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Identification and Plant Interaction of a *Phyllobacterium* sp., a Predominant Rhizobacterium of Young Sugar Beet Plants

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The second most abundant bacterium on the root surface of young sugar beet plants was identified as a *Phyllobacterium* sp. (*Rhizobiaceae*) based on a comparison of the results of 39 conventional identification tests, 167 API tests, 30 antibiotic susceptibility tests, and sodium dodecyl sulfate-polyacrylamide gel electrophoretic fingerprints of total cellular proteins with type strains of *Phyllobacterium myrsinacearum* and *Phyllobacterium rubiacearum*. It was found on 198 of 1,100 investigated plants between the 2nd and 10th leaf stage on three different fields in Belgium and one field in Spain. Densities ranged from 2×10^4 to 2×10^8 CFU/g of root. Five isolates exerted a broad-spectrum in vitro antifungal activity. DNA-DNA hybridizations showed that *Phyllobacterium* sp. does not contain DNA sequences that are homologous with the attachment genes *chvA*, *chvB*, the transferred-DNA (T-DNA) hormone genes *iaaH* and *ipt* from *Agrobacterium tumefaciens*, *iaaM* from *A. tumefaciens* and *Pseudomonas savastanoi*, or the nitrogenase genes *nifHDK* from *Klebsiella pneumoniae*. *Phyllobacterium* sp. produces indolylacetic acid in in vitro cultures and induces auxinlike effects when cocultivated with callus tissue of tobacco. When *Phyllobacterium* sp. was transformed with a Ti plasmid derivative, it gained the capacity to induce tumors on *Kalanchoe daigremontiana*. The potential role of *Phyllobacterium* sp. in this newly recognized niche is discussed.

The family *Rhizobiaceae* comprises four genera of plant-associated bacteria: *Agrobacterium*, *Rhizobium*, *Bradyrhizobium*, and *Phyllobacterium* (29). The first three genera have been extensively studied and exploited as tools in plant engineering (7, 8, 20, 51) and as seed inocula (3, 13, 41, 49). In contrast, the genus *Phyllobacterium* has received little attention because it has been found only occasionally in leaf nodules of the tropical plant species *Ardisia crispa*, *Ardisia crenata*, and *Pavetta zimmermania* (31). However, reinfection of plants with these original isolates did not result in leaf nodule formation. Thus, the question whether these bacteria are true leaf nodule-inducing bacteria and to what extent they are associated with nodules is still unanswered. Moreover, it is not possible to conclude whether one or more bacterial genera are involved in leaf nodulation. In the past, leaf nodule bacteria have been assigned to the genera *Chromobacterium*, *Phyllobacterium*, *Xanthomonas*, and *Klebsiella*. The symbiosis between the "leaf symbiont' *Bacterium folliicola* (9) (= *Phyllobacterium myrsinacearum* [31]) and *Ardisia crispa* was extensively studied by De Jongh (9). This author described a symbiotic cycle of this bacterium in this plant. Bacteria survive in the seed and colonize the buds of stems after germination of the seed and outgrowth of the plant. They later invade foliar nodules, inflorescence bud, flower, and finally the fruits. There is also much controversy about the possible role of this bacterium in the phytosphere. It was suggested that leaf nodule bacteria produce plant growth hormones, particularly cytokinins, which are necessary for the normal functioning of the host plant (18, 45). Others claimed that *Phyllobacterium* species are able to fix nitrogen, but this was contradicted by Van Hove (52) and Lersten and Horner (36).

The generic name *Phyllobacterium* was proposed by Knösel (31) who distinguished the species *Phyllobacterium myrsinacearum* and *Phyllobacterium rubiacearum*, both isolated from leaf nodules of greenhouse-grown tropical plants. These names were accepted as nomina revicta (32). Only three *Phyllobacterium* strains are kept in culture collections. DNA-rRNA hybridizations (10) have shown that *Phyllobacterium* is closely related to but distinct from *Agrobacterium*, *Brucella*, *Mycoplana*, *Rhizobium*, and CDC group Vd strains (= *Ochrobactrum anthropi*) (24).

In the present study, we report for the first time on the widespread occurrence of *Phyllobacterium* species on the root surface of young sugar beet plants and on its characteristics. This bacterium was found to be the second most abundant bacterium during a large-scale study of the bacterial communities of the rhizoplane of young sugar beet plants in Belgium and Spain (B. Lambert, P. Meire, P. Lens, H. Joos, and J. Swings, unpublished data). In this study, over 5,600 isolates were collected from 1,100 sugar beet plants by using 10% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) + 2% agar (TSBA) as a nonselective medium for the isolation of heterotrophic aerobic bacteria. All isolates were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total cellular proteins as previously described (35). This method yielded a characteristic, reproducible protein fingerprint for each isolate. These fingerprints were compared with one another. Identical fingerprints were classified in the same fingerprint type (FPT). The most frequently occurring FPT (no. 1) to appear on TSBA was identified as *Pseudomonas fluorescens*. The frequency of occurrence is the number of plants on which isolates of the particular FPT were found.

We obtained 240 isolates from 198 plants that were subsequently classified in FPT 3. This FPT was tentatively identified as "*Achromobacter* group Vd" by standard tests and API 20NE (API System, La Balme-les-Grottes, Monta-

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TABLE 1. List of bacteria used for characterization and identification

