Emended description of the genus *Phyllobacterium* and description of four novel species associated with plant roots: *Phyllobacterium bourgognense* sp. nov., *Phyllobacterium ifriqiyense* sp. nov., *Phyllobacterium leguminum* sp. nov. and *Phyllobacterium brassicacearum* sp. nov.

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Gram-negative bacteria were isolated from the rhizoplane of *Brassica napus* in France and from root nodules of *Argyrolobium uniflorum*, *Astragalus algerianus* and *Lathyrus numidicus* growing in the infra-arid zone of southern Tunisia. Based on phylogenetic analysis of the 16S rRNA gene sequences, the seven isolates belong to the *Alphaproteobacteria* and are related to *Phyllobacterium myrsinacearum* strains. The isolates formed three clusters; clusters A and C consist of Tunisian strains, whereas cluster B consists of two strains from *Brassica napus* from France. Phylogenetic reconstruction based on the *atpD* gene strongly supports their affiliation to the genus *Phyllobacterium*. DNA–DNA hybridizations revealed that (i) none of the isolates belong to the species *P. myrsinacearum*, (ii) clusters A and C represent two distinct genomospecies and (iii) the two strains of cluster B represent two separate genomospecies. Distinctive phenotypic features were deduced from numerical analysis of phenotypic data. Based on this polyphasic approach, four novel species are proposed: *Phyllobacterium leguminum* sp. nov. (type strain ORS 1419^T=CFBP 6745^T=LMG 22833^T), *Phyllobacterium ifriqiyense* sp. nov. (type strain STM 370^T=CFBP 6742^T=LMG 22831^T), *Phyllobacterium brassicacearum* sp. nov. (type strain STM 196^T=CFBP 5551^T=LMG 22836^T) and *Phyllobacterium bourgognense* sp. nov. (type strain STM 201^T=CFBP 5553^T=LMG 22837^T). The description of the genus *Phyllobacterium* is emended accordingly.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains STM 196^T, ORS 1419^T, STM 201^T and STM 370^T are respectively AY785319, AY785323, AY785320 and AY785325, and the accession numbers for the *atpD* gene sequences of strains STM 196^T, ORS 1419^T and STM 201^T are AY785335, AY785339 and AY785336.

Details of culture media compositions, sequence alignments in PIR format and extended phylogenetic trees for 16S rRNA gene and *atpD* analysis are available as supplementary material in IJSEM Online.

The first isolation of *Phyllobacterium* strains was reported by Zimmermann (1902), but the genus name *Phyllobacterium* was originally coined by Knösel (1962) for bacteria developing within leaf nodules of tropical ornamental plants. The genus description was based mainly on phenotypic features, and the genus included two species, *Phyllobacterium myrsinacearum* (the type species) and *Phyllobacterium rubiacearum* (Knösel, 1984). Later, on the basis of molecular characteristics (DNA–DNA hybridizations and fatty acid composition), Mergaert *et al.* (2002) merged the two species under the emended description of the type species *P. myrsinacearum*. At present, the genus *Phyllobacterium* lies

within the family *Phyllobacteriaceae* in the order *Rhizobiales* of the class *Alphaproteobacteria*, in the vicinity of *Mesorhizobium*, *Allorhizobium*, *Aminobacter*, *Aquamicrobium*, *Defluviobacter* and *Pseudaminobacter*. The genus includes *P. myrsinacearum* and two recently described species, *Phyllobacterium trifolii* (Valverde *et al.*, 2005) and *Phyllobacterium catacumbae* (Jurado *et al.*, 2005).

During the past 15 years, many bacteria have been assigned to the genus *Phyllobacterium* on the basis of molecular phylogeny (by using the 16S rRNA gene or *ssu*), fatty acid composition and phenotypic characterization. Identified in different environments (using molecular probes or after cultivation), a large majority of them are plant-associated bacteria and occupy diverse ecological niches: in the rhizosphere of *Picea abies* and *Lotus* spp. (Elo *et al.*, 2000; Oger *et al.*, 2004), in tight connection with roots in *Saccharum officinarum*, *Beta vulgaris* and *Brassica napus* (Lambert *et al.*, 1990; Lilley *et al.*, 1996; Bertrand *et al.*, 2001), endophytic in *Picea* spp., *Zea mays*, *Gossypium hirsutum* and *Trifolium pratense* (Chanway *et al.*, 1994; McNroy & Kloepper, 1995; Hallmann *et al.*, 1997; Sturz *et al.*, 1998) and in root nodules of *Trifolium pratense* (Sturz *et al.*, 1997; Valverde *et al.*, 2005) and *Dalbergia louvelli* (Rasolomampianina *et al.*, 2005). They have also been found as free-living bacteria in soil (Jurado *et al.*, 2005), in water (Mergaert *et al.*, 2001) and associated with unicellular organisms (Gonzalez-Bashan *et al.*, 2000; Alavi *et al.*, 2001). Their great variety of habitats suggests that phyllobacteria have developed important adaptive capacities to the environment. In addition, their non-pathogenic status and their ability to 'communicate' with plant tissues has made them attractive for examination of their plant-growth-promoting potential. Indeed, several strains have been characterized as plant-growth-promoting bacteria on different plants. Strain W3 stimulates initial root growth and de novo root development in *Picea* spp. (Chanway *et al.*, 1994), strain STM 196 (a synonym of isolate 29-15) was recognized as a plant-growth-promoting bacterium in plant culture of oilseed rape (*Brassica napus*) (Bertrand *et al.*, 2001; Larcher *et al.*, 2003) and *Arabidopsis thaliana* (Mantelin *et al.*, 2006) and strain BOG-1-98 promotes growth of black mangrove seedlings in artificial sea water when co-inoculated with *Bacillus licheniformis* (Rojas *et al.*, 2001). Interestingly, some strains have been isolated from root nodules (Sturz *et al.*, 1997; Rasolomampianina *et al.*, 2005), although the capacity of the isolates to induce nodulation was not clearly demonstrated. However, in early studies, van Veen *et al.* (1988) reported root-nodule formation in *Vicia sativa* by *P. myrsinacearum* after introduction of a *Rhizobium leguminosarum* symbiotic plasmid (pSym), indicating that chromosomal genes involved in nodule formation are functionally present in the bacterium. Furthermore, Valverde *et al.* (2005) recently isolated a *Phyllobacterium* strain that induced infective nodules on *Trifolium pratense* and *Lupinus albus* roots.

