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Diversity of grass-associated *Microbacteriaceae* isolated from the phyllosphere and litter layer after mulching the sward; polyphasic characterization of *Subtercola pratensis* sp. nov., *Curtobacterium herbarum* sp. nov. and *Plantibacter flavus* gen. nov., sp. nov.

Undine Behrendt,[1] Andreas Ulrich,[2] Peter Schumann,[3] Dieter Naumann[4] and Ken-ichiro Suzuki[5]

[1] Centre for Agricultural Landscape and Land Use Research (ZALF), Institute of Primary Production and Microbial Ecology, Gutshof 7, D-14641 Paulinenaue, Germany

[2] Centre for Agricultural Landscape and Land Use Research (ZALF), Institute of Primary Production and Microbial Ecology, Eberswalder Str. 84, D-15374 Müncheberg, Germany

[3] DSMZ – German Collection of Microorganisms and Cell Cultures, Mascheroder Weg 1b, D-38124 Braunschweig, Germany

[4] Robert Koch-Institute, Nordufer 20, D-13353 Berlin, Germany

[5] Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research), Wako-shi, Saitama 351-0198, Japan

Author for correspondence: Undine Behrendt. Tel: +49 33237 849357. Fax: +49 33237 849226. e-mail: ubehrendt@zalf.de

A representative selection of coryneform bacteria, isolated from the phyllosphere of grasses and the litter layer after mulching the sward, was characterized by a polyphasic approach to clarify their taxonomic position in the family *Microbacteriaceae*, with particular reference to potentially plant-pathogenic bacteria. On the basis of 16S rDNA analysis, the isolates can be classified into six genotypes representing the genera *Curtobacterium*, *Clavibacter*, *Subtercola* and a subgroup, which was not affiliated to a known genus. One genotype, belonging to the genus *Curtobacterium*, had an identical 16S rDNA sequence to reference strains of the *Curtobacterium flaccumfaciens* pathovars. Another genotype, closely related to the potentially pathogenic *Curtobacterium flaccumfaciens*, could be distinguished from known species of the genus on the basis of phylogenetic and phenotypic characterization and is consequently proposed as a novel species, *Curtobacterium herbarum* sp. nov. (type strain P 420/07^T = DSM 14013^T = LMG 19917^T). Two genotypes assigned to *Clavibacter* showed a close relationship to *Clavibacter michiganensis* subsp. *tessellarius*, a pathogenic bacterium causing foliar lesions on wheat. A further genotype, which clustered clearly in the genus *Subtercola* by comparison of 16S rDNA sequences, showed a hitherto undescribed B-type of peptidoglycan containing the diagnostic diamino acids ornithine and 2,4-diaminobutyric acid, in the cell wall; this genotype is proposed as *Subtercola pratensis* sp. nov. (type strain P 229/10^T = DSM 14246^T = LMG 21000^T). For one genotype, which formed a phylogenetically separate branch in the family of *Microbacteriaceae* showing chemotaxonomic similarities to the genus *Rathayibacter*, a novel genus, *Plantibacter* gen. nov., is proposed; the type species is *Plantibacter flavus* sp. nov. (type strain P 297/02^T = DSM 14012^T = LMG 19919^T).

Keywords: plant-associated bacteria, polyphasic analysis, *Subtercola pratensis* sp. nov., *Curtobacterium herbarum* sp. nov., *Plantibacter flavus* gen. nov., sp. nov.

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Abbreviations: DAB, 2,4-diaminobutyric acid; FT-IR spectroscopy, Fourier-transform infrared spectroscopy.

The EMBL accession numbers for the 16S rDNA sequences analysed in this study are AJ310412 (*Subtercola pratensis* P 229/10^T = DSM 14246^T = LMG 21000^T), AJ310413 (*Curtobacterium herbarum* P 420/07^T = DSM 14013^T = LMG 19917^T), AJ310414 (*Curtobacterium flaccumfaciens* P 259/26), AJ310415 (*Clavibacter michiganensis* P 202/10), AJ310416 (*Clavibacter michiganensis* P 250/01), AJ310417 (*Plantibacter flavus* P 297/02^T = DSM 14012^T = LMG 19919^T) and AJ312209 (*Curtobacterium flaccumfaciens* pv. *flaccumfaciens* LMG 3645^T).

INTRODUCTION

The phyllosphere of plants is a typical habitat for coryneform bacteria displaying the B-type of peptidoglycan. Several ecological studies have shown that this group forms a significant part of bacterial communities of a number of plant species (Austin *et al*., 1978; Bell *et al*., 1995; Ercolani, 1991; Legard *et al*., 1994; McInroy & Kloepper, 1995; Thompson *et al*., 1993). Because of difficulties in taxonomic characterization on the basis of phenotypic features, most of the isolated strains have been identified at the genus level or affiliated to *Curtobacterium flaccumfaciens* and *Clavibacter michiganensis* without further specification at the subspecies or pathovar level.

Studies of the microbial communities on grasses have also revealed a high abundance of coryneform bacteria, forming up to 80% of the culturable bacterial population (Behrendt, 2001). In accordance with Harris-Baldwin & Gudmestad (1996), use of the Biolog system, which is supported by some chemotaxonomic and physiological features, for identification has led to unsatisfactory taxonomic results leaving specific classification in comparison to potentially phytopathogenic species unresolved.

A variety of methods, proven to be valuable but somewhat limited for the differentiation of this bacterial group, has been used to classify coryneform bacteria on the basis of pheno- or genotypic features. In particular, plant-pathogenic coryneform organisms form a very homogeneous group and therefore, numerical analyses on the basis of phenotypic features are often inadequate to classify them at the species level (Kämpfer & Kroppenstedt, 1996; Kämpfer *et al*., 1993; Seiler, 1983). Classification by molecular methods like 16S rDNA restriction analysis have been shown to be useful for species and subspecies of the genera *Rathayibacter* and *Clavibacter* (Lee *et al*., 1997). However, temperature-gradient gel electrophoresis of 16S rRNA genes of coryneforms revealed a genus-dependent differentiation at the species or genus level or intermediate between them (Felske *et al*., 1999). In contrast, recent studies on coryneform bacteria based on Fourier-transform infrared (FT-IR) spectroscopy showed the high resolution power of this method at the species level, which also allowed an infraspecific classification, as demonstrated for *Brevibacterium linens*, *Rhodococcus erythropolis* and *Corynebacterium glutamicum* (Oberreuter, 2001). Thus, phenotypic classification by FT-IR spectroscopy, classical methods of physiological and chemotaxonomic characterization, in comparison with phylogenetic analysis by 16S rDNA and DNA–DNA hybridization, were used to study the grass-associated isolates with particular reference to plant-associated coryneforms.

Fifty strains representing major groups were selected based on rough physiological and chemotaxonomic classification to investigate the diversity of the grass-associated coryneforms. Members of the genus *Microbacterium* were excluded and studied separately (Behrendt *et al*., 2001). Inasmuch as 16S rDNA restriction analyses of selected isolates revealed six genotypes representing more than one strain, five representative strains of each genotype were chosen for analysis following a polyphasic strategy of classification to clarify their taxonomic position.

METHODS

Bacterial strains and cultivation. The bacterial strains examined in this study were isolated from grasses and surface litter as described previously (Behrendt, 2001). Numbers of the isolates and strains of reference species used for comparative studies are listed in Table 1. General laboratory cultivation was performed on nutrient agar II (SIFIN) or nutrient broth II (SIFIN) at 25 °C unless otherwise stated. Stocks of all cultures were maintained at −79 °C using the Microbank storage system (Pro-Lab Diagnostics).