Name	Strain no.[a]	Origin and yr of isolation (deposition)	Isolator
Phyllobacterium rubiacearum	LMG $1t_1^{Tb}$ = NCIB 12128^T	Leaf of *Pavetta zimmermania*, Stuttgart-Hohenheim, FRG, 1973	D. Knösel
Phyllobacterium myrsinacearum	LMG $2t_2^{Tb}$ = NCIB 12127^T	Leaf nodule of *Ardisia crispa*, Stuttgart-Hohenheim, FRG, 1973	D. Knösel
Phyllobacterium myrsinacearum	LMG $3t_1$	Leaf nodule of *Ardisia crenata*, Stuttgart-Hohenheim, FRG, 1973	D. Knösel
Phyllobacterium FPT 3	PGSB 3574[b]	Sugar beet rhizoplane, Belgium, 1986	B. Lambert
Phyllobacterium FPT 3	PGSB 3519[b]	Sugar beet rhizoplane, Belgium, 1986	B. Lambert
Phyllobacterium FPT 3	PGSB 6201	Sugar beet rhizoplane, Spain, 1986	B. Lambert
Phyllobacterium FPT 3	PGSB 6270	Sugar beet rhizoplane, Spain, 1986	B. Lambert
Phyllobacterium FPT 3	PGSB 6181[b]	Sugar beet rhizoplane, Spain, 1986	B. Lambert
Phyllobacterium FPT 3	PGSB 3971	Sugar beet rhizoplane, Belgium, 1986	B. Lambert
Phyllobacterium FPT 3	PGSB 3714	Sugar beet rhizoplane, Belgium, 1986	B. Lambert
Phyllobacterium FPT 3	PGSB 3720	Sugar beet rhizoplane, Belgium, 1986	B. Lambert
Phyllobacterium FPT 3	PGSB 3237	Sugar beet rhizoplane, Belgium, 1986	B. Lambert
Ochrobactrum anthropi (CDC group Vd)	LMG 3301[b] = CNS 2-75	Blood culture, France	
Ochrobactrum anthropi (CDC group Vd)	LMG 3331^T = CIP 14970^T	No information available	

[a] Abbreviations used: LMG, culture collection of Laboratory of Microbiology, Gent, Belgium; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; PGSB, Plant Genetic Systems Bacterial Collection, Gent, Belgium; CNS, Centre National de Salmonella, Institut Pasteur, Paris, France; CIP, Collection de l'Institut Pasteur, Paris, France; CDC, Centers for Disease Control, Atlanta, Ga.; FRG, Federal Republic of Germany.
[b] Strains used for DNA-DNA hybridizations.

lieu Vercieu, France). However, in the past, the genus *Achromobacter* has been a dumping ground for very different types of bacteria. Additional tests indicated that the sugar beet isolates were closely related to the genera *Agrobacterium* and *Phyllobacterium*. Thus, to determine the real identity of FPT 3 isolates, we performed an extensive characterization study including reference strains. This also enabled us to improve the characterization and taxonomic description of the genus *Phyllobacterium*, which is currently based on three isolates.

Furthermore, the taxonomic relationship between *Phyllobacterium* and the plant-associated genera *Agrobacterium* and *Rhizobium* stimulated us to investigate the possible interactions of *Phyllobacterium* species with plants. Indeed, plant infection by *Agrobacterium tumefaciens* and *Rhizobium* spp. comprises several analogous steps and is mediated by some common genes (4) that are involved in the early phases of infection. In *A. tumefaciens*, the genes *chvA*, *chvB* (14), *exoC* (4), *cel*, and *att* (37, 44), all involved in attachment, are located on the chromosome. *chvA*, *chvB*, *exoC*, and *att* are necessary for virulence. Several *Rhizobium* spp. contain chromosomal genes that are functional analogs of *chvA* (*ndvA*), *chvB* (*ndvB*), and several *exo* genes. DNA sequences that are homologous to *chvA* or *chvB* have already been found in *Rhizobium meliloti* (15), *Azospirillum* species (53), and *Azotomonas* species, *Pseudomonas putida*, and *Enterobacter agglomerans* (26).

The gene relationship between several species of plant-associated pathogenic or free-living bacteria suggests that the early phases in interactions between plants and infecting or root-colonizing bacteria are very analogous. Thus, other phytosphere bacteria such as *Phyllobacterium* species might harbor these early event genes. The presence of these genes was studied here by DNA-DNA hybridization and by infection tests with Ti plasmid carrying *Phyllobacterium* transconjugants. The latter experiment could reveal whether *Phyllobacterium* species can complement the chromosomal early infection genes of *A. tumefaciens* and teach us more about the nature of the association of *Phyllobacterium* species with plants. Maybe phyllobacteria represent the primitive end of a continuum running up to the specialized symbiotic bacteria such as the rhizobia and *Agrobacterium tumefaciens*.

MATERIALS AND METHODS

Phenotypic characterization and identification of FPT 3 isolates. A total of 167 API tests, 39 conventional tests, and susceptibility tests toward 30 antibiotics were done on the strains listed in Table 1. The following API test kits were used: API 20NE and 147 API carbon assimilation tests (API 50CH, 50AO, and 50AA). All API tests were performed according to the instructions of the manufacturer. The test methods and procedures for scoring the results have been described previously (38, 46). The results of the 147 carbon assimilation tests were compared by computer-assisted techniques (46) with the data on various gram-negative nonfermentative taxa, available in the Laboratory of Microbiology. Table 2 gives a list of the conventional tests and the API 20NE tests. In vitro susceptibility toward 30 antibiotics was determined by the Kirby-Bauer technique (1) on 1% glucose–0.5% yeast extract–0.5% Bacto-Peptone (Difco Laboratories, Detroit, Mich.)–2% agar with Oxoid sensitivity disks. All tests were done at 28°C unless otherwise stated. The tests were never incubated for more than 1 week.

In vitro antagonistic activities. All 240 sugar beet isolates of *Phyllobacterium* FPT 3 were first tested for antifungal activity against the phytopathogenic fungi *Phytium ultimum* PGSF 5 and *Phoma betae* PGSF 229 by a miniaturized bacterium-fungus confrontation test in tissue culture clusters (Lambert et al., in preparation). Isolates that inhibited at least one of these two fungi were then further tested against the following set of phytopathogenic fungi: *Rhizoctonia solani* PGSF 83, *Aphanomyces laevis* PGSF 226, *Pythium irregulare* PGSF 225, *Phoma betae* PGSF 79, *Sclerotinia minor* PGSF 97, *Botrytis* sp. strain PGSF 79, *Fusarium oxysporum* f. sp. *pisi* PGSF 9, and *Fusarium oxysporum* f. sp. *betae* PGSF 244. These fungi were tested by placing an

TABLE 2. Phenotypic features of *Phyllobacterium* species and *O. anthropi*[a]