In the past two decades, polyphasic taxonomic studies (Vandamme *et al.*, 1996), especially using methods for

analysing micro-organisms at the molecular level, have played a crucial role in improving the classification of many bacterial groups like the pseudomonads, rhizobia, *Burkholderia*, *Caulobacter* and *Acetobacter* (de Lajudie *et al.*, 1994; Gillis *et al.*, 1995; Abraham *et al.*, 1999; Catara *et al.*, 2002; Cleenwerck *et al.*, 2002). The 16S rRNA phylogeny has had a major influence on our current perception of evolutionary relationships among bacteria and rhizobia in particular (Willems & Collins, 1993; Young & Haukka, 1996), but other genes (*atpD*, *recA* or the glutamine synthetase I gene) are now also being examined and integrated in phylogenetic studies (Turner & Young, 2000; Gaunt *et al.*, 2001), and multilocus sequence analysis has been proposed for the delineation of genera and species (Martínez *et al.*, 2004; Gevers *et al.*, 2005).

Mergaert *et al.* (2002) emended the description of the genus *Phyllobacterium*, but the diversity and classification of bacteria assigned to *Phyllobacterium* still remain poorly documented. Here we report on a polyphasic taxonomic study that included 18 *Phyllobacterium* sp. strains originating from different ecological niches and geographical origins (Table 1): two reference strains described by Knösel (1984), nine additional strains assigned to *P. myrsinacearum* (Mergaert *et al.*, 2002) and isolated from *Saccharum officinarum* in Europe (Lambert *et al.*, 1990), two root-associated bacterial strains isolated from *Brassica napus* in France (Bertrand *et al.*, 2001) and five strains isolated from naturally occurring nodules of three wild legume species growing in the infra-arid zone of Tunisia. On the basis of published data and our molecular and phenotypic results (16S rRNA gene and *atpD* phylogenies, DNA–DNA hybridizations and numerical taxonomy of phenotypic characteristics), we describe four novel species of the genus *Phyllobacterium* and we propose an emended description of the genus.

The bacterial strains investigated in this study are listed in Table 1. Naturally occurring root nodules were collected *in natura* and either used directly for bacterial isolation or stored dried in CaCl₂. Upon utilization, nodules were rehydrated in sterile water and surface sterilized by immersion in 3% (w/v) calcium hypochlorite for 5 min. The nodules were then rinsed aseptically eight times in sterile water and then crushed in a drop of sterile water and the suspension was streaked on yeast extract/mannitol (YM) agar medium in Petri dishes (Vincent, 1970). Colonies appeared after incubation for 1 week at 28 °C under aerobic conditions and were checked for purity by repeated streaking on YM agar and by microscopic examination of living cells. Isolates were stored at –80 °C in YM broth adjusted to 20% glycerol (v/v).

To complete phylogenetic data, the following type strains were used, provided by the BCCM/LMG Bacteria Collection: *Agrobacterium tumefaciens* LMG 140^T (=ORS 1351^T), *Chelatobacter heintzii* LMG 2122^T (=STM 2150^T), *Mesorhizobium amorphae* LMG 18977^T (=STM 291^T), *Mesorhizobium chacoense* LMG 19008^T (=STM 2154^T), *Mesorhizobium plurifarum* LMG 11892^T (=ORS 1032^T),

Table 1. Studied strains

Strains of *P. myrsinacearum* were provided by the BCCM/LMG Bacteria Collection; other strains were isolated previously by our research group. Culture collection abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France; DSM, DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IAM, Culture Collection, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Mikrobiologie Universiteit Gent, Gent, Belgium; NCIB (now NCIMB), National Collections of Industrial, Food and Marine Bacteria, NCIMB Ltd, Aberdeen, UK; PGSB, Plant Genetic Systems Bacterial Collection, Gent, Belgium; STM and ORS, culture collection of the Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpellier, France. NK, Not known.