16S rDNA sequence determination and phylogenetic analysis. Restriction analysis of amplified 16S rDNA was performed as described by Behrendt *et al*. (1999). Representative strains of genotypes A, B1, B2, C, D and F, respectively, were used in 16S rDNA sequence determinations. The 16S rDNA fragments amplified with *Pfu* DNA polymerase (Promega) were cloned and sequenced using a cycle sequencing protocol with a Li-Cor Sequencer (model 4200; MWG Biotech) as previously described (Behrendt *et al*., 2001). The nearly complete 16S rDNA sequences were compared to sequences available from the Ribosomal Database Project and EMBL/GenBank. Similarity values were based on a pairwise comparison of sequences. For phylogenetic analyses, DNA sequences were aligned using the CLUSTAL w algorithm (program version 1.74; Thompson *et al*., 1994) and the trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms (PHYLIP, version 3.57; Felsenstein, 1993). Distance matrices were prepared using DNADIST with a transition/transversion ratio of 2·0 (Kimura, 1980). Tree topologies were evaluated by bootstrap analysis of the neighbour-joining tree using the original dataset and 1000 bootstrap datasets. The root position was estimated using the 16S rRNA sequence of *Brevibacterium linens* ATCC 9172^T (X77451) as outgroup.

DNA–DNA hybridization. DNA–DNA similarity was examined for P 420/07^T (genotype D) and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (LMG 3645^T), according to the method described by Martin *et al*. (1997).

Determination of chemotaxonomic characteristics. Methods used for the analyses of menaquinones and G+C values were described previously by Groth *et al*. (1999). Elucidation of the peptidoglycan structure was accomplished as described previously (Männistö *et al*., 2000). Analysis of enantiomeric diamino acid isomers was performed according to Sasaki *et al*. (1998). Fatty acid methyl esters and 1,1-dimethoxy-alkanes were analysed by GC and GC/MS as described by Schumann *et al*. (1997) using lyophilized biomass obtained after incubation of strains for 48 h at 28 °C. Determination of the glycolate content was performed according to the colorimetric method of Uchida *et al*. (1999).

Morphological, physiological and biochemical characterization. Cell morphology was determined by light microscopy of cells grown for 24 and 72 h. Motility of cells was

Table 1. Reference strains and isolated strains used in this study

Abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; CCM, Czech Collection Mikroorganism, Masaryk University, Brno, Czech Republic; CIP, Institut Pasteur, CIP-Collection de l'Institut Pasteur, Paris, France; IAM, Institute of Applied Microbiology, University of Tokyo, Institute of Molecular and Cellular Bioscience, Tokyo, Japan; ICMP, International Collection of Microorganisms from Plants, Plant Disease Division, DSIR, Auckland, New Zealand; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; CPB, International Collection of Phytopathogenic Bacteria, Davis, CA, USA; NCIB (NCIMB, NCMB), National Collection of Industrial and Marine Bacteria, Aberdeen, UK; LMG, Universiteit Gent, Laboratorium voor Mikrobiologie, Gent, Belgium; NCPPB, National Collection of Plant-pathogenic Bacteria, Central Science Laboratory, York, UK; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK; VKM, All-Russian Collection of Microorganisms, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia; P, culture at the collection in the Institute of Primary Production and Microbial Ecology, Centre for Agricultural Landscape and Land Use Research Müncheberg (ZALF), Paulinenaue, Germany.

Species/genotype of grass isolates	Collection no. (corresponding no.)
Curtobacterium flaccumfaciens	
pv. *flaccumfaciens*	LMG 3645^T (= ICMP 2584^T = NCPPB 1446^T); DSM 20129 (= ATCC 6887 = NCTC 4758)
pv. *flaccumfaciens* (*Corynebacterium flaccumfaciens* subsp. *aurantiacum*)	DSM 20135 (= ATCC 12813)
pv. *flaccumfaciens* (*Corynebacterium flaccumfaciens* subsp. *violaceum*)	LMG 7245 (= ICMP 2341)
pv. *betae*	LMG 3596 (= ICMP 2594 = ICPB CB104 = NCPPB 374); DSM 20141 (= ATCC 13437)
pv. *poinsettiae*	DSM 20149 (= ATCC 9682 = CCM 1587); LMG 3713 (= ICMP 2563 = NCPPB 849)
pv. *oortii*	LMG 3702 (= ATCC 25283 = ICMP 2632 = ICPB CO101 = NCPPB 2113); LMG 3704 (= ICMP 3498 = NCPPB 2240)
Curtobacterium luteum	DSM 20542^T (= ATCC 15830^T = CCM 2298^T = NCIB 11029^T)
Curtobacterium citreum	DSM 20528^T (= ATCC 15828^T = CCM 2297^T = IAM 1614^T = NCIB 10702^T)
Curtobacterium pusillum	DSM 20527^T (= ATCC 19096^T = IAM 1479^T = NCIB 10354^T)
Curtobacterium albidum	DSM 20512^T (= ATCC 15831^T = CCM 2296^T = IAM 1631^T = NCIB 11030^T)
Clavibacter michiganensis	
subsp. *tessellarius*	DSM 20741^T (= ATCC 33566^T = CIP 105364^T)
subsp. *nebraskensis*	DSM 7483^T (= ATCC 27794^T = CIP 105362^T = ICMP 3298^T = NCPPB 2581^T)
subsp. *michiganensis*	DSM 46364^T (= ICPB CM 177^T = LMG 7333^T = NCPPB 2979^T)
subsp. *insidiosus*	DSM 20157^T (= ATCC 10253^T = NCPPB 1109^T)
subsp. *sepedonicus*	DSM 20744^T (= ATCC 33113^T = ICPB CS101^T = NCPPB 2137^T)
Rathayibacter tritici	DSM 7486^T (= ATCC 11403^T = ICMP 2626^T = NCPPB 1857^T = VKM Ac-1603^T)
Rathayibacter iranicus	DSM 7484^T (= ICMP 3496^T = NCPPB 2253^T = VKM Ac-1602^T)
Rathayibacter rathayi	DSM 7485^T (= ICMP 2574^T = NCPPB 2980^T = VKM Ac-1601^T)
Rathayibacter toxicus	DSM 7488^T (= ATCC 49908^T = ICMP 9525^T = NCPPB 3552^T)
Genotype	
A	P 259/26; P 412/02; P 420/01; P 425/08; P 429/02
B1	P 202/10; P 211/03; P 219/02; P 221/03; P 231/03
B2	P 250/01; P 251/03; P 251/08; P 251/06; P 251/07
C	P 297/02^T (= DSM 14012^T = LMG 19919^T); P 309/02; P 259/33; P 294/03; P 334/04
D	P 420/07^T (= DSM 14013^T = LMG 19917^T); P 220/02; P 445/13; P 407/01; P 433/01
F	P 229/10^T (= DSM 14246^T = LMG 21000^T); P 209/03; P 209/05; P 243/03; P 243/10

tested by the hanging drop method of Rudolph & Marvidis (1990). The Gram reaction was tested by the classical staining procedure as described by Süßmuth *et al.* (1987), the rapid KOH string test (Ryu, 1938), growth on MacConkey agar (Merck) and by the presence of L-alanine aminopeptidase (Bactident test strips; Merck). Most tests for characterizing the biochemical profiles of studied strains were performed as described previously (Behrendt *et al.*, 1999). Hydrolysis of Tween and starch was assayed according to Sands (1990). The ability to grow anaerobically was tested using Anerocult A (Merck).

Fourier-transform infrared (FT-IR) spectroscopy of bacterial cells. Spectral analyses were performed on cells grown for 72 h on CASO agar (Merck) at 25 °C. A small amount of late-exponential-phase cells (approx. 10–60 µg) was removed with a platinum loop from confluent colonies in the second or third quadrant of growth on the agar surface, depending on intensity of growth, and suspended in 30 µl

distilled water. An aliquot (25 µl) was transferred to a zinc-selenite optical plate and dried under moderate vacuum between 2·5 and 7·5 kPa to a transparent film suitable for absorbance/transmission measurement. All spectra were recorded between 4000 and 500 cm^{-1} (wave numbers) on an IFS-48 FT-IR spectrometer (Bruker) equipped with an MCT (mercury-cadmium-telluride) detector by co-addition and averaging 256 scans. Spectral resolution was 8 cm^{-1}.

