probes corresponded to genes on transferred DNA T-DNA. The virulence (inter-*vir*) DNA probe was obtained with a pair of primers designed to amplify the *virB-virG* intergene using F749 (GCTAGCTTGGAAGATCGCAC) (this study) and FGP*vir*G15′ by using the sequence of a conserved region of *virB11* in order to amplify the *virB-virG* intergene of all of the Ti and Ri plasmids sequenced (data not shown). Two genomic DNAs were used to amplify this probe: the genomic DNA of C58 (pTiC58, nopaline type of Ti plasmid) and the genomic DNA of B6 (pTiB6, octopine type of Ti plasmid). The PCR conditions used were those described by Nesme et al. (21) and Picard et al. (27, 28). The PCR DIG probes were obtained by incorporating digoxigenin-11-dUTP (Roche Diagnostic, Basel, Switzerland) during PCR. Labelling was performed by using the reaction conditions recommended by the manufacturer. The specificities of the probes were tested with a collection of strains.

Colony hybridization. Pure colonies were transferred directly onto nylon membranes (GeneScreen Plus; NEN Research Products, Boston, Mass.). Colonies were lysed as described by Sambrook et al. (34), with some modifications. The filters were first wetted with 0.6% (wt/vol) lysozyme in 10 mM Tris-HCl–1 mM EDTA (dissodium salt dihydrate), (pH 8) for 15 min, and lysis was performed for 10 min in 10% (wt/vol) sodium dodecyl sulfate (SDS). The preparations were denatured for 10 min in denaturation solution (0.5 N NaOH, 1.5 M NaCl) and neutralized for 10 min in neutralizing solution (1 M Tris [pH 7.5], 1.5 M NaCl). Finally, the nylon membranes were soaked for 10 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). The transferred DNA was cross-linked by irradiation with UV light for 3 min, and the membranes were treated with proteinase K (2 mg ml^{-1} in 2× SSC) for 1 h at 37°C. For oligonucleotide hybridization, baked membrane filters were placed in 50 ml of prehybridization solution (1% [wt/vol] SDS, 10% [wt/vol] dextran sulfate, 2× SSC, 5 μg of denatured herring sperm DNA per ml, 2 μg of tRNA per ml and incubated for 2 h at the hybridization temperature (47°C). The prehybridization solution was discarded, and the membranes were incubated in the hybridization solution (prehybridization solution plus probe) overnight at the hybridization temperature. The membranes were washed twice in 2× SSC for 10 min at room temperature, once in 2× SSC–1% SDS for 10 min at the hybridization temperature, twice in 1× SSC–0.1% SDS at the hybridization temperature, and once in 0.1× SSC–0.1% SDS at the hybridization temperature. They were then exposed to X-ray film for autoradiography for 4 h to locate individual positive colony signals. Hybridization with PCR DIG probes was performed by using the reaction conditions recommended by the manufacturer (Roche Diagnostic).