Strain	Other strain designations	Geographical origin	Host plant	Reference(s)
<i>P. myrsinacearum</i>				
STM 948 ^T	LMG 2(t2) ^T =CFBP 5554 ^T =IAM 13584 ^T =CCUG 34962 ^T =NCIB 12127 ^T =DSM 5892 ^T =ATCC 43590 ^T	NK	Leaf nodule of <i>Ardisia crispa</i>	Knösel (1962, 1984)
STM 949*	LMG 1(t1)=CFBP 5555=IAM 13587 =CCUG 34964=NCIB 12128 =DSM 5893=ATCC 43591	NK	Leaf nodule of <i>Pavetta zimmermaniana</i>	Von Faber (1912), Knösel (1984)
STM 955	LMG 8227=CFBP 6705=PGSB 6201	Spain	Rhizoplane of <i>Saccharum officinarum</i>	Lambert <i>et al.</i> (1990)
STM 956	LMG 8228=CFBP 6706=PGSB 6270	Spain	Rhizoplane of <i>S. officinarum</i>	Lambert <i>et al.</i> (1990)
STM 957	LMG 8229=CFBP 6707=PGSB 6181	Spain	Rhizoplane of <i>S. officinarum</i>	Lambert <i>et al.</i> (1990)
STM 953	LMG 8225=CFBP 6703=PGSB 3574	Belgium	Rhizoplane of <i>S. officinarum</i>	Lambert <i>et al.</i> (1990)
STM 954	LMG 8226=CFBP 6704=PGSB 3519	Belgium	Rhizoplane of <i>S. officinarum</i>	Lambert <i>et al.</i> (1990)
STM 958	LMG 8230=CFBP 6708=PGSB 3971	Belgium	Rhizoplane of <i>S. officinarum</i>	Lambert <i>et al.</i> (1990)
STM 959	LMG 8231=CFBP 6709=PGSB 3714	Belgium	Rhizoplane of <i>S. officinarum</i>	Lambert <i>et al.</i> (1990)
STM 960	LMG 8232=CFBP 6710=PGSB 3720	Belgium	Rhizoplane of <i>S. officinarum</i>	Lambert <i>et al.</i> (1990)
STM 961	LMG 8233=CFBP 6711=PGSB 3237	Belgium	Rhizoplane of <i>S. officinarum</i>	Lambert <i>et al.</i> (1990)
<i>P. ifriqiense</i> sp. nov. (cluster A)				
STM 370 ^T	LMG 22831 ^T =CFBP 6742 ^T	Southern Tunisia	Root nodule of <i>Lathyrus numidicus</i>	This study
ORS 1420	LMG 22832=CFBP 6741	Southern Tunisia	Root nodule of <i>Astragalus algerianus</i>	This study
<i>P. brassicacearum</i> sp. nov. (cluster B)				
STM 196 ^T	LMG 22836 ^T =CFBP 5551 ^T =strain 29-15 ^T	France	Root of <i>Brassica napus</i> cv. Eurol	Bertrand <i>et al.</i> (2001)
<i>P. bourgognense</i> sp. nov. (cluster B)				
STM 201 ^T	LMG 22837 ^T =CFBP 5553 ^T =strain 31-25a ^T	France	Root of <i>Brassica napus</i> cv. Eurol	Bertrand <i>et al.</i> (2001)
<i>P. leguminum</i> sp. nov. (cluster C)				
ORS 1419 ^T	LMG 22833 ^T =CFBP 6745 ^T	Southern Tunisia	Root nodule of <i>Astragalus algerianus</i>	This study
ORS 1402	LMG 22834=CFBP 6743	Southern Tunisia	Root nodule of <i>Argyrolobium uniflorum</i>	This study
ORS 1403	LMG 22835=CFBP 6744	Southern Tunisia	Root nodule of <i>Argyrolobium uniflorum</i>	This study

*Type strain of *P. rubiacearum*.

Ochrobactrum anthropi LMG 3331^T (=STM 2148^T), *Rhodobacter sphaeroides* LMG 2827^T (=STM 2152^T), *Sinorhizobium adhaerens* LMG 20216^T (=STM 2072^T), *Sinorhizobium morelense* LMG 21331^T (=STM 2064^T) and *Sinorhizobium xinjiangense* LMG 17930^T (=STM 2071^T). All the strains were grown at 28 °C and, except for *C. heintzii*, *O. anthropi* and *R. sphaeroides*, strains were grown in YM medium. The compositions of the culture media are given as supplementary material in IJSEM Online.

Genomic DNAs were purified from an 800 ml 2-day culture. Bacterial cells were harvested by centrifugation (15 min, 3200 g, 20 °C) and washed twice with phosphate buffer (0.01 M, pH 7). Cell pellets (1.5–3 g) were suspended in 3 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8). Extraction and purification of genomic DNA were then performed as described by Pitcher *et al.* (1989). For other bacteria, genomic DNA was purified from a 5 ml 2-day culture, according to Chen & Kuo (1993). DNA quality and concentration were determined by UV spectrophotometry.

The primers used for DNA amplification and sequencing are described in Table 2. Nearly full-length 16S rRNA genes was amplified using the universal eubacterial primers FGPS6 and FGPS1509 (Sy *et al.*, 2001) adapted from Weisburg *et al.* (1991). Partial amplification and sequencing of the *atpD* gene were performed as described by Gaunt *et al.* (2001). PCR amplifications were performed in a reaction mixture of 25 µl (total volume) containing: 50 or 75 ng genomic DNA for *ssu* and *atpD* amplification, respectively, 0.2 mM each dNTP, 0.8 µM each primer, 1.25 U GoTaq DNA polymerase (Promega) and the buffer supplied with the enzyme. PCR amplifications were performed using a GeneAmp PCR System 2400 thermocycler (Applied Biosystems). A touch-down PCR program (Don *et al.*, 1991) was used for 16S rRNA gene amplification: 95 °C (5 min), 20 cycles of 94 °C (30 s), annealing temperature (30 s) from 60 to 50 °C and

72 °C (2 min), followed by 25 cycles of standard PCR (55 °C annealing temperature) and an additional cycle with a final 7 min chain elongation.

PCR products were visualized by electrophoresis on 1.8% agarose gel. Products of the expected size were cut from the agarose, purified with a QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's protocol and sequenced on both strands. Sequencing reactions were performed using the ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's protocol and analysed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Accession numbers for *ssu* and *atpD* sequences obtained in this study are shown by asterisks in Figs 1 and 2.

For phylogenetic analyses, nucleotide alignments of partial *ssu* and *atpD* sequences were performed with the CLUSTAL X windows interface, version 1.63b (Thompson *et al.*, 1997), and then optimized by hand under the GeneDoc alignment editor program (Nicholas *et al.*, 1997), especially for *atpD*, to obtain gene alignments in accordance with amino acid alignments. Five parts of the *ssu* sequences for which no unambiguous alignment could be achieved were removed from the final matrix. The *ssu* and *atpD* data matrices used in the phylogenetic analyses were finally 1410 and 447 bp, respectively, in length. Reduced alignments (without ambiguous regions) are available in PIR format as supplementary material in IJSEM Online.

Phylogenetic trees were generated by distance and maximum-likelihood methods using PAUP, version 4.0b10 (Swofford, 1998). We estimated the best likelihood model for each dataset using Winmodeltest (Posada & Crandall, 1998). We thus applied a GTR+G+I model for the *ssu* data matrix and estimated parameters (shape parameter of the gamma distribution of four rates at variable sites, base frequencies,

Table 2. Gene-specific primer sets for PCR amplification and sequencing

Primer position gives the first base amplified after the primer, relative to the start of the *ssu* or *atpD* gene of *Sinorhizobium meliloti* strain 1021 (sequenced genome; GenBank accession numbers NC_003047 and AL591792, respectively).