RESULTS

Phylogenetic characterization

All isolates yielded a single band of about 1500 bp after amplification with the universal eubacterial primers used (Weisburg *et al*., 1991). Digestion of the PCR products led to three to five different restriction patterns per enzyme, which were grouped into nine 16S rDNA genotypes as shown in Table 2. The six main groups, designated genotypes A–F, could be clearly differentiated by the enzymes *Alu*I and *Cfo*I. Restriction patterns of *Hae*III and *Msp*I revealed no additional information, whereas *Hinf*I and *Scr*FI revealed slight differences within genotypes C, D and F, each represented by one strain.

Restriction analysis of the *Curtobacterium flaccumfaciens* pathovars showed identical patterns for each endonuclease used (Table 2). This result points to nearly identical 16S rDNA sequences of the pathovars and, thus, the inability to differentiate them by this method. Furthermore, grass isolates of genotype A revealed the same restriction patterns as pathovars of *Curtobacterium flaccumfaciens* indicating that they also belong to this species. Sequencing of the type strain of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (LMG 3645^T) confirmed 16S rDNA identity with genotype A (strain P 259/26).

The phylogenetic relationship of genotypes A–F, as determined using sequences of representative isolates, is shown in Fig. 1. Isolate P 420/07^T (genotype D) unequivocally clustered into the genus *Curtobacterium*, supported by a high bootstrap value (100). Within the genus, however, the strain formed a separate branch that was found in both the neighbour-joining and maximum-likelihood trees. Optimum conformity of genotype D was found with *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* LMG 3645^T (similarity of 98·2 %). However, analysis of DNA–DNA reassociation of both strains resulted in a similarity of 35·6 %, clearly suggesting the novel species status of strain P 420/07^T (genotype D).

Reference strains of genotypes B1 and B2 clustered with *Clavibacter michiganensis* on the basis of 16S rDNA sequence similarity (Fig. 1). Genotype B1 (P 202/10) showed the highest similarity to *Clavibacter michiganensis* subsp. *tessellarius* (99·8 %). Genotype B2 (P 250/01) displayed a similarity of 99·6 % to *Clavibacter michiganensis* subsp. *tessellarius* and to *Clavibacter michiganensis* subsp. *insidiosus*. These results suggest that isolates of genotypes B1 and B2 belong to the species *Clavibacter michiganensis*.

Genotype F, represented by P 229/10^T, clustered with *Subtercola* using both neighbour-joining and maximum-likelihood algorithms. This branch was also supported by a high bootstrap value (96). Sequence similarities of P 229/10^T to *Subtercola boreus* and *Subtercola frigoramans* were 96·7 and 95·3 %, respectively, which was in the same range as the similarity between both *Subtercola* species (95·7 %). These results indicated that genotype F should be assigned to the genus *Subtercola* as a novel species.

The representative isolate of genotype C (strain P

Table 2. 16S rDNA restriction patterns of the grass isolates and *Curtobacterium flaccumfaciens* pathovars

Genotype	Strain	Restriction pattern using endonuclease:						
		*Alu*I	*Hae*III	*Msp*I	*Cfo*I	*Hinf*I	*Scr*FI	*Sau*3A
A	P 259/26; P 412/02; P 420/01; P 425/08; P 429/02; *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (LMG 3645^T; DSM 20129; DSM 20135; LMG 7245); *Curtobacterium flaccumfaciens* pv. *betae* (LMG 3596; DSM 20141); *Curtobacterium flaccumfaciens* pv. *poinsettiae* (DSM 20149; LMG 3713); *Curtobacterium flaccumfaciens* pv. *oortii* (LMG 3702; LMG 3704)	A	A	A	A	A	A	A
B1	P 202/10; P 211/03; P 219/02; P 221/03; P 231/03	B	B	B	B	B	B	B
B2	P 250/01; P 251/03; P 251/08; P 251/06; P 251/07	B	B	B	D	B	B	B
C	P 297/02^T (= DSM 14012^T); P 294/03; P 334/04; P 309/02	C	C	C	A	B	B	F
	P 259/33	C	C	C	A	C	B	F
D	P 420/07^T (= DSM 14013^T); P 445/13; P 407/01; P 433/01	D	A	A	A	A	D	D
	P 220/02	D	A	A	A	A	A	D
F	P 229/10^T (= DSM 14642^T); P 209/03; P 243/03; P 243/10	F	F	F	B	B	B	F
	P 209/05	F	F	F	B	C	B	F

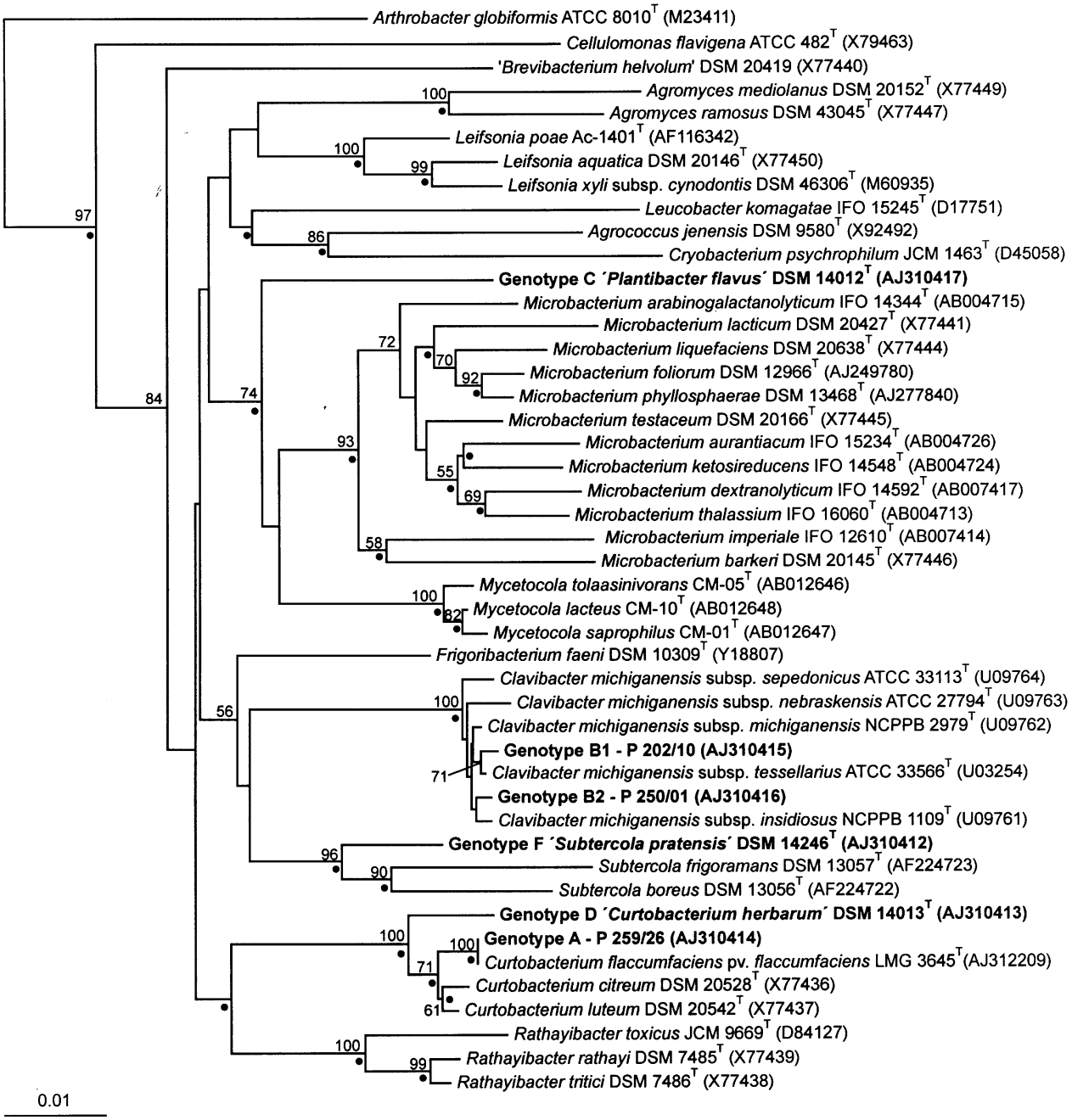

Fig. 1. Phylogenetic tree showing the relationship of the isolated genotypes within the family *Microbacteriaceae*. The tree is based on a 1486 bp alignment of 16S rDNA sequences and was constructed using the neighbour-joining method (Saitou & Nei, 1987). Dots indicate branches of the tree that were also formed using the maximum-likelihood method (Felsenstein, 1981). To estimate the root position of the tree, *Brevibacterium linens* (ATCC 9172^T; X77451) was used as an outgroup. The values are the number of times that a branch appeared in 100 bootstrap replications. Strains characterized in this study are in bold. Bar, relative sequence divergence.