TABLE 1. *Agrobacterium* strains used in this study

Strain[a]	Opine type produced in tumor[b]	Host plant or other properties	Primary designation and/or origin	MIC (mg · liter^{-1}) of:		16S rDNA probe[e]	Hybridization with plasmid Ti DNA probes[f]			
							vir			
				Na_2SeO_3[c]	K_2TeO_3[d]		*tmr*	*nos*	Plasmid Ti nopaline type	Plasmid Ti octopine type
Biovar 1										
Pathogenic strains										
CFBP2413^T	O/M	Apple tree	B6, United States	8,000	320	1	+	−	+	+
Ach5	O/M	*Prunus* sp.	United States (Y. Dessaux, France)[h]	8,000	320	1	+	−	+	+
CFPB2407	C/O	*Vitis vinifera* cv. Danom	France	8,000	320	1	+	−	+	−
CFBP1903	N	*Prunus cerasus*	C58, New York	8,000	320	1	+	+	+	+
CFBP1901	N	C58 with pTiT37	(Y. Dessaux, France)[h]	8,000	320	1	+	+	+	+
CFBP354	N	ND	B10, France	8,000	160	1	+	+	+	+
CFBP1904	N	*Vitis vinifera*	AG20, Greece	8,000	320	1	+	+	+	+
CFBP2410	N	*Populus* sp.	M22, ND	8,000	160	1	+	+	+	+
CFBP2411	N	*Salix purpurea*	CG4, New Zealand	8,000	160	1	+	+	+	+
CFBP2177 (6)	N	*Populus* section *Leuce*	France	8,000	160	1	+	+	+	+
CFBP2516 (5)	N	*Populus* section *Leuce*	France	8,000	320	1	+	+	+	+
A96.11	N	*Populus* section *Leuce*	France	ND	ND	1	+	+	+	+
A134.2 (5)	N	*Populus* section *Leuce*	France	ND	160	1	+	+	+	+
M10 (4)	N	*Populus* section *Leuce*	France	ND	320	1	+	+	+	+
S56	M	ND[g]	France (G1)[i]	ND	160	1	−	−	−	−
S377	ND	ND	France (G1)[i]	ND	160	1	−	−	−	−
S4	N	Black raspberry	France (G1)[i]	ND	160	1	+	+	+	+
ATCC 4720	ND	ND	United States (G1)[i]	ND	160	1	+	−	−	−
NCPPB925	ND	*Dahlia* sp.	South Africa (G6)[i]	ND	160	1	−	−	−	−
F/1Zutra	ND	*Dahlia* sp.	Israel (G6)[i]	ND	160	1	+	−	−	−
3/1Zutra	ND	Apple tree	Israel (G7)[i]	ND	160	1	−	−	−	−
NCPPB1641	ND	*Flacourtia ramontchi*	United Kingdom (G7)[i]	ND	160	1	−	−	−	−
T37	N	Walnut	United States (G8)[i]	ND	160	1	+	+	+	+
ICPB TT9	ND	Hop	United States (G8)[i]	ND	160	1	+	+	+	+
6 Mushin	ND	Hop	Australia (G8)[i]	ND	160	1	+	+	+	+
O362	ND	Soil	South Australia (G9)[i]	ND	160	1	+	+	+	+
2T3Pb (3)	ND	Walnut	Spain (M. Lopez, Spain)[h]	ND	160	1	+	+	+	+
6MS3	ND	Walnut	Spain (M. Lopez, Spain)[h]	ND	320	1	+	+	+	+
436.3SA	M	*Prunus* hybrid cv. GF677	Spain (M. Lopez, Spain)[h]	ND	320	1	+	−	+	+
CFBP296	N	*Lycopersicon esculentum*	111, France	8,000	160	1	−	−	−	−
M15	N	*Populus* section *Leuce*	France	8,000	160	1	−	−	−	−
Nonpathogenic strains										
CFBP2414^T	NA[j]	Unknown	3-24-2, The Netherlands	8,000	160	1	−	−	−	−
C58C1	NA	C58 cured of pTiC58		8,000	160	1	−	−	−	−
GMI9023	NA	C58 cured of pTiC58 and pAtC58	(Y. Dessaux, France)[h]	8,000	160	1	−	−	−	−
CFBP2518	NA	*Populus* section *Leuce*	France	8,000	160	1	−	−	−	−
O363	NA	Soil	Australia (G9)[i]	ND	160	1	−	−	−	−
CFBP2456	NA	ND	CDC B6016, United States	ND	ND	1	−	−	−	−
CFBP2457	NA	ND	CDC B3771, United States	ND	ND	1	−	−	−	−
RV3	NA	ND	ND (G7)[i]	ND	80	1	−	−	−	−
CIP28-75 (3)	NA	Human	France (G2)[i]	ND	ND	1	−	−	−	−
CIP111-78	NA	Human	France (G3)[i]	ND	ND	1	−	−	−	−
CFBP2454	NA	Human	France	ND	ND	1	−	−	−	−
CFBP2458	NA	ND	M2/1, ND	8,000	160	1	−	−	−	−
CFBP2241	NA	Human	CDC 7258, ND	ND	ND	1	−	−	−	−
CFBP2243	NA	Human	CDC A6597, United States	ND	ND	1	−	−	−	−
Biovar 2										
Pathogenic strains										
CFBP2408^T	A/M	ND	ND	ND	ND	2	−	−	+	+
CFBP450	ND	*Malus pumila* cv. M IX	France	2,000	1,280	2	−	−	−	−
CFBP1804	N	*Prunus persicae* cv. GF305	France	4,000	1,280	2	+	+	+	+
CFBP1905	N	*Vitis vinifera*	AG28, Greece	4,000	1,280	2	+	+	+	+
CFBP1936	N	*Rosa* sp.	Tahiti	2,000	1,280	2	+	+	+	+
CFBP1961	N	*Populus bolleana*	France	4,000	1,280	2	+	+	+	+
CFBP1962	N	*Prunus cerasus* cv. Mahaleb	Spain	4,000	1,280	2	+	+	+	+
CFBP2178	N	*Prunus avium*cv. F12-1	France	12,000	1,280	2	+	+	+	+
CFBP2417 (3)	N	Cherry hybrid cv. Colt	France	6,000	1,280	2	+	+	+	+
CFBP2519 (3)	N	*Populus* section *Leuce*	France	4,000	1,280	2	+	+	+	+
C104.12	N	*Populus* section *Leuce*	France	6,000	1,280	2	+	+	+	+
C104.22 (8)	N	*Populus* section *Leuce*	France	ND	1,280	2	+	+	+	+
M120	N	*Populus* section *Leuce*	France	ND	640	2	+	+	+	+

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TABLE 1—*Continued*

Strain[a]	Opine type produced in tumor[b]	Host plant or other properties	Primary designation and/or origin	MIC (mg · liter^{-1}) of:		16S rDNA probe[e]	Hybridization with plasmid Ti DNA probes[f]			
							tmr	*nos*	*vir*	
				Na_2SeO_3[c]	K_2TeO_3[d]				Plasmid Ti nopaline type	Plasmid Ti ocotpine type
Nonpathogenic strains										
CFBP1937	NA	Soil	K84, Australia	ND	ND	2	−	−	−	−
CFBP2520	NA	*Populus* section *Leuce*	France	4,000	640	2	−	−	−	−
Biovar 3										
Pathogenic strains										
CFBP2512	N	*Vitis vinifera*	565-5, Spain	10,000	1,280	−	+	+	+	(+)
CFBP2620	N	*Vitis vinifera*	K374, Australia	14,000	1,280	−	+	+	+	(+)
CFBP2618	C/O	*Vitis vinifera* cv. Cabernet	85-255, France	8,000	1,280	−	+	−	+	−
CFBP2622	C/O	*Vitis vinifera* cv. Sultana	Ag63, Greece	14,000	1,280	−	+	−	+	(+)
CFBP2621	C/O	*Vitis vinifera* cv. Sultana	Ag57, Greece	14,000	1,280	−	−	−	−	−
A. rubi										
Pathogenic strain										
CFBP999^T	N	*Rubus* sp.	TR2, United States	8,000	160	1	+	+	+	+
A. fici										
AF3.44		*Ficus benjamina*	United States (H. Bouzar, United States)	8,000	320	1	ND	ND	ND	ND