Phenotypic feature	Reaction or characteristic of:			Reference
	Phyllobacterium strain from sugar beet (FPT 3)	*Phyllobacterium* reference strains	*Ochrobactrum* reference strains	
Motility	+	+	+	43
Polar, subpolar, or lateral flagella	+	+	+	
Gram reaction	−	−	−	
Clumping and/or star formation in carrot juice	+	+	+	31
Colony very mucoid	+	+/−	−	
Colony dry (rough)	−	+/−	−	
API 20NE				
Indole formation (TRP)	−	−	−	
Glucose fermentation (GLU)	−	−	−	
Arginine dihydrolase (ADH)	−	−	−	
Urease (URE)	−	−	+/−	Manufacturer
β-Glucosidase (ESC)	+/−	+	−	
Gelatinase (GEL)	−	−	−	
β-Galactosidase (PNPG)	−	+/−	−	
Glucose assimilation (GLU)	+	+	+/−	
Arabinose assimilation (ARA)	+/−	+/±/−	+	
Mannose assimilation (MNE)	+	+	±	
Mannitol assimilation (MAN)	+	+	−	
N-Acetylglucosamine assimilation (NAG)	+	+	+	
Maltose assimilation (MAL)	+	+	+	
Gluconate assimilation (GNT)	±/−	±	±/−	
Caprate assimilation (CAP)	−	±/−	+/−	
Adipate assimilation (ADI)	−	−	−	
Malate assimilation (MLT)	+	+	+	
Citrate assimilation (CIT)	+/±/−	+/±	±	
Phenylacetate assimilation (PAC)	−	−	−	
Oxidase (OXI)	+	+	+	34
Catalase	+	+	+	
H_2S	+	+	−	
Oxidative metabolism of glucose	+	+	+	27
Pectate breakdown	−	−	−	42
Starch hydrolysis	−	−	−	
Tween 20 hydrolysis	−	−	−	47
Tween 60 hydrolysis	−	−	−	47
Ornithine decarboxylase	−	−	−	39
Lysine decarboxylase	−	−	−	39
Arginine decarboxylase	−	−	−	39
Nitrate reduction	−	+/−	−	
Reducing compounds from gluconate	−	−	−	
Methyl red reaction	−	−	−	
Voges-Proskauer reaction	−	−	−	
DNase activity	−	−	−	
3-Keto lactose test	−	−	−	2
Growth in basal medium	+/±	+/±/−	±	
Basal medium + NH_4Cl	+	+	±/−	
Basal medium + KNO_3	+	+	±	
Basal medium + sodium glutamate	+	+	+	
Yeast-mannitol agar	+	+	+	30
Peptone agar	+	+	+	
Glucose-yeast extract agar	+	+	+	
Nutrient agar	+	+	+	
Glucose (10%)	+	+	+	
Glucose (20%)	+	+/−	+	
Litmus milk	−	−	−	
NaCl (3%)	±	±	±	
pH 4.2	−	−	−	
pH 5.3	+	+	+	
pH 7.8	+	+	+	
Growth at 36°C	+	+	+	

[a] Strains are listed in Table 1. Symbols: +, positive reaction or good growth; ±, weak reaction or growth; −, negative reaction or no growth.

agar plug with mycelium in the center of an agar plate and streaking the bacterial isolates near the edge of the agar plate containing TSBA. Plates were incubated at 16°C and checked for inhibition zones after 2 to 3 days.

All *Phyllobacterium* strains listed in Table 1 were tested for antibacterial activity against several phytopathogenic bacteria. These tests were done as follows. About 15 ml of liquid TSBA (at 45°C) was seeded with approximately 10^4

viable cells of a target bacterium and poured in a petri dish. Strains to be tested for antibacterial activity were spotted on the surface after solidification of the agar. Plates were incubated at 25°C. Inhibitory strains showed clear inhibition zones after 2 to 3 days.

The following phytopathogenic target bacteria were used: *Erwinia carotovora* subsp. *atroseptica* LMG 2378, *Erwinia carotovora* subsp. *atroseptica* LMG 2385, *Erwinia carotovora* subsp. *carotovora* NCPPB 550, *Xanthomonas campestris* pv. *campestris* A 249, *Xanthomonas campestris* pv. *campestris* A 902, *Xanthomonas campestris* pv. *vesicatoria* LMG 905, *Pseudomonas syringae* pv. *tabaci* NCPPB 1237, *Pseudomonas syringae* pv. *tabaci* NCPPB 2706, *Pseudomonas syringae* pv. *maculicola* NCPPB 1776, and *Clavibacter michiganense* subsp. *michiganense* NCPPB 1573.

Transfer of Ti-plasmid derivatives from *Agrobacterium* species to *Phyllobacterium* species and testing for tumorigenicity or capacity to transform plant cells. The Ti-plasmid derivatives pGV2260::L136 and pBL001 were transferred by conjugation from *A. tumefaciens* C58CI Clm^r Ery^r into a rifampin-resistant mutant of *Phyllobacterium* strain PGSB 4038 from sugar beet. The plasmid pBL001 is a Sm^r Sp^r Su^r insertion mutant of pGV3100, which on its turn is a Tra^c derivative of the nopaline Ti plasmid pTiC58 (E. Van Haute, Ph.D. thesis, Laboratory of Genetics, State University, Gent, Belgium, 1984). The antibiotic resistance marker-carrying fragment of R702 was inserted (unpublished work) in the *Bam*HI site of *Hin*dIII fragment 23 of the pTiC58 transferred DNA (T-DNA) (11). pGV2260 is a nononcogenic derivative of the octopin Ti plasmid pTiB6S3 from which the T region is deleted and substituted by pBR322 (6). The plasmid pGVL136 carries, between the T-DNA borders, an Sm^r Sp^r gene and the *npt*II (kanamycin resistance) gene behind the nopaline synthase promoter (Pn). It also carries parts of pBR322. Cointegration of these two plasmids resulted in the plasmid pGV2260::L136 (23). The parental *Phyllobacterium* and *Agrobacterium* strains were grown in, respectively, Luria broth (LB) and LB containing streptomycin and spectinomycin (each 100 μg/ml). Exconjugants were selected on LB containing streptomycin, spectinomycin, and rifampin (50 μg/ml). Single colonies were selected and purified by subsequent transfer on a new TSBA plate with the selective antibiotics. Each cultured colony was transferred three times.