297/02^T) showed highest 16S rDNA similarity to *Microbacterium testaceum* (95·6%), followed by *Mycetocola saprophilus*, *Microbacterium keratanolyticum*, *Mycetocola tolaasinivorans* and *Microbacterium lacticum* at a 95% similarity level. Clustering on the basis of the neighbour-joining algorithm, as well as the maximum-likelihood method (Fig. 1), clearly revealed that strains of genotype C formed a separate branch connected with the cluster of genera *Microbacterium* and *Mycetocola*. This separate phylogenetic position indicates that genotype C deserves the status of a novel genus.

Table 3. Chemotaxonomic characteristics of type strains of the 16S rRNA restriction groups representing a new taxon

Noteworthy values are shown in bold. DMA, 1,1-Dimethoxy-anteiso-pentadecane.

Feature	Genotype D (P 420/07^T = DSM 14013^T)	Genotype C (P 297/02^T = DSM 14012^T)	Genotype F (P 229/10^T = DSM 14642^T)
Menaquinone composition (ratio of peak area):			
MK-7	6	–	–
MK-8	7	–	–
MK-9	**77**	9	13
MK-10	2	**60**	**51**
MK-11	–	**16**	**21**
MK-12	–	2	–
Fatty acid composition (%):			
14:0	–	–	0·25
16:0	1·57	2·14	**5·22**
i-14:0	0·40	1·73	0·53
i-15:0	1·22	**4·17**	1·00
i-16:0	**9·50**	**19·11**	**17·23**
i-17:0	1·06	0·90	0·51
a-15:0	**43·73**	**59·13**	**46·71**
a-17:0	**41·43**	**12·57**	**22·15**
a-15:1	0·41	–	–
a-17:1	0·69	–	–
a-15:0 DMA	–	–	4·17
Unknown	–	–	2·22
Diagnostic diamino acid	Orn	L-DAB	L-DAB; D-Orn
Acyl type	Acetyl	Acetyl	Acetyl
G+C content (mol%)	71	70	65

Chemotaxonomic characterization

As shown in Table 3, an extensive chemotaxonomic characterization was performed using type strains of the restriction types proposed to represent novel taxa (genotypes D, C and F). Chemotaxonomic properties of strain P 420/07^T (genotype D) corresponded with those of the genus *Curtobacterium*. The diamino acid ornithine (Orn) was found in the peptidoglycan, which was of the B2β type with acetyl residues, and the major respiratory menaquinone was MK-9. Analysis of fatty acid composition showed the occurrence of anteiso-methyl-branched, iso-methyl-branched and a minor component of straight-chain saturated fatty acids (Table 3).

Strain P 297/02^T (genotype C) had a cell wall composition comparable to those of the genera *Rathayibacter* and *Agromyces*. The peptidoglycan contained L-DAB (2,4-diaminobutyric acid), D-Glu, D-Ala and Gly in a molar ratio of 2:1:1:1, corresponding to type B2γ of Schleifer & Kandler (1972) with acetyl residues. Predominant menaquinones were MK-10 and MK-11. Fatty acid analysis revealed a composition typical of most genera of the family *Microbacteriaceae*. In addition to the major anteiso-methyl-branched and iso-methyl-branched fatty acids, straight-chain saturated fatty acids were found as minor components. The G+C content of strain P 297/02^T was 70 mol% and 68 mol% for an additional strain of genotype C (strain P 309/02). This range is typical for genera of the family *Microbacteriaceae*.

Strain P 229/10^T (genotype F) showed the peptidoglycan amino acids Gly, Ala and threo-3-hydroxyglutamic acid, but only traces of Glu and a hitherto unknown combination of diagnostic diamino acids in the peptidoglycan, L-DAB and D-Orn. To confirm this cell wall composition, hydrolysates were subjected to analyses by TLC, GC, GC/MS, HPLC and an automatic amino acid analyser. Two additional strains of genotype F (P 209/05 and P 243/03) were included in the analyses. All analytical techniques revealed the combined occurrence of Orn and DAB and both strains showed the same patterns as strain P 229/10^T, indicating that this peptidoglycan composition was typical of this group. Predominant menaquinones were MK-10 and MK-11. As shown in Table 3, fatty acid analyses revealed anteiso-methyl-branched and iso-methyl-branched fatty acids in major amounts. Straight-chain saturated fatty acids were also found and in a higher proportion than in isolates of genotypes C and D. As revealed by GC/MS analysis, the fatty acid methyl ester extract of strain P 229/10^T contained approximately 4·2% 1,1-dimethoxy-anteiso-pentadecane (ai-15:0 DMA). The G+C contents of strains P 229/10^T and P 209/05 were 65 and 66 mol%, re-

Table 4. Physiological and morphological characteristics of the 16S rRNA restriction types in comparison to reference strains of the genera *Curtobacterium*, *Clavibacter* and *Rathayibacter*

Abbreviations: +, positive reaction; +w, weakly positive; −, negative; d, reaction differs among strains; y, yellow; i, ivory; ly, light yellow; o, orange; y/b, blue pigment of the intracellular indigoidine granules sometimes produced in addition to the yellow pigment; yo, yellow-orange; w, white. Species/genotype: 1, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*; 2, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Corynebacterium flaccumfaciens* subsp. *aurantiacum*); 3, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Corynebacterium flaccumfaciens* subsp. *violaceum*); 4, *Curtobacterium flaccumfaciens* pv. *betae*; 5, *Curtobacterium flaccumfaciens* pv. *poinsettiae*; 6, *Curtobacterium flaccumfaciens* pv. *oortii*; 7, genotype A; 8, genotype D; 9, *Curtobacterium luteum*; 10, *Curtobacterium citreum*; 11, *Curtobacterium pusillum*; 12, *Curtobacterium albidum*; 13, *Clavibacter michiganensis* subsp. *tessellarius*; 14, *Clavibacter michiganensis* subsp. *nebraskensis*; 15, *Clavibacter michiganensis* subsp. *michiganensis*; 16, *Clavibacter michiganensis* subsp. *insidiosus*; 17, *Clavibacter michiganensis* subsp. *sepedonicus*; 18, genotype B1; 19, genotype B2; 20, genotype C; 21, *Rathayibacter tritici*; 22, *Rathayibacter iranicus*; 23, *Rathayibacter rathayi*; 24, *Rathayibacter toxicus*; 25, genotype F.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Number of strains	2	1	1	2	2	2	5	5	1	1	1	1	1	1	1	1	1	5	5	5	1	1	1	1	5
Colony colour	y	o	y	y	o	y	o	o	y	y	ly	i	o	o	y	y/b	w	o	y/yo	y	y	y	y	y	y
Motility	+	−	+	d	d	d	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Hydrolysis of:																									
Gelatin	+	w+	+	−	d	+	d	d/+*	−	−	+	+	w+	−	−	−	−	d	d	−	−	−	−	−	−
Aesculin	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	w	−	+
Casein	+	+	+	−	d	+	d	d/+*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Starch	−	−	−	d	d	d	d	−	−	−	−	−	−	−	−	−	−	−	d	d/+†	−	−	−	−	−
Tween 80	−	w+	−	d	+	d	+	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	+
Tween 60	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	d	−	+	+	+	+	−	+
DNase	w+	w+	w+	−	−	−	−	−	−	−	−	−	+	+	+	+	−	w+	+	+	−	−	−	−	−
Levan	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Oxidative acid production from:																									
Adonitol	+	+	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−	d	−	−	−	−	−	−
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	d	+	−	−	−	−	−	d/−‡
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	d/−†	+	+	−	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−
D-Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	+
D-Melezitose	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+	−	−	−	−	−
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	d/−†	−	−	−	−	w+
D-Sorbitol	+	+	+	+	+	+	+	+	−	−	−	−	+	w+	−	−	−	+	+	d/−†	w+	w+	w+	w+	−
L-Sorbose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	d/−†	−	−	−	−	−
Raffinose	+	+	+	+	+	+	+	+	−	−	+	+	−	−	−	−	−	+	+	+	−	−	−	−	+
L-Rhamnose	+	+	+	+	−	+	d	d/−*	+	+	+	+	−	−	−	−	−	d	d	+	−	−	−	−	d/+‡
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	−	−	−	+