[a] CFBP, Collection Française de Bactéries Phytopathogènes, Institut National de la Recherche Agronomique, Angers, France; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom; ICPB, International Colleciton of Phytopathogenic Bacteria, Davis, Calif.; CIP, Collection de l'Institut Pasteur, Paris, France; ATCC, American Type Culture Collection, Manassas, Va. The numbers in parentheses indicate the numbers of strains with the same origin and the same relevant characteristics that gave the same MIC and hybridization results. The groups of strains were as follows: CFBP2177, B100.11, M9, 85.2, 85.6, and 85.104; CFBP2516, A134.6, M292, X88.283, and 85.66; A134.2, A134.3, M214, X88.299, and 85.49; M10, X88.293, X88.303, and 85.52; 2T3Pb, 1C3Pb, and 2T3Sa; CIP28-75, CIP43-76, and CIP127-76; CFBP2417, CFBP2418, and CFBP2419; CFBP2519, 85.100, and 85.186; and C104.22, M3, M32, M84, M111, 85.30, 85.120, and 85.123.

[b] O/M, octopine/mannopine; N, nopaline; M, mannopine; C/O, cucumopine/octopine; A/M, agropine/mannopine.

[c] All Se MICs were determined on MG medium.

[d] Te MICs were determined by using 1A medium for biovar 1, 2E medium for biovar 2, and MG medium for biovar 3, *A. rubi*, and *A. fici.*

[e] 1, hybridization with the biovar 1 cluster chromosomal probes; 2, hybridization with the biovar 2 chromosomal probe for 16S DNA analysis.

[f] +, hybridization; −, no hybridization; (+), weak hybridization.

[g] ND, not determined.

[h] The information in parentheses indicates the person who provided the strain.

[i] G1 to G3 and G6 to G9 correspond to genomic species defined by Popoff et al. (31).

[j] NA, not applicable.

RESULTS AND DISCUSSION

Direct counting of agrobacterial populations and studies of the genetic structure of isolated strains require new selective media that ensure recovery of sparse populations without producing any significant differences between agrobacterial genotypes. We investigated the suitability of selenite and tellurite for this purpose by testing the responses of strains chosen to obtain the greatest possible biodiversity of *Agrobacterium* spp.

Resistance of agrobacteria to Na_2SeO_3. As indicated by Lippincott et al. (15), agrobacterial colonies growing on 1A and 2E media develop an orange-brown to red-brown pigmentation. This red coloration is produced by all of the agrobacteria tested and is presumably due to reduction of the added Se compound to its elemental form. Selenite reduction was thus used to improve medium selectivity. However, the original concentration of Na_2SeO_3 in 1A and 2E media (0.1 g · liter^{-1}) was not high enough to effectively control the competing microflora when the density of agrobacteria in the soil was low (less than 10^{-4} CFU · g of soil^{-1}). The selenite concentration in the medium could not be increased because MIC studies have shown that the resistance of agrobacterial strains to Na_2SeO_3 varies considerably (the MICs range from 2 to 14 g · liter^{-1}) (Table 1), while complete control of the competing microflora requires at least 10 g · liter^{-1} (data not shown). As reduction of the Se compound is generally associated with reduction of other metal salts that are much more toxic, such as K_2TeO_3 (14), the latter compound was used to improve the selectivity of the 1A and 2E media used to isolate agrobacteria directly from soils.

Resistance of agrobacteria to K_2TeO_3. Here, we show for the first time that agrobacteria are resistant to K_2TeO_3 (Table 1). Agrobacteria growing on amended media had the typical colony morphology (convex, glistening, circular with entire edges) but a typical black color (Fig. 1), probably due to intracellular accumulation of black crystals of metallic tellurium (16, 38). Other members of the alpha subdivision of the *Proteobacteria*, such as *Rhodobacter* spp. *Rhodopseudomonas palustris*, *Bradyrhizobium* spp., and *Rhizobium* spp., were also found to be resistant to tellurite (19).

Resistance to tellurite was studied by determining the MICs of K_2TeO_3 for 76 strains selected to represent most of the diversity presently known in the genus *Agrobacterium*. Special emphasis was placed on representatives of biovar 1 and biovar 2 because these agrobacteria are the organisms most frequently isolated from crown gall tumors in fruit and forest tree nurseries. The MICs of K_2TeO_3 varied from 640 to 1,280 μg ml^{-1} for biovar 2 and from 80 to 320 μg ml^{-1} for biovar1, *A. rubi*, *A vitis*, and *A. fici* (Table 1) in all of the amended media (MG, 1A, and 2E media). Thus, the closely related organisms *A. tumefaciens*, *A. rubi* and *A. fici* could be selectively isolated by using the same K_2TeO_3 concentration with the same medium (1A medium). Hence, K_2TeO_3 concentrations of 60 to 80 μg · ml^{-1} should allow growth of almost all agrobacteria.