The identity of exconjugants (*Phyllobacterium*) was checked by comparison of their protein fingerprints with those of donor and acceptor strains. The presence of the plasmids was confirmed by Southern blotting (48) with a nonradioactive labeling kit (Boehringer GmbH, Mannheim, Federal Republic of Germany). Total DNA was prepared by a modification of the method of Dhaese et al. (12). A 1-ml sample of a 48-h-old culture (in 4 ml of medium) was centrifuged in an Eppendorf centrifuge for 5 min. The pellet was suspended in 300 μl of Tris (10 mM; pH 7.9)–EDTA (0.1 mM) (TE) buffer, and 20 μl of lysozyme (2 mg/ml) was added. The mixture was incubated overnight at 37°C. Then, 100 μl of Sarkosyl (5%) and 100 μl of pronase (2.5 mg/ml) were added. The DNA was purified by phenol and chloroform extraction (each time with an equal volume) and precipitated by adding 1 volume of isopropanol and 0.1 volume of $NaClO_4 \cdot H_2O$ (5 M). The precipitated DNA was pelleted by centrifugation and washed with cold ethanol (80%). The DNA was then suspended in 25 μl of TE buffer. For detection of the presence of pBL001, the plasmid pGV0319 was used as a probe. pGV0319 carries a subclone of the T-DNA (including *Hin*dIII fragments 14, 19, 41, 22, and 31). For detection of pGV2260::L136, pGVL136 was used as a probe. Probes were labeled with a nonradioactive label according to the protocol of the labeling kit (Boehringer) and hybridized overnight at 68°C to *Eco*RI (for pBL001)- and *Pst*I (for pGV2260::L136)-digested total DNA of parental strains and transconjugants. The membranes were subsequently washed two times at room temperature for 5 min and two times at 68°C for 15 min in 2× SSC (NaCl, 0.3 M; sodium citrate, 0.03 M; pH 7.0). Blots were developed by immunodetection as described in the protocol. One exconjugant, *Phyllobacterium* strain PGSB 4038 Rif^r (pBL001), was tested for tumor induction on *Kalanchoe daigremontiana* by wounding the stem with a toothpick infected with cells of the bacterium. Nine plants were inoculated. The parental donor and acceptor strains were used as positive and negative controls, and each was inoculated on five plants. *Phyllobacterium* strain PGSB 4038 Rif^r(pGV2260::L136) was tested for capacity to transform tobacco by a leaf disk assay (7). In this assay, *Phyllobacterium* strain PGSB 4038 Rif^r(pGV2260::L136) was cocultivated with leaf disks which were subsequently transferred to Murashige and Skoog (40) agar plates supplemented with 100 mg of kanamycin per liter, 1 mg of benzylaminopurine per liter, 0.1 mg of naphthylacetic acid per liter, and 500 mg of Claforan (Hoechst) per liter. *A. tumefaciens* C58CI Rif^r (pMP90), *A. tumefaciens* C58CI Clm^r Ery^r(pGV2260::L136), and *Phyllobacterium* strain PGSB 4038 Rif^r were used as controls. The plasmid pMP90 is a pTiC58 derivative carrying a gentamicin resistance marker in which the total T region, including the borders, is deleted (33). The whole set of cocultivated leaf disks was also incubated on the same medium without kanamycin (without selection for transformed plant cells).

Detection of genes that are potentially involved in plant-bacterium interactions. *Phyllobacterium* strains LMG $1t_1^T$, LMG $2t_2^T$, PGSB 3519, PGSB 3574, and PGSB 6181 and *O. anthropi* LMG 3301 (Table 1) were screened by Southern blotting for the presence of different genes known to be involved in plant-bacterium interactions. The genes and restriction fragments that were used are listed in Table 3. Total DNA was prepared as described above and digested with *Pst*I. The gene fragments were labeled and hybridized to the target DNA as described above.

RESULTS

Distribution of FPT 3. *Phyllobacterium* species was isolated from 198 (18%) of a total of 1,100 investigated sugar beet plants. It occurred on, respectively, 14 and 37% (average) of the plants in Belgium and Spain and was found on all the fields. It was found at each investigated growth stage between the 2nd and 10th leaf stage, except at the 2nd leaf stage in Spain. It occurred on the two investigated sugar beet varieties. Densities ranged from 2×10^4 to 2×10^8 CFU/g of root.

Identification of FPT 3. The numerical analysis of 147 carbon source assimilation tests (see below for results) of the nine strains of FPT 3 indicated that they cluster together above 89% similarity with the three reference strains of *Phyllobacterium* (Fig. 1). The protein fingerprints of the sugar beet isolates were highly similar to fingerprints of the type strain of *P. rubiacearum* (Fig. 2) and clearly different from those of the type strains of *A. tumefaciens* and *O. anthropi*. The patterns of the two *P. myrsinacearum* strains (LMG $2t_2^T$ and LMG $3t_1$ differed from those of all the other *Phyllobacterium* strains by the presence of a heavy protein band in the upper part of the gel.

TABLE 3. Plasmids and gene fragments used for DNA probing

Gene	Gene product	Function	Origin	Vector and fragment(s) (kilobases)	Reference
iaaH	Hydrolase	Conversion of indole-3-acetamide into IAA	*A. tumefaciens*	pG2, *Eco*RI (1)	28
iaaM	Tryptophan-2-mono-oxygenase	Synthesis of indole-3-acetamide from L-tryptophan	*A. tumefaciens*	pG1, *Eco*RI-*Pvu*I (3, 4)	28
			P. savastanoi	pLUC2, *Eco*RI (2, 7)	5
ipt	Isopentenyl transferase	First step in cytokinin biosynthesis	*A. tumefaciens*	pG41, *Bgl*II-*Hin*dIII (1, 5)	28
chvA	Unknown	Attachment to plant cells	*A. tumefaciens*	pCD523:Tn*3*Hoho107, *Bam*HI (6, 7)	14
chvB	Protein involved in attachment	Synthesis of cyclic-1,2-glucan	*A. tumefaciens*	pCD523::Tn*3*Hoho107, *Bam*HI (6, 7)	14
nifHDK	Structural genes for nitrogenase	N_2 fixation	*K. pneumoniae*	pRS2, *Bgl*II-*Xho*I (3, 6)	16

These data provide sufficient evidence to definitely assign the sugar beet isolates to the genus *Phyllobacterium*.

Phenotypic characterization of *Phyllobacterium*. The taxonomic description of the genus *Phyllobacterium* could be improved since 236 phenotypic features of each of 12 strains, listed in Table 1, were investigated for the first time.

(i) Colony and cellular morphology. After 1 to 2 days of incubation on nutrient agar, *Phyllobacterium* colonies are white-greyish, punctiform or circular with a colony diameter of less than 1 mm, and regularly edged. Most cultures are mucoid and confluent. After 4 days, colonies reach a maximum diameter of 4 mm. All *Phyllobacterium* cultures examined are gram negative. Cells are rod shaped, ovoid, or reniform, with a maximum width of 1.1 μm and length of 2.2 μm. Pleomorphism occurs in a few strains. All cultures are motile.