Gene	Position	Direction	Sequence (5'–3')	Reference
<i>ssu</i>	28	Forward ^a *	GGAGAGTTAGATCTTGGCTCAG	Sy <i>et al.</i> (2001)
<i>ssu</i>	336	Forward	GGCAGCAGTGGGGAATATTG	Sy <i>et al.</i> (2001)
<i>ssu</i>	440	Reverse	GGCACGAAGTTAGCCGGGGC	Sy <i>et al.</i> , (2001)
<i>ssu</i>	825	Reverse	AGCCTTGCGGCCGTACTCCC	This study
<i>ssu</i>	843	Forward	CCTGGGGAGTACGGTCGCAAG	Sy <i>et al.</i> (2001)
<i>ssu</i>	1023	Reverse	GGGACTTAACCAACATCT	Sy <i>et al.</i> (2001)
<i>ssu</i>	1041	Forward	ATGTTGGGTTAAGTC	This study
<i>ssu</i>	1464	Reverse ^b	AAGGAGGGGATCCAGCCGCA	Sy <i>et al.</i> (2001)
<i>atpD</i>	390	Forward	ATCGGCGAGCCGGTCGACGA	Gaunt <i>et al.</i> (2001)
<i>atpD</i>	923	Reverse	GCCGACACTTCCGAACCNGCCTG	Gaunt <i>et al.</i> (2001)

*Primers correspond to universal eubacterial primers FGPS6 (a) and FGPS1509 (b).

Fig. 1. Phylogeny for *ssu* (16S rRNA) genes of *Phyllobacterium* strains among representatives of the family *Phyllobacteriaceae* and relatives. The distance tree is rooted using the sequence of *Bordetella bronchiseptica* RB50 (not shown). A complete tree with outgroup members is available as Supplementary Fig. S1 in IJSEM Online. Distances used were based on maximum-likelihood estimated parameters (see text for details). Filled circles at nodes indicate clades also found in the maximum-likelihood reconstruction. Numbers at branching points indicate bootstrap percentages (when $\geq 70\%$) derived from 1000 replicates. Bar, 0.01 % sequence dissimilarity (evolutionary distance). The designation of the original sequenced strain is given after the species name. GenBank/EMBL/DBJ accession numbers are indicated in parentheses; asterisks indicate sequences obtained in this study.

proportion of invariable sites) from our data. A similar model (GTR + G + I, but with different parameters) was used for the *atpD* analyses, as a result of Winmodeltest choice tests. The distance trees were obtained by using a heuristic search implemented in PAUP. The distance matrix used was based on the maximum-likelihood model chosen with the parameters estimated previously. Bootstrap values of each node were calculated from 1000 replicates. Maximum-likelihood analysis was similarly conducted with a heuristic search using the model defined for each data matrix.

In the current bacterial taxonomy, genus delineation is mainly based upon the phylogenetic relationships of 16S rRNA genes. The 16S rRNA phylogeny reconstructed by the distance method placed the strains listed in Table 1 unambiguously in the *Alphaproteobacteria* and grouped all of them and *P. myrsinacearum*, *P. catacumbae* and *P. trifolii* in a clade with 88 % bootstrap support (Fig. 1; a complete version of this tree is available as Supplementary Fig. S1 in IJSEM Online). Although highly supported in the distance

tree, the *Phyllobacterium* strains made a polyphyletic group in the maximum-likelihood tree (indicated in Fig. 1). The *atpD* phylogeny (Fig. 2; a complete version of this tree is available as Supplementary Fig. S2 in IJSEM Online) also grouped all studied strains with *P. myrsinacearum*, although not with high bootstrap support (less than 70 %). For technical reasons, we could not obtain *atpD* sequences for strains belonging to 16S rRNA cluster A. The failure to amplify *atpD* sequences from these strains could be due to a point mutation in the sequence corresponding to one of the two primers used for amplification. Although their phylogenetic position could therefore not be confirmed by the *atpD* phylogeny, we are confident that cluster A strains are closely related to *P. myrsinacearum*, since their position in the *ssu* phylogeny is highly supported in both phylogenetic analyses (distance and maximum-likelihood). Altogether, these results strongly support the conclusion that the strains investigated in this study form a monophyletic group with the previously defined *Phyllobacterium* species and that they may all be affiliated to *Phyllobacterium*.

Fig. 2. Phylogeny for partial *atpD* sequences of *Phyllobacterium* strains among representatives of the family *Phyllobacteriaceae* and relatives. Bar, 0.1% sequence dissimilarity. Other details are similar to Fig. 1. A complete tree with outgroup members is available as Supplementary Fig. S2 in IJSEM Online.

As observed previously by Gaunt *et al.* (2001), the placement of the *P. myrsinacearum* clade relative to other genera differs in the two trees (*ssu* and *atpD*) and is thus uncertain. The close relationship with *Mesorhizobium* seen in the *ssu* phylogeny was not supported by the *atpD* phylogeny, which suggested a deeper placement. In the *atpD* tree (Fig. 2), *Phyllobacterium* appears closer to the *Sinorhizobium*–*Rhizobium* clade than to *Mesorhizobium*. This uncertainty may originate from ancestral lateral gene transfer among alphaproteobacteria. More molecular and phylogenetic analyses should be performed to resolve this.