We estimated the sampling bias caused by adding tellurite to

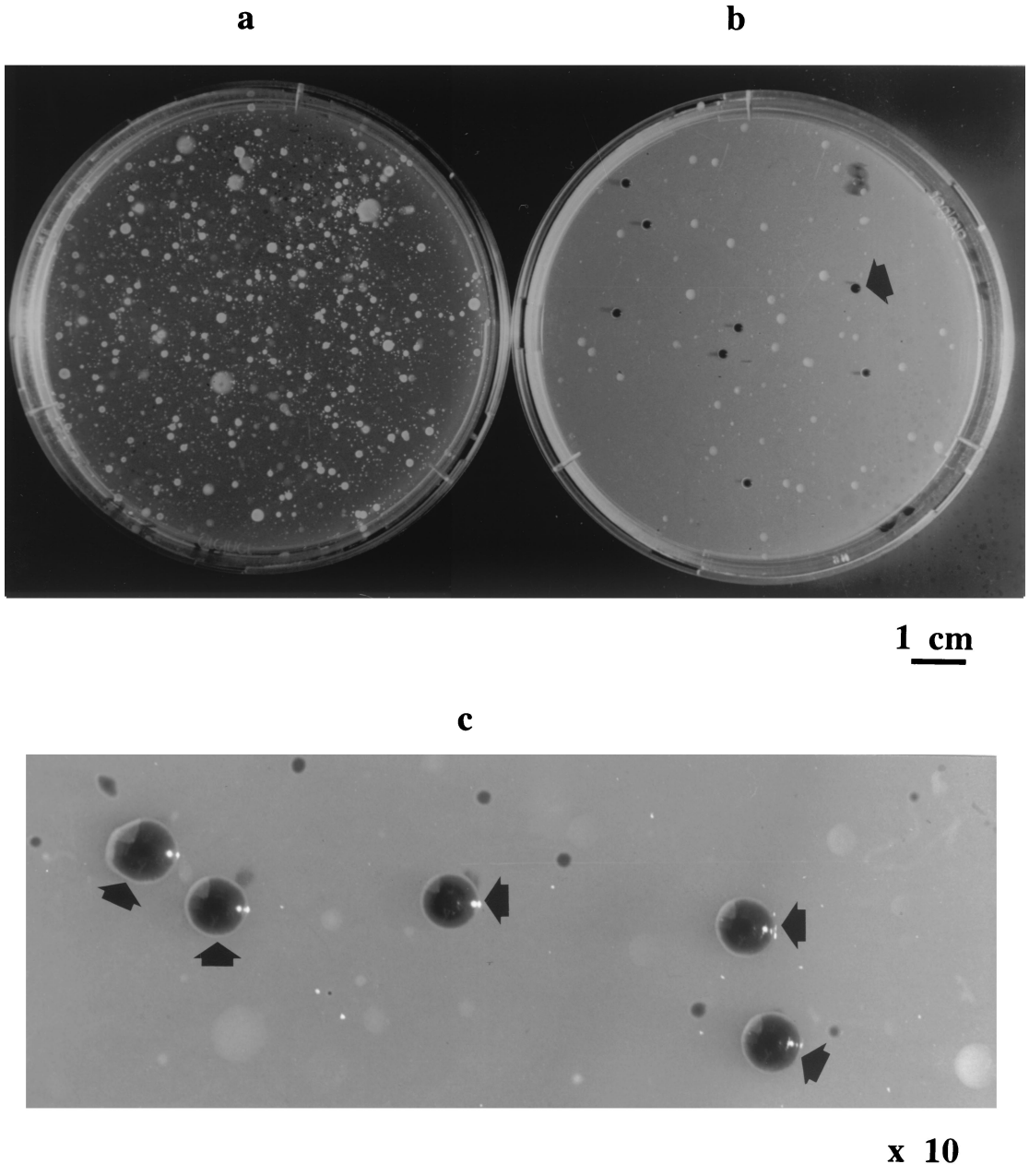

FIG. 1. Plating of a 10^{-1} dilution suspension of soil on 1A medium (a) or 1A medium amended with 60 ppm of K_2TeO_3 (b). (c) Enlarged (magnification, × 10) typical black colonies of *A. tumefaciens* on the amended medium. Some agrobacterial colonies are indicated by arrows.

the medium by determining the percentage of cells recovered in amended medium to see if this value depended upon the agrobacterial genotype. The recoveries of 38 biovar 1 and 2 strains with amended and unamended media were compared. Two concentrations of K_2TeO_3 were tested with 1A medium containing K_2TeO_3 (60 and 80 $\mu g \cdot ml^{-1}$), and two concentrations were tested with 2E medium containing K_2TeO_3 (80 and 160 $\mu g \cdot ml^{-1}$). The percentage of cell recovery was determined by dividing the average number of CFU obtained with amended medium by the number obtained with unamended medium, based on three independent experiments. The average levels of cell recovery were 95% ± 3% (mean ± standard error of the mea and 85% ± 8% for 1A medium containing 60 and 80 μg of K_2TeO_3 per ml, respectively, and 75% ± 16% and 61% ± 12% for 2E medium containing 80 and 160 μg of K_2TeO_3 per ml, respectively. No significant interactions between medium and genotype and no genotype or medium effects were detected ($P > 0.05$, as determined by analysis of variance). This suggests that almost all strains of agrobacteria can be isolated by using media amended with these concentrations of K_2TeO_3 without any significant bias for individual genotypes. The relative levels of the various agrobacterial genotypes isolated from a given population should be identical in amended and unamended media. However, the amended media should facilitate sampling of agrobacteria in heavily contaminated environments.

There are several determinants of resistance to tellurite, and there is little or no link among them (39). Nothing is known about resistance to tellurite in agrobacteria. However, the characteristic garlicky odor of the volatile dimethyl telluride resulting from tellurite reduction by a thiopurine methyltransferase found, for instance, in *Pseudomonas syringae* pathovar pisi (8) has never been reported for agrobacteria, suggesting that the mechanism of resistance is different. However, Summers and Jacoby (37) showed that resistance to tellurite (Te^r) is plasmid mediated in several bacterial species, while the tellurite resistance of *Rhodobacter sphaeroides* is borne on the chromosome (23). Tellurite resistance is probably chromosomal in agrobacteria, since the resistance of C58 and the resistance of its derivatives C58C1 (cured of Ti plasmid pTiC58) and GMI9023 (cured of both pTiC58 and cryptic plasmid pAtC58) were identical (Table 1).