(ii) Conventional biochemical and physiological features. These features are listed in Table 2.

(iii) API carbon assimilation tests (API 50CH, API 50AO, API 50AA). The following carbohydrates (API 50CH) were metabolized by all *Phyllobacterium* strains: glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, *meso*-inositol, D-mannitol, sorbitol, methyl-α-D-glucoside, *N*-acetylglucosamine, D-cellobiose, maltose, sucrose, trehalose, xylitol, β-gentiobiose, D-turanose, D-lyx-

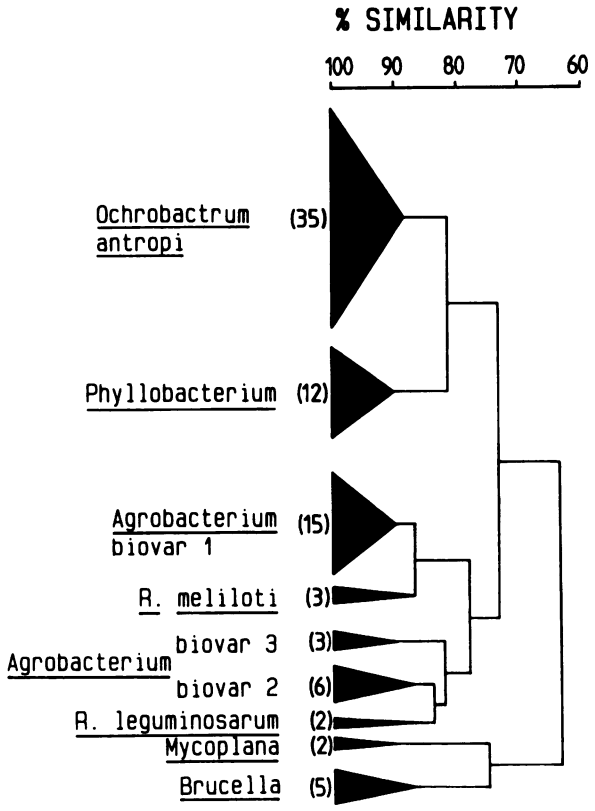

FIG. 1. Simplified dendrogram showing the phenotypic relationships between *Phyllobacterium* (including the isolates from sugar beet and type strains), *O. anthropi*, *Mycoplana*, *Brucella* (24), and various *Agrobacterium* and *Rhizobium* taxa (K. Kersters, unpublished data). The Gower similarity coefficient and the unweighted pair group average linkage clustering were used. Numbers of investigated strains are given in parentheses.

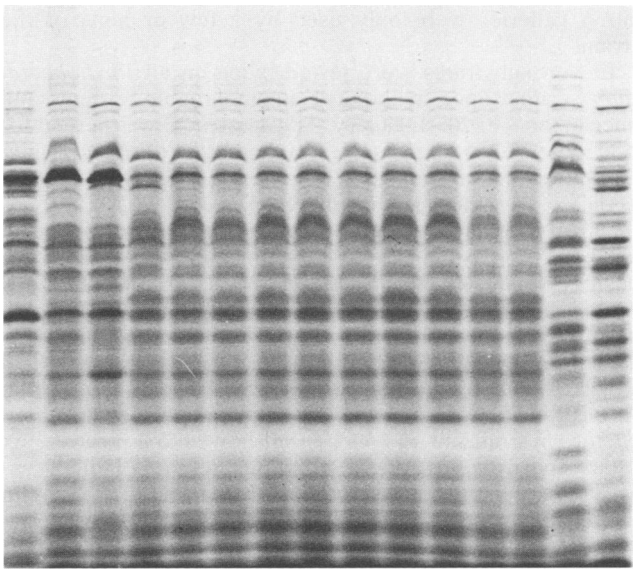

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic protein fingerprints of *Phyllobacterium* isolates from sugar beet and reference strains. Lanes: 1, *A. tumefaciens* B6^T; 2 and 3, *P. myrsinacearum* LMG 3t_1 and LMG 2t_2^T, respectively; 4, *P. rubiacearum* LMG 1t_1^T; 5 to 13, sugar beet isolates PGSB 3519, PGSB 3574, PGSB 6270, PGSB 3971, PGSB 3237, PGSB 3714, PGSB 3720, PGSB 6201, and PGSB 6181, respectively; 14 and 15, *O. anthropi* LMG 3331^T and LMG 3301, respectively.

TABLE 4. Antifungal activity spectrum of *Phyllobacterium* FPT 3 isolates from sugar beet[a]

Target fungus	Antifungal activity of:				
	PGSB 1408	PGSB 3247	PGSB 3519	PGSB 3524	PGSB 3715
Rhizoctonia solani F83	−	−	±	−	±
Aphanomyces laevis F226	+	+	+	−	−
Phythium ultimum F5	−	−	±	−	−
Phythium irregulare F225	±	−	+	−	±
Phoma betae F229	+	+	+	+	+
Phoma betae F79	+	+	−	+	+
Sclerotinia minor F97	+	+	+	+	+
Botrytis sp. strain F29	+	+	+	+	+
Fusarium oxysporum f. sp. *pisi* F9	±	±	±	±	−
Fusarium oxysporum f. sp. *betae* F224	±	±	±	−	±

[a] Symbols: +, inhibition; −, no inhibition; ±, weak and temporary inhibition.

ose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, D-gluconate, and 2-ketogluconate.

The following organic acids (API 50AO) were used by all *Phyllobacterium* strains: acetate, propionate, butyrate, isobutyrate, *n*-valerate, isovalerate, succinate, fumarate, glycolate, DL-lactate, DL-glycerate, DL-3-hydroxybutyrate, D-malate, L-malate, pyruvate, aconitate, citrate, and *p*-hydroxybenzoate.

The following amino acids or amines (API 50AA) were used as a C source by all *Phyllobacterium* strains: L-α-alanine, L-leucine, L-isoleucine, L-serine, L-tryptophan, trigonelline, L-threonine, L-aspartate, L-glutamate, L-proline, betaine, DL-4-aminobutyrate, ethanolamine, and glucosamine.

All other substrates of the API 50CH, API 50AO, and API 50AA galleries were only used by a few or none of the strains.