Within the *Phyllobacterium* clade, the studied strains are divided into four well-supported clusters (in the 16S rRNA phylogeny), obtained by both phylogenetic methods (distance and maximum-likelihood), three of which are confirmed by the *atpD* phylogeny (Figs 1 and 2). The *P. myrsinacearum* cluster includes recognized members of the species, together with the strains studied by Lambert *et al.* (1990), with 99% internal sequence similarity [all isolates of Lambert *et al.* (1990) display the same *ssu* sequence]. The strains originating from *Brassica napus* (Bertrand *et al.*, 2001) are genetically different, but share more than 98% identity, and fall within cluster B. Clusters A and C consist of strains from Tunisia, having identical *ssu* gene sequences

within each cluster. Cluster A is part of a larger cluster, supported by a high bootstrap value (86%), that includes the type strains of the recently described species *P. catacumbae* and *P. trifolii* (Jurado *et al.*, 2005; Valverde *et al.*, 2005). In this clade, *P. trifolii* is a sister branch of the subclade including *P. catacumbae* and cluster A strains. Although the *P. catacumbae* type strain and the two cluster A strains can not be distinguished phylogenetically, the overall divergence of their sequences suggests that *P. catacumbae* is genuinely different from the two studied strains found in cluster A. This relationship may warrant further DNA–DNA hybridization studies, however. The relationships of the studied strains in the *atpD* phylogeny are similar to those constructed by 16S rRNA gene sequences (Fig. 1), and all nodes are recovered in both distance and maximum-likelihood trees (indicated in Fig. 2). In conclusion, according to the phylogenetic analyses, all the strains investigated in this study form a monophyletic group relative to the genus *Phyllobacterium* and form well-defined clusters.

To determine the species status of the three clusters identified in our phylogenetic approach, DNA–DNA hybridizations were performed (Table 3). Native DNAs were labelled *in vitro* by random priming with tritium-labelled nucleotides using the Megaprime DNA labelling

system (Amersham Biosciences). The S1 nuclease/trichloroacetic acid method for hybridization has been described previously (Crosa *et al.*, 1973; Grimont *et al.*, 1980). Reassociation was performed at 70 °C in 0.42 M NaCl. DNA–DNA hybridization tests were carried out using labelled DNA from *P. myrsinacearum* STM 948^T and from representative strains of each phylogenetic cluster. Each value is a mean of two to four replicate experiments. Thermal stability of reassociated DNA was determined by using the method of Crosa *et al.* (1973) when the percentage of DNA–DNA hybridization was between 65 and 75 % (ΔT_m given in Table 3).

The DNA relatedness between the type strains of *P. myrsinacearum* and *P. rubiacearum* (77 %) was above the species delineation value of 70 %. This result is in accordance with the conclusion of Mergaert *et al.* (2002) using a different method, thus confirming the synonymy of these two species. DNA hybridization values between representative strains of phylogenetic clusters A, B and C and the type strain of *P. myrsinacearum* ranged from 5 to 19 % (Table 3). Reciprocal hybridizations gave the same results. These values are not significant and indicate that these clusters may represent novel *Phyllobacterium* species. Genetic homogeneity inside clusters A and C was verified by high internal DNA hybridization values between members of each cluster. These results indicated unambiguously that clusters A and C represent two distinct genomospecies. In contrast, DNA reassociation between the two strains of cluster B was only 20 %; strains STM 201^T and STM 196^T therefore represent two additional genomospecies.

The DNA base composition was determined by the thermal denaturation temperature protocol (Marmur & Doty, 1962) and was calculated by using the equation of Owen & Lapage (1976). *Escherichia coli* K-12 [DNA G + C content of 50.6 mol% (T_m)] was used as a control. The DNA base compositions are indicated in Table 3. The DNA G + C contents of the studied strains are in the range 51–58.5 mol% (T_m), which corresponds to a lower range of values than those reported by Gillis & De Ley (1980) for recognized members of the genus *Phyllobacterium*, i.e. 60–61 mol% (T_m) for *P. myrsinacearum* strains. The DNA base composition is homogeneous between strains belonging to the same genomospecies.

Twenty-two conventional biochemical and physiological tests were performed. Gram determination was performed with 3 % KOH solution (Suslow *et al.*, 1982). Oxidase activity was assessed with dimethyl *p*-phenylenediamine reagent (Kovacs, 1956). Oxidative or fermentative glucose metabolism was determined by using Hugh & Leifson medium (Hugh & Leifson, 1953) in Yvan Hall tubes. Urease activity and indole formation were tested by using commercial urea-indole medium (bioMérieux) and β -galactosidase activity by using ONPG discs (bioMérieux). Hydrolysis of gelatin and Tween 80 was tested on gelatin and Tween 80 agar, respectively (Sands, 1990). The presence of DNase was tested on commercial DNA agar (Diagnostic Pasteur). Production of 3-ketolactose was assessed according to Bernaerts & De Ley (1963). Strains were also investigated for their ability to grow under different conditions. The ability of the strains to grow in Luria–Bertani (LB) medium, YM

Table 3. DNA base composition, DNA–DNA relatedness (%) and ΔT_m among *Phyllobacterium* strains

Values in parentheses are values of ΔT_m . ND, Not determined.

Source of unlabelled DNA	DNA G + C content (mol%)	Hybridization (%) with labelled DNA from strain:			
		1	2	3	4
<i>P. myrsinacearum</i>					
1. STM 948 ^T	58.5	100	9	12	9
STM 949	ND	77	ND	ND	8
<i>P. ifriqiense</i> sp. nov.					
2. STM 370 ^T	52	7	100	9	3
ORS 1420	51	5	74 (2 °C)	5	5
<i>P. brassicacearum</i> sp. nov.					
STM 196 ^T	55.5	15	15	20	16
<i>P. bourgognense</i> sp. nov.					
3. STM 201 ^T	54	19	13	100	8
<i>P. leguminum</i> sp. nov.					
4. ORS 1419 ^T	57	9	15	ND	100
ORS 1402	57	11	3	4	100
ORS 1403	58	5	3	4	100

broth without CaCl_2 , YM broth with 1, 2 or 3 % NaCl and YM broth at pH 4, 5, 9 or 10 was determined in liquid medium as described previously (Nour *et al.*, 1994). All the tests were incubated at 28 °C and the presence or absence of growth was recorded at 3, 6, 9, 12 and 15 days. Maximum growth temperature was tested on YM with agar (15 g l^{-1}) at 35, 37 and 40 °C. Metabolic profiles based on 99 carbon sources were studied by using Biotype 100 strips with biomedium 1 (biomedium 2 was used for strains of cluster C), as recommended by the manufacturer (bioMérieux). Strips were incubated at 28 °C and read at 2, 4 and 6 days after inoculation.