* Reaction of the type strain P 420/07^T (= DSM 14013^T = LMG 19917^T).

† Reaction of the type strain P 297/02^T (= DSM 14012^T = LMG 19919^T).

‡ Reaction of the type strain P 229/10^T (= DSM 14642^T = LMG 21000^T).

spectively. This range is in accordance with that of the family *Microbacteriaceae*.

Analysis of diagnostic diamino acids of genotype A, phylogenetically highly related to *Curtobacterium flaccumfaciens*, revealed Orn as being typical of the genus. The acetyl residue of the peptidoglycan and the predominant menaquinone, MK-9, observed for the representative strain, P 259/26, also corresponded with *Curtobacterium*. Strains of genotypes B1 and B2 had DAB in their cell wall, corresponding with the genus *Clavibacter*.

Morphological and physiological characterization

Strains listed in Table 1 were studied with respect to their phenotypic features. Reference species compared with the grass-associated isolates were chosen for their affiliation to genera known for their potential occurrence in the phyllosphere of plants.

In spite of the taxa they belong to, all strains were negative for spore formation, anaerobic growth, oxidase and the Voges–Proskauer reaction, as well as hydrolysis of cellulose; however, they were catalase-positive and able to form acid from D-mannitol, D-galactose, D-fructose, sucrose, L-arabinose and D-trehalose (excluding strain P 251/07). Furthermore, they oxidatively produced acid from D-mannose, D-xylose and D-glucose, with the exception of *Clavibacter michiganensis* subsp. *sepedonicus*, which was negative for these characteristics. The tested strains were Gram-positive but, by the rapid KOH test, a lot of grass isolates behaved like Gram-negative strains. Furthermore, the classical staining procedure gave uncertain results as most of the isolates decolourized easily. The absence of L-alanine aminopeptidase was also difficult to determine because borderline reactions often occurred. Thus, cell wall analysis was the method of choice to make certain of the Gram assignment. To select these problematic strains for cell wall analyses from a collection of isolates also containing Gram-negative strains, a comparison of results of the KOH

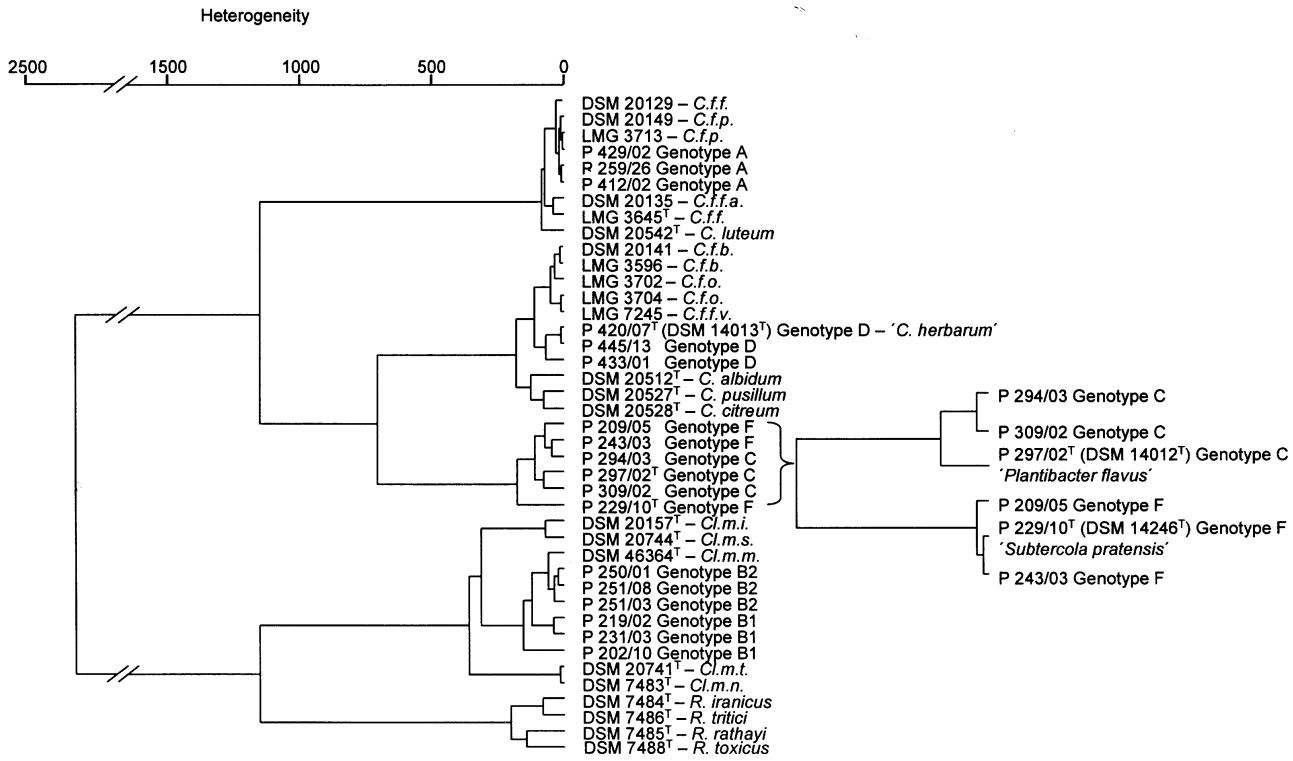

Fig. 2. Classification of coryneform bacteria by FT-IR spectroscopy. Cluster analysis (Wards algorithm) was performed using the mean spectrum of the first derivatives of four spectra determined per strain considering the equally weighted spectral ranges (windows) 901–699 cm^{-1} and 1201–900 cm^{-1} for all the tested strains and window 1111–1089 cm^{-1} to separate genotypes F and C. Abbreviations: *C.*, *Curtobacterium*; *f.*, *flaccumfaciens*; *b.*, *betae*; *p.*, *poinsettiae*; *o.*, *oortii*; *a.*, *aurantiacum*; *v.*, *violaceum*; *Cl.*, *Clavibacter*; *m.*, *michiganensis*; *i.*, *insidiosus*; *s.*, *sepedonicus*; *t.*, *tessellarius*; *n.*, *nebraskensis*; *R.*, *Rathayibacter*.

test and the ability to grow on MacConkey agar proved to be useful. Isolates that showed a Gram-negative reaction and did not grow on MacConkey agar usually contained diagnostic diamino acids typical of coryneform bacteria.

The optimal growth temperature for all grass isolates was 21 °C, but growth was also observed at 4 and −2 °C; the colder the temperature, the slower the growth.