Eleven substrates were tested twice on all *Phyllobacterium* strains: once in the API auxanographic systems and once in the API 20NE gallery. The agreement was perfect for D-glucose (+), D-mannose (+), D-mannitol (+), *N*-acetylglucosamine (+), D-maltose (+), caprate (−), adipate (−), L-malate, (+) and phenylacetate (−).

Three substrates yielded several weakly positive results in the API 20NE systems, e.g., L-arabinose, which was clearly positive for all *Phyllobacterium* strains in API 50CH and which showed nine weakly positive, one positive, and two negative reactions in API 20NE. Another example is D-gluconate, which was uniformly positive in API 50CH and which showed 10 weakly positive and 2 negative results in API 20NE. Citrate was also clearly positive for all *Phyllobacterium* strains in API 50AO and showed four positive, six weakly positive, and two negative results in API 20NE.

(iv) Susceptibility toward antibiotics. Only for doxycyclin (30 μg), novobiocin (30 μg), framycetin (100 μg), and tetracycline (30 μg) were inhibition zones with a diameter of 7 mm (= disk diameter) or more noted for all *Phyllobacterium* strains. For all other antibiotics, at least one strain showed an inhibition zone of less than 7 mm.

Antifungal activity. *Pythium ultimum* PGSF 5 and *Phoma betae* PGSF 229 were inhibited by, respectively, 79 and 31 isolates. Five of the *Phoma betae*-inhibiting isolates were also active against other fungi. Their inhibition spectrum is shown in Table 4.

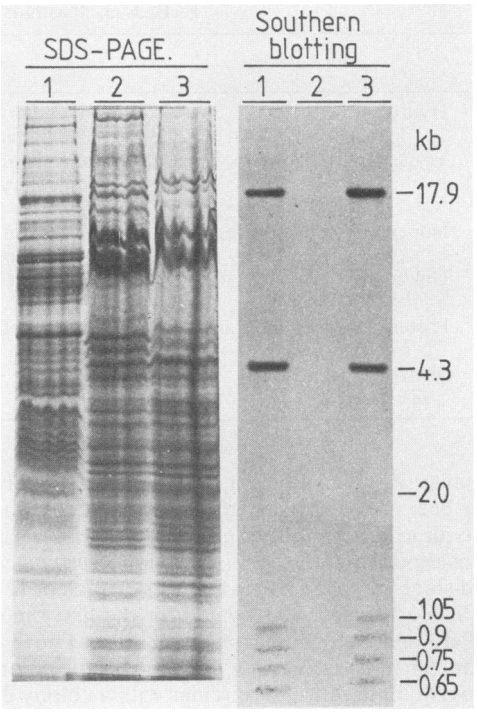

FIG. 3. Comparison of wild-type (lane 2) and transformed (lane 3) *Phyllobacterium* strain PGSB 4038 Rif^r with donor strain *A. tumefaciens* C58CI Ery^r Clm^r(pBL001) (lane 1) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Southern blotting with pGVO319 (see text). Note the presence of the seven *Eco*RI fragments common to pGVO319 and pBL001 in *A. tumefaciens* and the transformed *Phyllobacterium* strain. kb, Kilobases.

Antibacterial activity. Only *P. rubiacearum* LMG $1t_1^T$ and *P. myrsinacearum* LMG $2t_2^T$ weakly inhibited the growth of *X. campestris* pv. *campestris* A 249. None of the tested strains inhibited the growth of the other phytopathogenic bacteria.

Evidence for interaction between *Phyllobacterium* species and plants. To assay whether *Phyllobacterium* species carries some of the genes involved in plant-bacterium interactions, we determined the presence of several chromosomal virulence and other genes (Table 3). We first tested the *Agrobacterium* attachment genes *chvA* and *chvB*. These gene probes did not hybridize to the target DNA of the tested strains, while positive controls did so. This negative result prompted us to test whether *Phyllobacterium* species when harboring a Ti plasmid could induce tumors or transform plant cells. This would indicate that *Phyllobacterium* species carries genes that are functionally equivalent to the common chromosomal genes of *Agrobacterium* and *Rhizobium* species, particularly *chvA* and *chvB*. Therefore, two Ti-plasmid derivatives were conjugated to *Phyllobacterium* strain PGSB 4038 Rif^r and tested for tumorigenicity or capacity to transform plant cells. The plasmids pBL001 and pGV2260::L136 were transferred to *Phyllobacterium* strain PGSB 4038 Rif^r at frequencies of 0.5×10^{-3} and 0.5×10^{-6}, respectively. A comparison of the protein fingerprints and the Southern blots of exconjugants with donor and acceptor strains confirmed that the exconjugants were *Phyllobacterium* species and that they carried pBL001 (Fig. 3) or pGV2260::L136. *Phyllobacterium* strain PGSB 4038 Rif^r(pBL001) induced tumors on 9 of the 10 *K. daigremontiana* plants tested. All five plants