Design of chromosomal DNA probes. Molecular probes were developed to hybridize colony DNA blots and therefore verify that *Agrobacterium*-like colonies growing on selective media were bona fide agrobacterial colonies. Specific regions of the *rrs* (16S rRNA) gene were identified from previously published sequences and used to define three 20-mer oligonucleotides, F639*rrs*AT41 and F640*rrs*AT42 specific for biovar 1, *A. rubi*, and *A. fici*, and F641*rrs*AR5 specific for biovar 2. When pooled F639*rrs*AT41 and F640*rrs*AT42 were used as radioactive DNA probes, they hybridized with colony DNA blots of all of the strains of biovar 1, *A. rubi*, and *A. fici* tested but did not hybridize with the strains of biovar 2 and *A. vitis* used in this study (Fig. 2 and Table 1). The 20-mer oligonucleotide F641*rrs*AR5 hybridized with colony DNA blots of only *A. rhizogenes*.

Design of Ti plasmid probes. Molecular probes were also designed to detect wild *Agrobacterium* strains harboring a Ti plasmid. Four nonradioactive DNA probes were prepared from PCR products corresponding to the following conserved regions of Ti plasmids: the *tmr* region, two inter-*vir* regions, and the *nos* regions. The results of a hybridization analysis confirmed the predicted specificities of the probes based on the Ti plasmid contents of control strains. There was no hybridization with nonpathogenic (i.e., Ti plasmid-free) agrobacteria, whatever probe was used (Fig. 3 and Table 1), confirming the results obtained by PCR (data not shown).

The *tmr* probe is 173 bp long and is found in nopaline and octopine types of Ti plasmids (20). Positive hybridization signals were obtained with this probe only with DNAs of agrobacteria known to harbor a Ti plasmid (69 strains) (Table 1), which confirmed the results obtained by PCR and suggested that the *tmr* region is a good indicator of the presence of a Ti plasmid (9, 21, 30). However, the *tmr* probe did not give positive results with 10 strains described as pathogenic. In three instances (CFBP296, M15, CFBP450), the strains had probably lost the Ti plasmids, since they were not amplified in the present study although they had been amplified in previous studies (data not shown). Ti plasmids are generally stable in agrobacteria, but incubation at a high temperature can result in loss of these plasmids (10). A lack of *tmr* hybridization was expected in two instances because *A. vitis* CFBP2621 (= Ag57) and strain CFBP2408 are known to have no *tmr* gene (7, 26). Two strains (S56 and 3/1Zutra) showed no DNA hybridization, while PCR products of the expected size were obtained, sug-