A total of 121 tests, including 22 conventional and physiological characters and assimilation of 99 carbon sources, were used for numerical taxonomy analysis for the 18 *Phyllobacterium* strains listed in Table 1. The distance matrix was calculated using the Jaccard coefficient (Sneath & Sokal, 1973). Cluster analysis was performed by using the unweighted pair group method with arithmetic averages (Sneath & Sokal, 1973). Discriminatory tests were selected using the diagnostic ability coefficient deduced from the numerical analysis (Descamps & Véron, 1981). The dendrogram of phenotypic distances is shown in Fig. 3.

At a distance of 0.15, three phenons were delineated and two strains were in a separate position. Phenon 1 included all 11 *P. myrsinacearum* strains. Phenons 2 and 3 corresponded to

ssu phylogenetic clusters A and C, respectively. The two separate strains are the strains that represent distinct genomospecies within phylogenetic cluster B. The phenotypic characteristics that differentiate the four identified genomospecies and *P. myrsinacearum* are presented in Table 4. The major features that differentiate *P. myrsinacearum* from the four novel genomospecies are its ability to grow at pH 4 and to assimilate glutarate and L-tyrosine. Strains of cluster C were distantly related to the other *Phyllobacterium* strains and are distinguished by assimilation of few Biotype 100 substrates, even with the use of biomedium 2 (bioMérieux), which contains 31 growth factors, as the inoculation medium. Strains of cluster C are the only *Phyllobacterium* strains that assimilate mucate and saccharate on Biotype 100 (strain STM 201^T is the other exception, as it assimilates D-saccharate). Strains of clusters A and C are the only *Phyllobacterium* strains able to grow at 37 °C. This phenotypic trait is probably related to their common geographical origin (southern Tunisia), where high temperatures occur. Assimilation of i-erythritol is an exclusive character of strains from cluster A. Like some strains of *P. myrsinacearum*, strains of cluster A are able to grow in 3 % NaCl. Strains of cluster B, isolated from roots of *Brassica napus*, share common properties that differentiate them from other *Phyllobacterium* strains; they cannot grow at 35 °C and do not assimilate D-glucuronate or D-galacturonate. Among the *Phyllobacterium* strains studied, strain STM 196^T shares common features with strains of

Fig. 3. Dendrogram of phenotypic characteristics of 18 *Phyllobacterium* strains based on the unweighted pair group method with averages. Distance = 1 – Jaccard coefficient.

Table 4. Phenotypic characteristics that differentiate *Phyllobacterium* clusters and species

Phenons: 1, *P. myrsinacearum* (11 strains tested); 2, *P. ifriqiyense* sp. nov.; 3, *P. leguminum* sp. nov.; a, *P. brassicacearum* sp. nov.; b, *P. bourgognense* sp. nov. +, Positive; –, negative; v, variable. The following substrates of the Biotype 100 strip (bioMérieux) are assimilated by all strains: α -(+)-D-glucose, α -(-)-L-fucose, α -L-rhamnose, β -(+)-D-fructose, β -gentiobiose, (+)-D-arabitol, (+)-D-cellobiose, (+)-D-galactose, (+)-D-mannose, (+)-D-trehalose, (+)-D-xylose, D-gluconate, D-glucosamine, DL-lactate, D-lyxose, D-mannitol, D-sorbitol, aesculin, (+)-L-arabinose, L-proline and N-acetyl-D-glucosamine. The following are not assimilated by any strain: 3-O-methyl D-glucopyranose, 3-phenylpropionate, α -(+)-D-melibiose, benzoate, caprate, caprylate, (-)-D-tartrate, (+)-D-raffinose, D-alanine, gentisate, histamine, hydroxyquinoline β -glucuronide, itaconate, L-histidine, (+)-L-tartrate, meso-tartrate, L-tryptophan, m-coumarate, m-hydroxybenzoate, phenylacetate, putrescine, tricarballoylate and tryptamine. Other characteristics shared by all strains: positive for oxidase, urease, Hugh & Leifson oxidative metabolism, growth in 1% NaCl, in YM broth without CaCl₂ and at pH 5, 9 and 10; negative for Gram stain, indole, 3-ketolactose, gelatinase, DNase, hydrolysis of Tween 80 and growth at 40 °C.

Characteristic	Phenon 1 (n = 11)	Cluster A (phenon 2)		Cluster C (phenon 3)			Cluster B	
		ORS 1420	STM 370 ^T	ORS 1402	ORS 1403	ORS 1419 ^T	Phenon a	Phenon b
							STM 196 ^T	STM 201 ^T
Growth in/at:								
pH 4	+	–	–	–	–	–	–	–
35 °C	+	+	+	+	+	+	–	–
37 °C	–	+	+	+	+	+	–	–
2 % (w/v) NaCl	+	+	+	+	+	+	+	–
3 % (w/v) NaCl	v (5+; +)*	+	+	–	–	–	–	–
LB broth	+	+	+	+	+	+	+	–
Assimilation of:								
Glutarate	+	–	–	–	–	–	–	–
L-Tyrosine	+	–	–	–	–	–	–	–
1-O-Methyl α -D-glucopyranoside	+	–	–	–	–	–	–	+
p-Hydroxybenzoate	+	–	–	–	–	–	+	–
α -Ketoglutarate	+	–	–	–	–	–	+	–
Xylitol	+	+	+	–	–	–	–	–
Maltotriose	+	–	–	–	–	–	+	+
Protocatechuate	+	–	–	–	–	–	+	+
Citrate	+	–	–	–	–	–	+	+
(–)-Quinate	+	–	–	–	–	–	+	+
D-Tagatose	+	+	+	–	–	–	–	+
Ethanolamine	+	+	+	–	–	–	+	–
(–)-L-Arabitol	+	+	+	–	–	–	–	+
D-Saccharate	–	–	–	+	+	+	–	+
Adonitol	+	+	+	–	–	–	–	+
DL- α -Amino-n-butyrate	+	+	+	–	–	–	+	–
DL-Glycerate	+	+	+	–	–	–	+	–
L-Serine	+	–	+	–	–	–	+	–
(+)-D-Melezitose	v (10+; –)	–	–	–	–	–	–	–
cis-Aconitate	+	–	+	–	–	–	+	+
Trigonelline	+	+	–	–	–	–	+	+
Succinate	+	–	+	–	–	–	+	+
Maltitol	+	+	+	–	–	–	+	+
Mucate	–	–	–	+	+	+	–	–
trans-Aconitate	+	+	+	–	–	–	+	+
Betaine	+	+	+	–	–	–	+	+
Dulcitol	+	+	+	–	–	–	+	+
Fumarate	+	+	+	–	–	–	+	+
(+)-D-Malate	+	+	+	–	–	–	+	+
(–)-L-Malate	+	+	+	–	–	–	+	+
5-Keto-D-gluconate	+	+	+	–	–	–	+	+
DL- β -Hydroxybutyrate	+	+	+	–	–	–	+	+