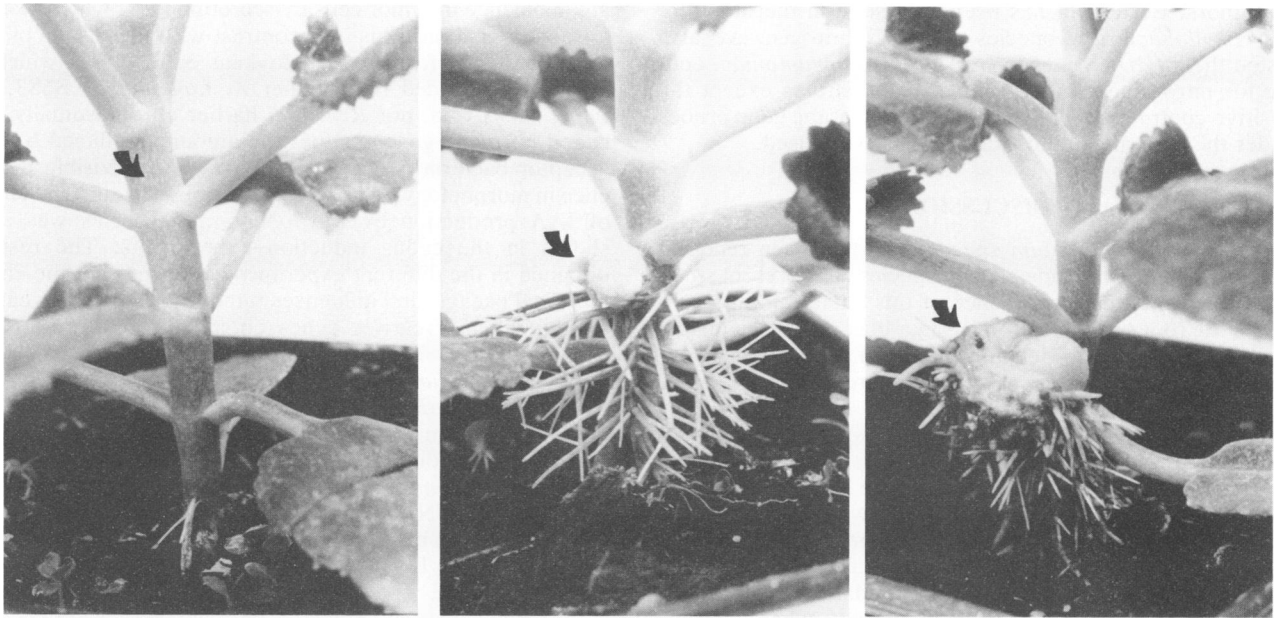

FIG. 4. Virulence tests on *K. daigremontiana* with *Phyllobacterium* strain PGSB 4038 Rif^r (left), *Phyllobacterium* strain PGSB 4038 Rif^r (pBL001) (center), and *A. tumefaciens* C58CI Ery^r Clm^r(pBL001) (right). Note the tumor morphology: no tumor for PGSB 4038 Rif^r, a rough tumor for C58CI Ery^r Clm^r(pBL001), and a smooth tumor with elongated root formation only at the tumor base for PGSB 4038 Rif^r(pBL001).

infected with *Agrobacterium* species generated tumors, while none of the plants infected with *Phyllobacterium* strain PGSB 4038 Rif^r showed any sign of hypertrophy (Fig. 4). The morphology of the tumors induced by *Phyllobacterium* strain PGSB 4038 Rif^r(pBL001) was different from those induced by the *Agrobacterium* parental strain. The *Phyllobacterium* species-induced tumors were smooth with a small number of elongated roots sprouting at the base. The *Agrobacterium* tumors were rough with many short rootlets all over the tumor.

Phyllobacterium strain PGSB 4038 Rif^r(pGV2260::L136), in contrast to the parental *Agrobacterium* strain carrying the same plasmid, was not able to transform tobacco cells in a leaf disk assay; no shoots regenerated on selective medium containing kanamycin. Nonetheless, an interesting phenomenon was observed on the control calluses that were placed on nonselective medium without kanamycin (Fig. 5). Leaf disks treated with *A. tumefaciens* C58CI Rif^r(pMP90) showed only shoot formation, while shoot formation was strongly reduced on disks that were cocultivated with *Phyllobacterium* strain PGSB 4038 Rif^r. The same strain carrying pGV2260::L136 induced root and some shoot formation after cocultivation of leaf disks and placing these on nonselective medium.

Plating tests showed that all leaf disks were colonized by the respective bacteria. Comparison with *A. tumefaciens* C58CI Rif^r(pMP90)-treated disks indicated that *Phyllobacterium* strain PGSB 4038 Rif^r may produce auxins (which are known to inhibit shoot formation) and that the addition of a Ti plasmid even causes a root induction effect. Thus, in both plant experiments we obtained indications that *Phyllobacterium* species produces auxins. Subsequent analysis of pure culture supernatants confirmed that *Phyllobacterium* strain PGSB 4038 Rif^r produces indole-3-acetic acid (IAA) (H. Van Onckelen, unpublished data). Consequently, we tested whether *Phyllobacterium* species carries genes encoding phytohormone biosynthesis. We screened the six strains mentioned in Table 1 for the presence of the following genes: the gene *iaaM* present both in *A. tumefaciens* and *Pseudomonas savastanoi* which catalyzes the synthesis of indole-3-acetamide from L-tryptophan; the genes *iaaH* and *ipt* from *A. tumefaciens*, encoding the synthesis of isopentenyltransferase, which is involved in the inhibition of root formation

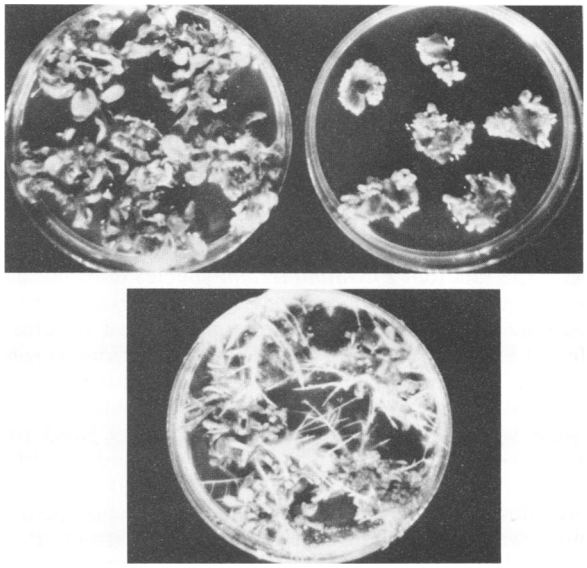

FIG. 5. Callus induction on tobacco leaf disks on Murashige and Skoog (40) agar supplemented with 1 mg of benzylaminopurine per liter, 0.1 mg of naphthylacetic acid per liter, and 500 mg of Claforan per liter after cocultivation with *A. tumefaciens* C58CI Rif^r(pMP90), (upper left), *Phyllobacterium* strain PGSB 4038 Rif^r (upper right), and *Phyllobacterium* strain PGSB 4038 Rif^r(pGV2260::L136) (lower). Note the reduction of shoot formation by PGSB 4038 Rif^r compared with C58CI Rif^r(pMP90) and the induction of root formation by PGSB 4038 Rif^r(pGV2260::L136) compared with PGSB 4038 Rif^r.

on tumors. Because it has been suggested in the literature that *Phyllobacterium* species could fix nitrogen, we also tested the *nifHDK* genes from *Klebsiella pneumoniae*, coding for nitrogenase. Again, none of the strains except the positive control strains hybridized to any of the gene probes under the experimental conditions that were used.