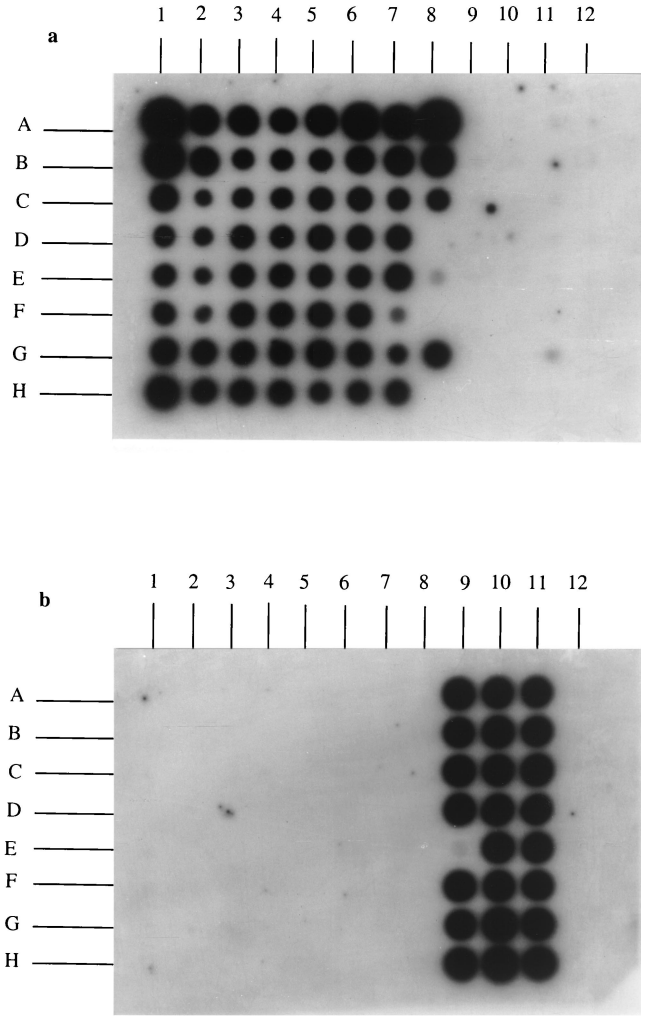

FIG. 2. 16S rDNA slot blot analyses of PCR products of agrobacterium collection with the biovar 1 cluster DNA probes F639*rrs*AT41 and F640*rrs*AT42 (a) and the biovar 2 DNA probe F641*rrs*AR5 (b). See Table 1 for a description of the strains. Biovar 1 DNAs were obtained from strains CFBP2413^T (Spot A1), Ach5 (B1), CFBP1903 (C1), GMI9023 (D1), CFBP1901 (E1), CFBP296 (F1), CFBP354 (G1), CFBP1904 (H1), CFBP2241 (A2), CFBP2243 (B2), CFPB2407 (C2), CFBP2410 (D2), CFBP2411 (E2), CFBP2414^T (F2), CFBP2454 (G2), CFBP2456 (H2), CFBP2457 (A3), CFBP2458 (B3), CFBP2177 (C3), CFBP2516 (D3), CFBP2518 (E3), S56 (F3), S377 (G3), S4 (H3), ATCC 4720 (A4), CIP28-75 (B4), CIP43-76 (C4), CIP127-76 (D4), CIP111-78 (E4), NCPPB925 (F4), F/1Zutra (G4), RV3 (H4), 3/1Zutra (A5), NCPPB1641 (B5), T37 (C5), ICPB TT9 (D5), 6 Mushin (E5), O362 (F5), O363 (G5), A96.11 (H5), A134.2 (A6), A134.3 (B6), A134.6 (C6), B100.11 (D6), M9 (E6), M10 (F6), M15 (G6), M214 (H6), M292 (A7), X88.283 (B7), X88.293 (C7), X88.299 (D7), X88.303 (E7), 85.2 (F7), 85.6 (G7), 85.49 (H7), 85.52 (A8), 85.66 (B8), and 85.104 (C8). *A. fici* DNA was obtained from strain AF3.44; (E8). *A. rubi* DNA was obtained from strain CFBP999^T (G8). Biovar 2 DNAs were obtained from strains CFBP450 (A9), CFBP1804 (B9), CFBP1905 (C9), CFBP1936 (D9), CFBP1937 (E9), CFBP1961 (F9), CFBP1962 (G9), CFBP2178 (H9), CFBP2408^T (A10), CFBP2417 (B10), CFBP2418 (C10), CFBP2419 (D10), CFBP2519 (E10), CFBP2520 (F10), C104.12 (G10), C104.22 (H10), M3 (A11), M32 (B11), M84 (C11), M111 (D11), M120 (E11), 85.30 (F11), 85.110 (G11), and 85.120 (H11). Biovar 3 DNAs were obtained from strains CFBP2512 (A12), CFBP2618 (B12), CFBP2620 (C12), CFBP2621 (D12), and CFBP2622 (E12).

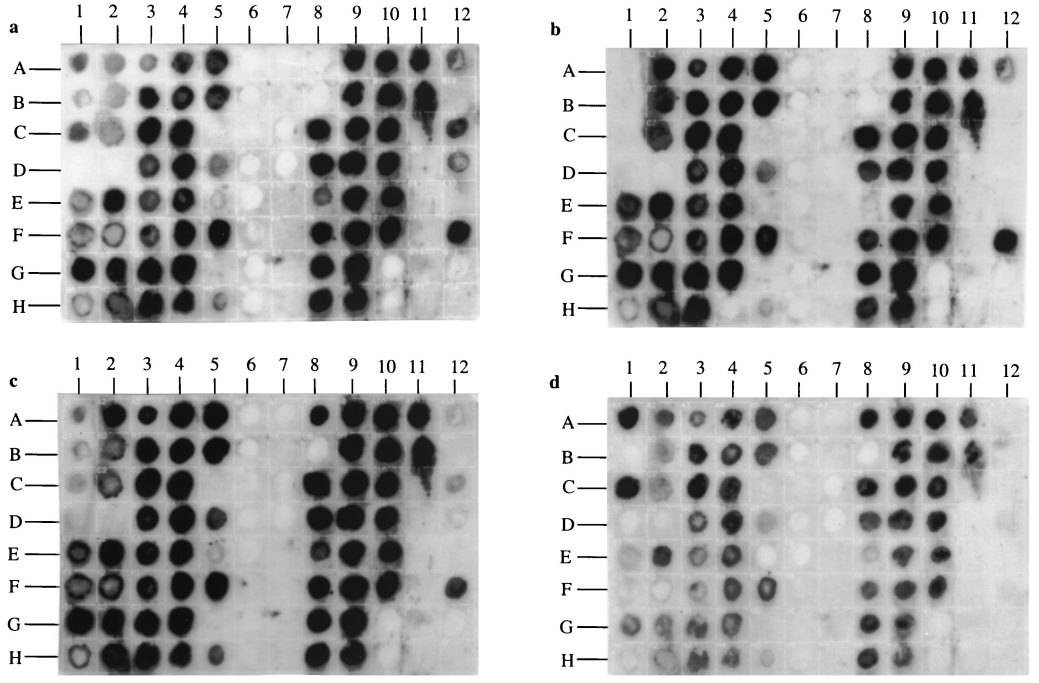

FIG. 3. Pathogenicity status as determined by cell blot analyses of *Agrobacterium* spp. (see Materials and Methods). The DNA probes used were the *tmr* probe (a), the *nos* probe (b), and *vir* probes obtained with pTiC58 (c) and with pTiB6 (d). The biovar 1 pathogenic strains with the octopine type of Ti plasmid used were strains CFBP2413^T (Spot A1), CFBP2407 (B1), and Ach5 (C1). The biovar 1 pathogenic strains with the nopaline type of Ti plasmid used were strains CFBP296 (D1), CFBP354 (E1), CFBP1904 (F1), CFBP2410 (G1), CFBP2177 (H1), CFBP2516 (A2), A134.6 (B2), A134.2 (C2), M15 (D2), B100.11 (E2), M292 (F2), 85.104 (G2), 85.66 (H2), 85.52 (A3), X88.283 (B3), A134.3 (C3), 85.2 (D3), 85.49 (E3), X88.293 (F3), M9 (G3), M10 (H3), 85.6 (A4), X88.299 (B4), M214 (C4), X88.303 (D4), A99.11 (E4), CFBP1903 (D5), CFBP1901 (F5), and CFBP999^T (H5). The biovar 1 pathogenic strains with an unknown opine type of Ti plasmid used were strains 2T3Pb (F4), 6MS3 (G4), 436.3SA (H4), 1C3Pb (A5), and 2T3Sa (B5). The biovar 1 nonpathogenic strains used were strains CFBP2414 (A6), GMI9023 (B6 and E5), CFBP2241 (C6), CFBP2243 (D6), CFBP2454 (E6), CFBP2456 (F6), CFBP2457 (G6), CFBP2458 (H6), and CFBP2518 (A7). The biovar 2 pathogenic strains with the nopaline type of Ti plasmid used were strains CFBP1804 (C8), CFBP1905 (D8), CFBP1936 (E8), CFBP1961 (F8), CFBP1962 (G8), CFBP2178 (H8), CFBP2417 (A9), CFBP2418 (B9), 85.186 (C9), C104.12 (D9), C104.22 (E9), 85.110 (F9), 85.30 (G9), 85.123 (H9), M3 (A10), M32 (B10), M120 (C10), M111 (D10), M84 (E10), 85.120 (F10), CFBP2419 (A11), and CFBP2519 (B11). The biovar 2 pathogenic strain with the agropine/mannopine type of Ti plasmid used was strain CFBP2408^T (A8). The biovar 2 pathogenic strain with an unknown opine type of Ti plasmid used was strain CFBP450 (B8). The biovar 2 nonpathogenic strains used were strains CFBP1937 (C7 and G10) and CFBP2520 (D7 and H10). The biovar 3 pathogenic strains with the nopaline type of Ti plasmid used were strains CFBP2512 (A12) and CFBP2620 (G12). The biovar 3 pathogenic strains with the cucumopine/octopine type of Ti plasmid used were strains CFBP2618 (C12), CFBP2622 (D12), and CFBP2621 (G12).