Physiological properties which were appropriate for classification are shown in Table 4. The orange-pigmented strains of genotypes A and D could only be differentiated from one another by acid formation from adonitol. In particular, genotype A was physiologically highly similar to the pathovars of *Curtobacterium flaccumfaciens*. Separation on the basis of tested features was not possible. Pathovars of *Curtobacterium flaccumfaciens* were also highly similar to each other and no feature was evident for a corresponding classification. In contrast, the remaining species of the genus *Curtobacterium* could be differentiated from genotypes A and D by comparison of the following characteristics: hydrolysis of aesculin and Tween 80 in combination with acid production from adonitol and raffinose.

Strains of genotypes B1 and B2, which were phylogenetically highly related to subspecies of *Clavibacter michiganensis*, differed from one another only in acid formation from D-melezitose and colony colour. Apart from the slow growing *Clavibacter michiganensis* subsp. *sepedonicus*, which was mainly physiologically inactive, comparison between type strains of the subspecies and the grass-associated isolates revealed more differences in acid production from carbohydrates than when the subspecies were compared to each other (Table 4). Accordingly, the grass isolates of genotype B formed a physiologically separate group which cannot be affiliated to a known subspecies of *Clavibacter* on the basis of tested characteristics.

Strains of genotype C that phylogenetically represent a novel genus could be differentiated from the chemotaxonomically similar species of *Rathayibacter* by the hydrolyses of Tween 80 and DNase. Furthermore, the genotype C organisms oxidatively formed acid from D-melezitose, raffinose, L-rhamnose and ribose, in contrast to *Rathayibacter* species. Higher physiological similarity was revealed to the yellow-pigmented strains of genotype B2. As shown in Table 4, genotype C strains differed only in Tween hydrolyses and production of acid from inositol and ribose. Similar results were obtained for a comparison with genotype F,

which can be distinguished by DNase and acid formation from lactose and D-melezitose.

Genotype F could be differentiated from the remaining 16S rDNA genotypes, as well as from *Clavibacter* and *Curtobacterium*, by acid production from inulin. Species of *Rathayibacter* were dissimilar in hydrolyses of Tween 80 and acid production from L-raffinose and ribose.

Finally, it can be concluded that the tested grass isolates and reference strains of related species were physiologically very similar. There were only a few differentiating characteristics, which were useful for a grouping corresponding to the phylogenetic classification.

FT-IR spectroscopy

Classification on the basis of FT-IR spectra was performed using three representative isolates of each restriction type and reference strains listed in Table 1. As shown in Fig. 2, cluster analysis on the basis of the selected spectral regions, 901–699 cm^{-1} and 1201–900 cm^{-1}, showed a phenetic classification where most of the tested strains were grouped according to the genus affiliation based on 16S rDNA similarity. Only strains of genotypes C and F formed a mixed cluster close to some *Curtobacterium* species. Analysing this cluster by another spectral window (1111–1089 cm^{-1}) allowed a clear classification according to phylogenetic typing (Fig. 2).

Strains belonging to the genus *Curtobacterium* were divided up into two clusters in the spectral range shown in Fig. 2. In particular, the pathovars of *Curtobacterium flaccumfaciens* were subdivided. One cluster included *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, *Curtobacterium flaccumfaciens* pv. *poinsettiae* and the tested isolates of genotype A. In the other group, *Curtobacterium flaccumfaciens* pv. *betae*, *Curtobacterium flaccumfaciens* pv. *oortii* and the genotype D isolates clustered together. Furthermore, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, formerly assigned to *Corynebacterium flaccumfaciens* subsp. *violaceum*, was found in this group, whereas the strain of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, formerly assigned to *Corynebacterium flaccumfaciens* subsp. *aurantiacum*, clustered in the first group. Separate analysis of further spectral regions of the *Curtobacterium flaccumfaciens* pathovars revealed no differences in clustering according to pathovar classification. Strain-specific dissimilarities were higher than pathovar-specific spectral vibrations. Accordingly, genotype A organisms could not be distinguished from this group, whereas genotype D strains and representatives of further *Curtobacterium* species could be separated by analysing additional spectral windows.

DISCUSSION

Taxonomic analyses of coryneform bacteria associated with the phyllosphere of grasses and the surface litter after mulching the sward revealed a phylogenetically highly diverse assemblage. In addition to groups closely related to potentially phytopathogenic species, bacteria representing novel taxa have been isolated.

Characterization of strains belonging to 16S rDNA genotype A showed that they are epiphytically living strains of the species *Curtobacterium flaccumfaciens*, which comprises phytopathogenic pathovars of several plant species (Collins & Jones, 1983). In contrast to the subspecies of *Clavibacter michiganensis*, which can be grouped on the basis of 16S rDNA restriction patterns as shown by Lee *et al*. (1997), genotype A and *Curtobacterium flaccumfaciens* pathovars displayed no dissimilarities in 16S rDNA sequence. As with results of other studies (Dye & Kemp, 1977; Vidaver & Davis, 1994), analysis of morphological and physiological properties revealed no phenotypic markers for a valid classification of pathovars. Davis *et al*. (1984) showed a combination of differential physiological characteristics, which are partly in contrast to the results of the studies mentioned above. Evaluation of the reliability of the Biolog identification system to classify pathovars of *Curtobacterium* by utilization of 95 carbon sources (Harris-Baldwin & Gudmestad, 1996) confirmed the difficulties in distinguishing pathovars on the basis of physiological features. Furthermore, comparison of FT-IR spectra, a method proven to show infraspecific diversity between strains possessing high 16S rDNA conformity (Oberreuter, 2001), enabled differentiation between a subset of two pathovars only. Further specific pathovar differences to enable complete classification were not detected as strain-specific spectral vibrations were dominant. These results indicate that all four pathovars, as well as the grass isolates of genotype A, are highly similar genetically and their biochemical and physiological traits reflect these close relationships.

The representative strain of genotype D was assigned to the genus *Curtobacterium* on the basis of 16S rDNA sequence comparison. Chemotaxonomic features like respiratory quinones, fatty acid composition, G+C content and peptidoglycan type of the cell wall also matched the genus description. Phylogenetic analysis significantly demonstrated the separate position within the monophyletic branch of *Curtobacterium*, indicating novel species status (Fig. 1). DNA–DNA hybridization studies supported this finding. Analysis of FT-IR spectra confirmed the separate position of genotype D on the basis of phenotypic characteristics. However, physiological properties of this group are very similar to those of grass isolates of genotype A, as well as the *Curtobacterium flaccumfaciens* pathovars, a general problem becoming more and more noticeable in the description of novel taxa (Behrendt *et al*., 2001; Pukall *et al*., 1999). The only effective feature to distinguish them from each other was the ability to

Table 5. Differential characters of genera of the family *Microbacteriaceae*

Genera: 1, *Agrococcus*; 2, *Agromyces*; 3, *Clavibacter*; 4, *Cryobacterium*; 5, *Curtobacterium*; 6, *Frigoribacterium*; 7, *Leifsonia*; 8, *Leucobacter*; 9, *Microbacterium*; 10, *Mycetocola*; 11, *Subtercola*; 12, *Rathayibacter*;13, Type C '*Plantibacter*'. Data obtained in this study and extracted from following references: Davis *et al.* (1984), Evtushenko *et al.* (2000), Groth *et al.* (1996), Kämpfer *et al.* (2000), Männistö *et al.* (2000), Sasaki *et al.* (1998), Takeuchi *et al.* (1996), Takeuchi & Hatano (1998), Tsukamoto *et al.* (2001), Wieser *et al.* (1999). Abbreviations: C, cocci; F, filaments; R, rods; DAB, diaminobutyric acid; GABA, γ-aminobutyric acid; Orn, ornithine; Lys, lysine; S, straight-chain saturated; A, anteiso-methyl-branched; I, iso-methyl-branched; (H), cyclohexyl fatty acids sometimes present; ND, not determined; d, different reaction.