Table 4. cont.

Characteristic	Phenon 1 (n=11)	Cluster A (phenon 2)		Cluster C (phenon 3)			Cluster B	
		ORS 1420	STM 370 ^T	ORS 1402	ORS 1403	ORS 1419 ^T	Phenon a	Phenon b
							STM 196 ^T	STM 201 ^T
L-Aspartate	+	+	+	–	–	–	+	+
L-Alanine	+	+	+	–	–	–	+	+
Propionate	v (10+; –)	–	–	–	–	–	–	–
i-Erythritol	–	+	+	–	–	–	–	–
D-Glucuronate	+	+	+	+	+	+	–	–
D-Galacturonate	+	+	+	+	+	+	–	–
Malonate	v (2+; –)	+	+	–	–	–	+	–
Palatinose	+	+	+	+	–	–	+	+
1-O-Methyl β-D-galactopyranoside	+	+	+	+	+	+	+	–

*For variable characters, the number of strains testing positive and the result for the type strain (STM 948^T) obtained in this study are given in parentheses.

cluster C, which are unable to assimilate (–)-L-arabitol, D-tagatose and adonitol. Strain STM 201^T is characterized by its inability to assimilate 1-O-methyl β-D-glucopyranoside and its inability to grow in LB broth and in 2 % NaCl YM broth.

For the description of novel bacterial species, the need for a polyphasic approach has been emphasized, combining phylogenetic, genetic and phenotypic aspects. Our DNA–DNA hybridization results (Table 3) indicated that *Phyllobacterium* includes four novel genomospecies that can be clearly differentiated from each other by phylogenetic and phenotypic analyses. As a consequence, we propose the creation of four novel species: *Phyllobacterium leguminum* sp. nov. for cluster C strains, *Phyllobacterium ifriqiense* sp. nov. for cluster A strains, *Phyllobacterium brassicacearum* sp. nov. for cluster B strain STM 196^T and *Phyllobacterium bourgognense* sp. nov. for cluster B strain STM 201^T, and an emended description of the genus *Phyllobacterium*. Among the novel species, *P. leguminum* was phylogenetically and phenotypically the most distant from *P. myrsinacearum*, but it shares sufficient common properties (both phenotypic and genotypic) that allow it to be included in the genus *Phyllobacterium*.

We observed that the strains isolated by Lambert *et al.* (1990) are closely associated with *P. myrsinacearum* by both phylogenetic (Figs 1 and 2) and phenotypic (Table 4 and Fig. 3) characteristics. These results strengthen the conclusion of Mergaert *et al.* (2002), who proposed to assign the sugar-beet isolates to *P. myrsinacearum* based on results from two representative strains (LMG 8225 and LMG 8229).

Since *Phyllobacterium* strains are geographically widespread in a great variety of habitats, they could be soil residents that may occasionally become associated specifically with plant roots. In addition, the fact that some bacteria were isolated

from either leaf or root nodules indicates that phyllobacteria might be a general phytosphere colonizer able to evolve and adopt different life styles. This important adaptive capacity to various environments developed by phyllobacteria was foreseen by Lambert *et al.* (1990) on the basis of the nutritional versatility of *P. myrsinacearum* strains and their antifungal activity. They reported that these features ‘probably support [their] competitive growth and abundant proliferation in the rich environment of the root surface where various compounds present in the root exudates attract diverse micro-organisms’.

**Emended description of the genus
Phyllobacterium (ex Knösel 1962) Knösel 1984**

This description takes into account results from Valverde *et al.* (2005) and Jurado *et al.* (2005) together with those from this study. Cells are Gram-negative rods, motile by means of polar, subpolar or lateral flagella. Colonies grown on YM agar medium are circular, white or cream-coloured with regular margins. Most strains are highly mucoid on YM medium. The optimal growth temperature is 28 °C. Growth occurs in 1 % NaCl and does not occur at 40 °C. Glucose metabolism is oxidative. Cultures are oxidase- and urease-positive. They lack the following exoenzyme activities: β-galactosidase, gelatinase, DNase (not tested for *P. trifolii*) and Tween 80 hydrolase (not tested for *P. trifolii*). Aesculin is hydrolysed (weak reaction for *P. trifolii*). Indole and 3-ketolactose are not produced (not tested for *P. trifolii*). Assimilation of α-(+)-D-glucose, (+)-D-mannose, maltose, (+)-L-arabinose, D-mannitol and N-acetyl-D-glucosamine is positive. Additional features common to the 18 strains studied in this paper, representing five of the seven species of the genus *Phyllobacterium*, are presented in the legend to Table 4. The G+C content of the DNA is 51–61 mol% (*T_m*). The type species is *Phyllobacterium myrsinacearum*.