gesting that the *tmr* sequences of these strains differ significantly enough to hinder probe hybridization. The cause of the lack of hybridization with the three remaining strains (NCPPB1641, NCPPB925, S377) is not well understood, but these strains belong to rare genomic groups of agrobacteria (31) and could harbor unusual Ti or Ri plasmids with no or divergent *tmr* sequences.

The *nos* probe corresponds to a region of the T-DNA encoding nopaline synthase. This probe hybridized with the DNAs of strains harboring a Ti plasmid known to produce tumors containing nopaline. There was no hybridization with strains that did not form nopaline in tumors. Strain CFBP2618, which uses nopaline but does not synthesize nopaline in tumors, did not hybridize with the nopaline synthetase probe. This probe is thus adequate for identifying the nopaline type of Ti plasmid.

Two inter-*vir* probes, one amplified from pTiC58 (nopaline type of Ti plasmid) and the other amplified from pTiB6 (octopine type of Ti plasmid), were generated. These two probes hybridized with DNAs of all typical nopaline and octopine types of Ti plasmids. The intensities of the hybridization signals varied according to the similarity to the Ti plasmid and also to the mannopine-agropine type of Ri plasmid of CFBP2408 (Fig. 3 and Table 1). The octopine cucumopine type of Ti plasmids hybridized only with the PCR-amplified probe from pTiC58. The other hybridization patterns were obtained with strains having an undetermined opine type.

Densities of *Agrobacterium* spp. populations in bulk soil and necrosed tumors. Amended media were tested for the ability to isolate bona fide agrobacteria from plant tumors. Pathogenic isolates were recovered from old or necrosed plant material (data not shown), for which unamended media are inappropriate because of the high density of competing bacteria (mainly fluorescent *Pseudomonas* sp.). The tellurite-amended media were developed under a program funded by the European Community (Integrated Control of Crown Gall in Mediterranean Countries). Our findings represented such a marked improvement that the other partners in this program rapidly adopted the method for isolation of agrobacteria from various materials.

The other reservoirs of agrobacteria are the soil and rhizospheres. It is laborious to determine agrobacterial densities

TABLE 2. Soil bacterial densities determined with media supplemented or not supplemented with tellurite

Bacteria	Medium	Density (log CFU · g [dry wt] of soil^{-1}) in[a]:	
		June	August
Total cultivable bacteria	TSA	6.89 ± 0.07	6.99 ± 0.04
	TSA + K_2TeO_3	6.21 ± 0.04	6.43 ± 0.06
	1A	5.89 ± 0.23	5.76 ± 0.11
	1A + K_2TeO_3	5.00 ± 0.15	4.79 ± 0.03
	2E	4.63 ± 0.07	5.47 ± 0.05
	2E + K_2TeO_3	3.00 ± 0.11	4.42 ± 0.04
Agrobacterium like colonies	1A	2.90 ± 0.31	4.23 ± 0.11
	1A + K_2TeO_3	2.85 ± 0.42	3.80 ± 0.16
	2E	<2.3[b]	<2.3
	2E + K_2TeO_3	<2.3	<2.3
Bona fide *Agrobacterium*	1A	2.81 ± 0.31 (0.08)[c]	4.10 ± 0.11 (2.2)
	1A + K_2TeO_3	2.85 ± 0.42 (0.7)	3.80 ± 0.16 (10)

[a] Values are means ± standard errors of the means based on three independent counts.
[b] No *Agrobacterium*-like colonies were recovered at the threshold indicated.
[c] Bona fide *Agrobacterium* values were determined after colony blot hybridization of *Agrobacterium*-like colonies with chromosomal probes specific for *Agrobacterium* biovar 1 (see text). The values in parentheses are the percentages of bona fide *Agrobacterium* colonies among all of the colonies growing on the same medium.

with unamended media, because delineation of bona fide agrobacteria from *Agrobacterium*-like colonies always requires additional tests to determine identities. These tests include biochemical or molecular assays but are generally limited to pathogenicity trials. Consequently, very few data on the ecology of agrobacteria in soils and rhizospheres are available.