Genus	1	2	3	4	5	6	7	8	9	10	11	12	13
Morphology	C	F, R	R	R	R	R	R, F	R	R	R	R	R	R
Motility	−	−	−	−	+	+	+	−	d	−	−	−	−
Diamino acid	L-DAB	L-DAB	DL-DAB	L-DAB	D-Orn	D-Lys	DL-DAB	L-DAB, GABA	L-Lys/D-Orn	Lys	DAB	L-DAB	L-DAB
Acyl type	Acetyl	Acetyl	Acetyl	ND	Acetyl	Acetyl	ND	Acetyl	Glycolyl	Acetyl	Acetyl	ND	Acetyl
G+C content (mol%)	74	70–76	65–78	65	68–75	71·7	66–73	66	65–76	64–65	64–68	63–72	68–70
Fatty acid type	S, A, I	S, A, I	S, A, I	S, A, I	S, A, I (H)	S, A, I	S, A, I	S, A, I	S, A, I	A, I	S, A, I	S, A, I	S, A,I
Major MK	12, 11	12, 13	9, 10	10	9	9	11, 10	11	11, 12, 13, 14	9, 10	9, 10	10	10, 11

form acid from adonitol (Table 4). Furthermore, genotype D could also be distinguished from the other described species of *Curtobacterium* (*Curtobacterium luteum*, *Curtobacterium citreum*, *Curtobacterium pusillum* and *Curtobacterium albidum*). On the basis of physiological characterization (Table 4), differential properties like acid formation from adonitol and raffinose or hydrolyses of aesculin and Tween 80 are revealed. As a consequence of this phenotypic and phylogenetic characterization, it is concluded that genotype D isolates deserve a separate species status, for which the name *Curtobacterium herbarum* sp. nov. is proposed.

Phylogenetic analyses of genotypes B1 and B2 revealed their assignment to the species *Clavibacter michiganensis* (Fig. 1). Both genotypes were highly related to *Clavibacter michiganensis* subsp. *tessellarius* but, on the basis of slight differences in their 16S rDNA sequences, they could be separated by restriction analyses, a method that has been proven by Lee *et al.* (1997) to be effective in classification of *Clavibacter michiganensis* subspecies. This result indicates that both genotypes form a separate group of strains that should not be assigned to a known subspecies. Differences in physiological properties support this assumption.

The representative strain of genotype C constituted a separate phylogenetic branch in the family *Microbacteriaceae*. The nearest phylogenetic neighbours are the genera *Microbacterium* and *Mycetocola*, which clustered with genotype C by both neighbour-joining and maximum-likelihood methods, supported by high bootstrap values (Fig. 1). Phylogenetic distances between *Mycetocola* and *Microbacterium* are much smaller than to genotype C, indicating novel genus status. Chemotaxonomic properties commonly used for differentiation at the genus level also differed for both genera and genotype C (Table 5). Thus, the type of peptidoglycan is a prominent feature to distinguish them from one another. Comparison of the cell wall composition of genotype C, which corresponds to type B2γ of Schleifer & Kandler (1972), with those of the remaining genera of the family *Microbacteriaceae* revealed agreement with *Clavibacter*, *Leifsonia*, *Agromyces*, *Cryobacterium* and *Rathayibacter* (Evtushenko *et al.*, 2000; Sasaki *et al.*, 1998). Whereas *Clavibacter* and *Leifsonia* represent a subgroup displaying L and D isomers of DAB in almost equal amounts, genotype C isolates, *Rathayibacter*, *Cryobacterium* and *Agromyces* shared the same amino acid ratio, with L-DAB as the predominant isomer (Sasaki *et al.*, 1998), and these species form another subgroup. Within this subgroup, *Agromyces* species are characterized by filamentous cells fragmenting to yield coccoid forms in older cultures and the predominant menaquinones MK-12 and MK-11 and thus, they can easily be distinguished from genotype C by phenotypic features without requiring phylogenetic studies. In contrast, the genus *Rathayibacter* shows similar phenotypic properties in terms of chemotaxonomic and physiological characteristics (Tables 4 and 5). Only acid production from raffinose, L-rhamnose and ribose, hydrolysis of DNA and Tween 80 are features that can be used to discriminate genotype C strains and all currently described *Rathayibacter* species. In spite of this phenotypic similarity, assignment to *Rathayibacter* is not possible because of the high phylogenetic distance of at least 5%, based on 16S rDNA sequences. *Cryobacterium psychrophilum* is obligately psychrophilic (Suzuki *et al.*, 1997) and represents a phylogenetic lineage separate from that of genotype C strains (Fig. 1). A further genus containing L-DAB in the peptidoglycan, but incorporated in an unknown structure is *Agrococcus* (Groth *et al.*, 1996; Wieser *et al.*, 1999). In addition to the amino acids found in genotype C strains, threonine and aspartic acid can be shown in the peptidoglycan. The coccoid cell form and menaquinone composition are additional features for discrimination on the basis of phenotypic characteristics (Table 5).

In conclusion, regarding the separate phylogenetic position of the genotype C, which is also reflected by phenotypic differences to the nearest related genera, it is proposed that this grass-associated genotype should

be placed in a novel genus of the family *Microbacteriaceae*, *Plantibacter* gen. nov., with the type species *Plantibacter flavus* sp. nov.

The last grass-associated genotype under investigation, genotype F, was closely related to species of the genus *Subtercola* (Fig. 1). Sequence conformity to *S. boreus* and *S. frigoramans* below 97 % clearly revealed a demarcation from species of this genus (Stackebrandt & Goebel, 1994). The chemotaxonomic traits of *Subtercola* recently described by Männistö *et al.* (2000) are characterized by peptidoglycan type B2γ of Schleifer & Kandler (1972), although nearly all of the glutamic acid residues were replaced by hydroxyglutamic acid. In contrast, the peptidoglycan of genotype F contained a hitherto undescribed combination of the diagnostic amino acids L-DAB and D-Orn, and the glutamic acid is also replaced by hydroxy-glutamic acid. Furthermore, the isoprenoid quinones of *Subtercola* are dominated by MK-9 and MK-10; those of genotype F comprised predominantly MK-10 and MK-11. These chemotaxonomic properties commonly used in the past for generic assignment are drastically different. However, following the taxonomic strategy to attribute most importance to analyses of partial 16S rDNA sequence similarity for determining evolutionary relationships at the genus level, as shown for unification of the genera *Microbacterium* and *Aureobacterium* (Takeuchi & Hatano, 1998), it is proposed that genotype F be assigned to the genus *Subtercola*.

The fatty acid composition of genotype F was predominantly anteiso- and iso-branched acids, which is similar to the situation in genera of the family *Microbacteriaceae* (Table 5). In contrast to the described species of *Subtercola*, a relatively high level of saturated fatty acids is found. On the other hand, strains of genotype F and *Subtercola* correspond in all containing 1,1-dimethoxy-anteiso-pentadecane.

Species of the genus *Subtercola* are particularly characterized by optimal growth temperatures 10 °C lower than the majority of *Microbacteriaceae* and the ability to grow partially down to −2 °C, which is similar to the genus *Frigoribacterium* (Kämpfer *et al.*, 2000; Männistö *et al.*, 2000). Both genera, along with *Cryobacterium psychrophilum* (Suzuki *et al.*, 1997), have been assigned as a psychrophilic group of *Microbacteriaceae*. Strains of genotype F also grow in this temperature range, but optimal growth was observed at room temperature. Thus, strains of genotype F should not be considered as psychrophilic.

As a consequence of phylogenetic and chemotaxonomic analyses, which clearly indicate a separate species status for genotype F strains in the genus *Subtercola*, a novel species, *Subtercola pratensis* sp. nov., is proposed.

Application of FT-IR spectroscopy to the classification of grass-associated coryneforms, with regard to bacteria of the genera *Curtobacterium*, *Clavibacter* and *Rathayibacter* associated with plants, showed that this method is reliable to distinguish coryneform bacteria at the species level, which is similar to results presented by Oberreuter (2001).