DISCUSSION

So far, *Phyllobacterium* species has only been reported from leaf nodules of tropical plant species. The present study revealed the abundant presence of this bacterium in a new niche, the root surface of young sugar beet plants originating from different fields in Belgium and Spain. Inventories in our laboratory of the rhizosphere of other crops from the same area and the surface of sugar beet seeds did not reveal any phyllobacteria. Thus, *Phyllobacterium* species could be a soil resident that becomes specifically associated with sugar beet roots. Another possibility is that the bacteria survive in particular tissues inside the seeds as shown by De Jongh (9). The nutritional versatility of *Phyllobacterium* species, as shown in the API tests, and the antifungal activity probably support its competitive growth and abundant proliferation in the rich environment of the root surface where various compounds present in the root exudates attract diverse microorganisms. The fact that the rhizosphere isolates from sugar beet are nearly identical to those obtained from leaf nodules indicates that *Phyllobacterium* species might be a general phytosphere colonizer. In contrast to previous suggestions which claimed (in analogy with the root nodule-inducing rhizobia) that the leaf nodule bacterium *Phyllobacterium* is able to fix nitrogen, our experiments showed that they cannot exert this activity; none of the isolates including the reference strains hybridized to *nifHDK*. All known nitrogen-fixing bacteria so far harbor these genes.

Tumor induction by the Ti-plasmid-carrying *Phyllobacterium* strains on *Kalanchoe* plants shows that the *Phyllobacterium* genome carries genes that are functionally equivalent to the chromosomal genes of *A. tumefaciens* and also demonstrates its ability to associate closely with plant tissues. Tumor induction involves attachment of bacterial cell sites to plant cell wall sites and mutual signaling before the induction of T-DNA transfer. *chvA*, *chvB*, *exoC*, and *att* genes are the only known chromosomal genes to be involved in the early infection events and to be necessary for virulence. The fact that *Phyllobacterium* species does not carry DNA sequences that hybridized to *chvA-chvB* under the experimental conditions implies the presence of structurally different genes. Transfer of Ti or Ri plasmids from *Agrobacterium* to *Rhizobium* spp. has already been done by others (22, 25, 50). In one of these experiments, the octopin-type Ti plasmid was transferred from *A. tumefaciens* B6S3 to *A. tumefaciens* C58CI (cured of its Ti plasmid) and to a *Rhizobium trifolii* strain. Both acceptor strains gained the ability to induce tumors on *K. daigremontiana*. The phenotype of the tumors was identical to that of the donor strain. It was concluded that the plasmid determines the phenotype of the tumor that will develop irrespective of the chromosomal background.

Tumors induced by *Phyllobacterium* strain PGSB 4038 Rif^r(pBL001) generated a small number of elongated roots from the base of a smooth tumor, but the same Ti plasmid in *A. tumefaciens* C58CI Ery^r Clm^r induced rough tumors with many short rootlets. This difference in tumor morphology can be explained by assuming that *Phyllobacterium* cells proliferating in the tumor tissue influence the cytokinin-auxin balance in tumor cells by secreting IAA, which results in root elongation. This is in contrast with the results of the experiments performed by Hooykaas et al. (25) in which it must be concluded that neither *A. tumefaciens* B6S3, *A. tumefaciens* C58, nor *R. trifolii* harbor chromosomally encoded hormone genes or that the amount produced by the acceptor bacteria is equivalent and gives no visible difference in morphology compared to the donor strain. The effect of IAA production by *Phyllobacterium* species was also shown in the callus induction experiments. The results obtained in the different experiments showed that the chromosomal background influences tumor morphology or callus growth when it carries genes coding for biosynthesis of phytohormones. The fact that *Phyllobacterium* species does not hybridize to *iaaM* gene fragments from *P. savastanoi* nor to *iaaM* and *iaaH* from *A. tumefaciens* indicates that the genes involved in IAA synthesis in *Phyllobacterium* species are structurally different from this class of auxin biosynthesis genes. *A. tumefaciens* also contains other auxin genes outside the T-DNA, and IAA is also produced by several plant-associated bacteria (17), but nothing is known about the interspecies DNA-DNA homology of these auxin biosynthesis genes.

In conclusion, the screening of *Phyllobacterium* species for the presence of several genes that are known to be involved in plant-bacterium interactions did not give any suggestion about the potential role or influence of this bacterium in plant associations. The fact that none of the tested genes involved in plant-bacterium interactions were found in *Phyllobacterium* species shows that this bacterium is, in this respect, genetically different from *Agrobacterium* species and rhizobia. In fact, the numerical analysis of the carbon assimilation tests demonstrated that *Phyllobacterium* species shows only 73% homology with the *Rhizobiaceae* cluster, which must indeed be reflected on the DNA level.

With the present work, we also improved the characterization of *Phyllobacterium* species. The initial identification of the nine sugar beet isolates by the API 20NE test system as "*Achromobacter*" was unsatisfactory. This generic designation has been used for a long time for aerobic, peritrichously flagellated rods, oxidase positive, producing acid from xylose and occasionally from other sugars, but not from lactose, not producing indole, not producing extracellular enzymes for the hydrolysis of Tween 80, DNA, gelatin, starch, or casein (19). The sugar beet isolates have these properties except for the flagellation, which is not peritrichous, but polar, subpolar, or lateral. In fact, the genus *Achromobacter* is one of the "dumping grounds" (30) in which a heterogeneous assemblage of different microorganisms can be found. Hendrie et al. (21) have proposed to discontinue the use of "*Achromobacter*."

The application of auxanography has solved the exact identity of the sugar beet isolates. A search in the data base available in the Laboratory of Microbiology revealed that the isolates belong to the genus *Phyllobacterium*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins confirmed this identification (Fig. 2).

The application of various API systems also allowed us to considerably extend the current phenotypic characterization of *Phyllobacterium* species (32). This work also demonstrates the value of a correct identification, which in this case led to the discovery of a new niche of *Phyllobacterium* species.

The abundant presence on the root surface, the indirect evidence for its communication capacity with plant cells, and the fact that it is apparently nonpathogenic make this

bacterium an interesting new study object for fundamental as well as for applied research. Subsequent research should give us a better idea about the status and molecular ecology of this bacterium. This could provide us with new insights in the evolution from a primitive parasitic plant-bacterium interaction to the complex mechanisms of symbiotic or genetic plant colonization, as shown by *Rhizobium* spp. and *A. tumefaciens*, respectively.

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