The assay described below was designed to check whether amended media could be used for direct plate counting of soil agrobacteria by visual inspection alone. Samples of the La-Côte-Saint-André soil taken at 2-month intervals were used to isolate heterotrophic culturable bacteria (TSA), biovar 1 and related agrobacteria (medium 1A), and biovar 2 agrobacteria (medium 2E), with or without K_2TeO_3. The efficacies of the amended media were tested by directly plating soil suspensions and by verifying the bona fide *Agrobacterium* status of the isolates by colony DNA blot hybridization with chromosomal oligonucleotide probes. Significantly fewer bacterial colonies were recovered with all tellurite-amended media. However, this was not true when *Agrobacterium*-like colonies alone were considered, at least with 1A medium since no *Agrobacterium*-like colonies were detected with 2E medium (Table 2). Plating soil suspensions on K_2TeO_3-amended media also resulted in the typical black color of *Agrobacterium*-like colonies together with strong inhibition of the competing microflora, which facilited both visualization and isolation of *Agrobacterium* candidates (Fig. 1).

Agrobacterium-like colonies were recovered from 1A medium (40 isolates) and from 1A medium containing K_2TeO_3 (180 isolates). Isolates obtained from 1A medium were characterized by hybridization with 16S ribosomal DNA (rDNA) probes, and nine of them were shown to be fluorescent pseudomonads. All 180 isolates obtained from 1A medium containing K_2TeO_3 were identified as bona fide *Agrobacterium* isolates. The densities of agrobacteria in bulk soil could thus be determined directly by visual inspection of plates containing Te-amended medium. The percentages of bona fide *Agrobacterium* isolates in *Agrobacterium*-like colonies (78% with unamended medium and 100% with amended 1A medium) were used to estimate the densities of bona fide agrobacteria in bulk La-Côte-Saint-André soil (Table 2). Analysis of variance showed that there was no significant effect of added tellurite on determination of agrobacterial density in soil (Table 3), confirming the usefulness of this amended medium for direct counting of soil populations of *Agrobacterium*.

Ecology of *Agrobacterium* spp. in the La-Côte-Saint-André soil. In this work, we discovered three significant ecological features about agrobacteria from La-Côte-Saint-André soil. One was the lack of culturable agrobacteria when was used 2E medium that was amended or not amended with K_2TeO_3 (Table 2). This indicated that the density of culturable biovar 2 organisms in the bulk La-Côte-Saint-André soil was less than 200 CFU · g^{-1}. However, members of this taxon were present, since 2E medium containing K_2TeO_3 allowed direct counting of many bona fide biovar 2 isolates from the rhizospheres of plants grown in the same soil (unpublished results). The question of the predominance of *Agrobacterium* biovars has been studied in several instances (17), but the data never applied to nonpathogenic agrobacteria in bulk soil. The low density of biovar 2 in the La-Côte-Saint-André bulk soil is an interesting ecological trait of this taxon and is probably related to a property of the local La-Côte-Saint-André environment. The density of biovar 1 agrobacteria reported in the present study, 10^3 to 10^4 CFU · g (dry weight) of soil^{-1}, is similar to the values reported for other soils (22, 36). This value is lower than the 10^7 agrobacterium-like colonies per g of soil reported by

TABLE 3. Variance analysis of the densities of bona fide *Agrobacterium* soil isolates in 1A medium amended or not amended with tellurite on two arbitrary soil sampling dates

Source of variation	df	Square sum	Mean square	F value	*P* value
Medium	1	0.050	0.050	0.543	1
Sampling date	1	3.776	3.776	16.44	0.0037
Medium × date	1	0.092	0.092	0.402	1
Error	8	1.838	0.230		

Bouzar et al. (4) under favorable conditions. Thus, even if agrobacteria are present in the sandy loam soil which we studied, environmental factors, especially the low organic matter content, were probably not optimal for growth of agrobacteria in the La-Côte-Saint-André bulk soil.

The second important ecological feature was the effect of sampling on the soil density of biovar 1 (Table 3). This could have been due to a temporal effect. Temporal variations in soil agrobacteria can occur, and the amended media used in the present work should facilitate study of seasonal variations in agrobacterial populations.

The third ecological feature was the lack of a Ti plasmid in any of the agrobacteria isolated from the bulk soil, as determined by DNA hybridization with the Ti plasmid probes described above and confirmed by the lack of tumor formation in *Kalanchoe daigremontiana* plants (data not shown). Similar results were obtained with rhizosphere agrobacteria from the same soil (unpublished results). The frequency of *Agrobacterium* harboring a Ti plasmid in the La-Côte-Saint-André soil was less than 1/1,300. This agrees with previous reports showing that the population of tumor-inducing agrobacteria in natural or cultivated soil is low to undetectable except in the vicinity of infected plants (22, 36). The ratio was highest (1/13) in soils in which host plants had been growing and lowest (1/500) in soils that had never been cultivated or supported host plants other than dicotyledonous weeds (17). This was the case for La-Côte-Saint-André soil, which had been cultivated with maize for many years.

In conclusion, the selectivity and sensitivity of the 1A and 2E media were increased by taking advantage of the intrinsic resistance of agrobacteria to tellurite. Agrobacteria could be reliably counted directly on plates by using media amended with tellurite. Isolation of agrobacterium strains from soil and characterization of isolates by using biovar-specific oligonucleotide probes designed by using the 16S rDNA allowed direct study of natural populations in a bulk soil. The amended media and the PCR DNA probes for pathogenicity were also used to determine the density of pathogenic agrobacteria in contaminated soil. This procedure should be useful for sanitary inspection of soils before planting.

ACKNOWLEDGMENTS

We thank M. A. Poirier for technical assistance.

This research was supported by project ERB1C18CT970198 (Intergrated Control of Crown Gall in Mediterranean Countries) funded by the European Union INCO-DC Programme to X.N.

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