Summarizing the results of this study and the characterization of grass-associated strains of the genus *Microbacterium* (Behrendt *et al.*, 2001) isolated from the same habitat, it was shown that coryneform bacteria living in the phyllosphere of grasses are relatively similar in their physiological properties, which may be due to adaptation to environmental conditions (Table 4). In contrast, phylogenetic analysis reveals a high diversity between grass-associated coryneforms. It has also to be taken into account that the isolates studied only represent a part of the coryneform community as genotypes standing for the majority of isolated strains were analysed, indicating that the phyllosphere is a reservoir of more coryneform genotypes. Comparing phylogenetic relationships and phenotypic properties revealed contradictory results. On the one hand, at the genus level, a strain group (genotype C) was found showing similar chemotaxonomic traits to other genera, but was phylogenetically highly divergent. This was also observed at the species level for strains that can be hardly differentiated by means of phenotypic characterization, whereas DNA–DNA hybridization studies clearly reveal a separate species status, as shown for genotype D and *Microbacterium* spp. (Behrendt *et al.*, 2001). On the other hand, a genotype (F) was found showing a high phylogenetic relationship to known species, but some chemotaxonomic properties that generally prove useful to discriminate between genera were extremely different.

Description of *Plantibacter* gen. nov.

Plantibacter (plan.ti.bac′ter. L. fem. n. *planta* plant; N.L. masc. n. *bacter* equivalent of Gr. neut. n. *baktron* rod; N.L. masc. n. *Plantibacter* rod of/from plants).

Cells are Gram-positive, pleomorphic, non-motile rods that are sometimes arranged at an angle to give V-formations. Endospores are not formed. Growth is obligately aerobic. Cell wall peptidoglycan contains L-DAB, D-Glu, D-Ala and Gly, corresponding to type B2γ, and muramic acid is acetylated. Non-hydroxylated fatty acids are predominantly anteiso- and iso-methyl-branched. Minor amounts of straight-chain saturated acids are found. Respiratory quinones are MK-10 and MK-11. DNA G+C composition is about 70 mol %. Type species is *Plantibacter flavus*.

Description of *Plantibacter flavus* sp. nov.

Plantibacter flavus (fla′vus. L. adj. *flavus* yellow, referring to the colony colour).

Morphological and physiological description of the species is based on five strains. Cells are Gram-positive, strictly aerobic, non-spore-forming, non-motile, irregularly shaped rods that sometimes form V-shapes. Colonies are yellow, shiny, slightly convex and round with entire margins. Oxidase and Voges–Proskauer

reaction are negative, whereas catalase is positive. Aesculin, DNA, Tween 60 and Tween 80 are hydrolysed. Utilization of starch is strain dependent; the type strain is positive for hydrolysis of starch. None of the strains hydrolyses casein, gelatin or cellulose. Formation of levan from sucrose is negative. Strains produce acid oxidatively from L-arabinose, D-fructose, D-galactose, D-glucose, lactose, D-maltose, D-mannitol, D-mannose, D-melezitose, raffinose, L-rhamnose, sucrose, D-trehalose, ribose and D-xylose. Acid formation from inulin, melibiose, D-sorbitol and L-sorbose is strain dependent, whereas the type strain is negative for these carbohydrates. All strains are negative for adonitol and inositol. Optimum growth temperature is about 25 °C. At 4 °C, grows slowly; at −2 °C, growth also occurs, but is even slower. Major menaquinones and structure of peptidoglycan are the same as described for the genus. The major cellular fatty acids are 12-methyl tetradecanoic acid (a-15:0), 14-methyl pentadecanoic acid (i-16:0), 14-methyl hexadecanoic acid (a-17:0) and 13-methyl tetradecanoic acid (i-15:0). DNA G+C composition for the type strain is 70 mol%. Strains were isolated from the phyllosphere of grasses and from the litter layer after mulching the sward. The type strain, isolated from the phyllosphere, is DSM 14012^T (= P 297/02^T = LMG 19919^T).

Description of *Curtobacterium herbarum* sp. nov.

Curtobacterium herbarum (her.ba′rum. L. gen. pl. fem. n. *herbarum* of plants).

Morphological and physiological description of the species is based on five strains. Cells are Gram-positive, strictly aerobic, non-spore-forming, motile, irregularly shaped rods that sometimes form V-shapes. Colonies are orange, shiny, slightly convex and round with entire margins. Oxidase and Voges–Proskauer reaction are negative, whereas catalase is positive. Aesculin, Tween 60 and Tween 80 are hydrolysed. Utilization of casein and gelatin is strain dependent; the type strain is positive. None of the strains hydrolyses starch, DNA or cellulose. Formation of levan from sucrose is negative. Strains produce acid oxidatively from L-arabinose, D-fructose, D-galactose, D-glucose, inositol, lactose, D-maltose, D-mannitol, D-mannose, D-melezitose, melibiose, D-sorbitol, raffinose, sucrose, D-trehalose, ribose and D-xylose. Acid formation from L-rhamnose is strain dependent and positive for the type strain. All strains are negative for inulin and L-sorbose. Optimum growth temperature is about 25 °C. At 4 °C, grows slowly; at −2 °C, growth also occurs, but is even slower. The diagnostic diamino acid of the peptidoglycan is Orn; peptidoglycan is of the B2β type with acetyl residues as described for the genus. The major menaquinone is MK-9. The predominant cellular fatty acids are 12-methyl tetradecanoic acid (a-15:0), 14-methyl hexadecanoic acid (a-17:0) and 14-methyl pentadecanoic acid (i-16:0). DNA G+C composition for the type strain is 71 mol%. Isolated from the phyllosphere of grasses and from the litter layer after mulching the sward. The type strain, isolated from the litter layer, is DSM 14013^T (= P 420/07^T = LMG 19917^T).

Description of *Subtercola pratensis* sp. nov.

Subtercola pratensis (pra.ten′sis. L. adj. *pratensis* pertaining to meadows/grassland).

Morphological and physiological description of the species is based on five strains. Cells are Gram-positive, strictly aerobic, non-spore-forming, non-motile, irregularly shaped rods that sometimes form V-shapes. Colonies are yellow, shiny, slightly convex and round with entire margins. Oxidase and Voges–Proskauer reaction are negative, whereas catalase is positive. Aesculin, Tween 60 and Tween 80 are hydrolysed. None of the strains hydrolyses starch, casein, gelatin, DNA or cellulose. Formation of levan from sucrose is negative. Strains produce acid oxidatively from L-arabinose, D-fructose, D-galactose, D-glucose, inulin, D-maltose, D-mannitol, D-mannose, raffinose, sucrose, D-trehalose, ribose and D-xylose. Acid production from melibiose is weak and is strain dependent from inositol and L-rhamnose. Type strain is negative for inositol and positive for L-rhamnose. Formation of acid from adonitol, lactose, D-melezitose, D-sorbitol and L-sorbose is negative for all strains. Optimum growth temperature is about 25 °C. At 4 °C, grows slowly; at −2 °C, growth also occurs, but is even slower. Diagnostic diamino acids of the peptidoglycan are L-DAB and D-Orn; glutamic acid is 3-hydroxylated to a high degree and muramic acid is acetylated. Major isoprenoid quinones are MK-10 and MK-11. Predominant cellular fatty acids are 12-methyl tetradecanoic acid (a-15:0), 14-methyl hexadecanoic acid (a-17:0), 14-methyl pentadecanoic acid (i-16:0) and hexadecanoic acid (16:0). 1,1-Dimethoxy-anteisopentadecane occurs in the whole-cell methanolysate. DNA G+C composition of the type strain is 65 mol%. Isolated from the phyllosphere of grasses. Type strain is DSM 14246^T (= P 229/10^T = LMG 21000^T).

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NOTE ADDED IN PROOF

While this paper was being reviewed, Evtushenko *et al.* (2001) proposed a novel genus, *Agreia*, which showed chemotaxonomic features corresponding to those of *Sub-*

tercola pratensis. As a consequence, a necessary reclassification of *Subtercola pratensis* is in preparation. Thus, the authors refrain from emending the genus *Subtercola* in the present paper to avoid a revocation after valid reclassification of *Subtercola pratensis*.

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