Description of *Phyllobacterium leguminum* sp. nov.

Phyllobacterium leguminum (le.gu'min.um. L. gen. pl. neut. n. *leguminum* of legumes, referring to its isolation from root nodules of legumes).

Strains share the general properties of the genus *Phyllobacterium*. Colonies on YM agar are white and highly convex. The optimal growth temperature is 28 °C. Growth on YM agar occurs at 37 °C but not at 40 °C. Strains are able to grow in YM broth, YM broth without CaCl₂ and LB broth. Growth occurs in YM broth with 2 % NaCl, but not 3 %, and in YM broth at pH 5–10, but not at pH 4. Strains assimilate few substrates on Biotype 100 strips, which differentiates the species from other *Phyllobacterium* species. Assimilation of D-galacturonate, D-glucuronate, D-saccharate, mucate and 1-O-methyl β-D-galactopyranoside are discriminatory characters. Assimilation of the following substrates is weak: (+)-D-trehalose, maltose, 1-O-methyl β-D-glucopyranoside, (–)-D-ribose, glycerol, *myo*-inositol, (+)-D-turanose, 2-keto-D-gluconate, D-glucosamine, L-glutamate and L-proline. Can be differentiated from the other *Phyllobacterium* species by DNA–DNA hybridization, 16S rRNA gene or partial *atpD* gene sequencing and phenotypic tests (Table 4). The G + C content of the DNA is 57–58 mol% (*T_m*).

The type strain is strain ORS 1419^T (=CFBP 6745^T=LMG 22833^T). Strains have been isolated from root nodules of *Argyrolobium uniflorum* and *Astragalus algerianus*.

Description of *Phyllobacterium ifriqiense* sp. nov.

Phyllobacterium ifriqiense (if.ri'qi.yen.se. N.L. neut. adj. *ifriqiense* pertaining to Ifriqiya, the earliest Arabic name of the North African territory that included Tunisia, where the first strains were isolated).

Strains have the general characteristics of the genus *Phyllobacterium*. The optimal growth temperature is 28 °C. Growth on YM agar occurs at 37 °C but not at 40 °C. Strains are able to grow in YM broth, YM broth without CaCl₂ and LB broth. Growth occurs in YM broth with 1, 2 or 3 % NaCl and in YM broth at pH 5–10, but not at pH 4. Assimilation of the following substrates is variable or weak: α-lactose, lactulose, 1-O-methyl β-galactopyranoside, 1-O-methyl α-galactopyranoside, *cis*-aconitate, succinate, DL-α-amino-n-valerate, trigonelline and L-serine. Assimilation of i-erythritol is an exclusive character of strains of this species. Lack of assimilation of maltotriose, citrate, protocatechuate and quinate on Biotype 100 strips are discriminatory characters for strains of *P. ifriqiense*, shared with *P. leguminum* strains; other discriminatory characters within the genus are listed in Table 4. At the molecular level, the species is differentiated by DNA–DNA hybridization, by 16S rRNA gene sequencing and by its low DNA G + C content, 51–52 mol% (*T_m*).

The type strain is strain STM 370^T (=CFBP 6742^T=LMG 22831^T). Strains have been isolated from root nodules of *Astragalus algerianus* and *Lathyrus numidicus*.

Description of *Phyllobacterium brassicacearum* sp. nov.

Phyllobacterium brassicacearum (bras.si.ca.ce.ar'um. N.L. gen. pl. fem. n. *brassicacearum* of the Brassicaceae, referring to the isolation of the type strain from *Brassica napus* and its growth-promoting effect on *B. napus* and *Arabidopsis thaliana*, members of the Brassicaceae).

Strains share the general properties of the genus *Phyllobacterium*. The optimal growth temperature is 28 °C. Growth on YM agar does not occur at 35 °C. Strains are able to grow in YM broth, YM broth without CaCl₂ and LB broth. Growth occurs in YM broth with 2 % NaCl, but not 3 %, and in YM broth at pH 5–10, but not at pH 4. This species can be differentiated from other *Phyllobacterium* species by its auxanotrophic characteristics (Table 4), mainly its inability to assimilate (–)-L-arabitol, D-tagatose and adonitol, like strains of *P. leguminum*. The inability of the type strain to grow at 37 °C is one of the discriminatory characters between this species and *P. leguminum*. At the molecular level, DNA–DNA hybridization and 16S rRNA gene and/or partial *atpD* gene sequencing can be used to differentiate the strain. The G + C content of the DNA is 55.5 mol% (*T_m*).

The type strain, strain STM 196^T (=CFBP 5551^T=LMG 22836^T), was isolated from the rhizoplane of *Brassica napus*.

Description of *Phyllobacterium bourgognense* sp. nov.

Phyllobacterium bourgognense (bour.gogn.en'se. N.L. neut. adj. *bourgognense* pertaining to Bourgogne, a region of central France, where the type strain was isolated).

Strains have the general characteristics of the genus *Phyllobacterium*. The optimal growth temperature is 28 °C. Growth on YM agar does not occur at 35 °C. Growth occurs in YM broth at pH 5–10, but not at pH 4. In contrast to other *Phyllobacterium* species, it does not grow in LB broth or in YM broth at 2 % NaCl. Can be differentiated from other *Phyllobacterium* species by its auxanotrophic characteristics (Table 4), mainly its inability to assimilate 1-O-methyl β-D-glucopyranoside, DL-glycerate and ethanolamine and its ability to assimilate D-saccharate. Most of these characters are shared by *P. leguminum*, but these two species are easily differentiated by temperature and NaCl tolerance. At the molecular level, DNA–DNA hybridization and 16S rRNA gene and/or partial *atpD* gene sequencing can be used to differentiate the species. The G + C content of the DNA is 54 mol% (*T_m*).

The type strain, strain STM 201^T (=CFBP 5553^T=LMG 22837^T), was isolated from the rhizoplane of *Brassica napus*.

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