Herbaspirillum chlorophenolicum sp. nov., a 4-chlorophenol-degrading bacterium

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A 4-chlorophenol-degrading bacterial strain, formerly designated as a strain of *Comamonas testosteroni*, was reclassified as a member of the genus *Herbaspirillum* based on its phenotypic and chemotaxonomic characteristics, as well as phylogenetic analysis using 16S rDNA sequences. Phylogenetic inference based on 16S rDNA sequences showed that strain CPW301^T clusters in a phylogenetic branch that contains *Herbaspirillum* species. 16S rDNA sequence similarity of strain CPW301^T to species of the genus *Herbaspirillum* with validly published names is in the range 98.7–98.9%. Despite the considerably high 16S rDNA sequence similarity, strain CPW301^T could be distinguished clearly from type strains of *Herbaspirillum* species with validly published names by DNA–DNA relatedness values, which were <15.7%. The genomic DNA G+C content of strain CPW301^T is 61.3 mol%. The predominant ubiquinone is Q-8 and the major cellular fatty acids are C_{16:0} and cyclo-C_{17:0}. The strain does not fix nitrogen and is not plant-associated. It is an aerobic rod with one unipolar flagellum. On the basis of these characteristics, a novel *Herbaspirillum* species, *Herbaspirillum chlorophenolicum* sp. nov., is proposed. The type strain of the novel species is strain CPW301^T (=KCTC 12096^T = IAM 15024^T).

The bacterial genus *Herbaspirillum* includes four species with validly published names: *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans*, *Herbaspirillum frisingense* and *Herbaspirillum lusitanum*. *H. seropedicae* Z67^T (=IAM 14977^T), isolated from cereal roots, was the first species to be reported in this genus (Baldani *et al.*, 1986). Later, a mild plant pathogen, formerly named *Pseudomonas rubrisubalbicans*, was reclassified as *H. rubrisubalbicans* LMG 2286^T (=IAM 14976^T) (Baldani *et al.*, 1996). Kirchhof *et al.* (2001) identified a nitrogen-fixing bacterium, isolated from C4-fibre plants, as *H. frisingense* GSF30^T (=IAM 14974^T). More recently, *H. lusitanum* LMG 21710^T was isolated from root nodules of *Phaseolus vulgaris* plants and proposed as a novel species by Valverde *et al.* (2003). Although the unnamed *Herbaspirillum* species 3 (Baldani *et al.*, 1996) did not fix nitrogen, all type strains of species with validly

published names have been recognized as nitrogen-fixing micro-organisms.

Chlorophenols are common environmental pollutants that arise from extensive use of wood preservatives, herbicides and fungicides and they are found frequently in pulp-bleaching effluents and industrial wastewater (Kringstad & Lindström, 1984; Valo *et al.*, 1984, 1985). Due to the environmentally hazardous properties of chlorophenols, intensive attention has been paid to processes of microbial chlorophenol degradation. Many micro-organisms have been reported to degrade chlorophenols as their sole carbon and energy source, including a *Pseudomonas* species (Knackmuss & Hellwig, 1978; Radehaus & Schmidt, 1992), *Arthrobacter* species (Stanlake & Finn, 1982; Li *et al.*, 1991), *Rhodococcus chlorophenolicus* (Apajalahti *et al.*, 1986) and some *Flavobacterium* strains (Saber & Crawford, 1985). In our laboratory, Bae *et al.* (1996) previously reported a 4-chlorophenol-degrading bacterium, strain CPW301^T, that was isolated from soil sediment collected in a stream near an industrial region in Cheongju, Korea, via selective enrichment with 4-chlorophenol as the sole carbon and energy source. It was identified tentatively as a strain of *Comamonas testosteroni* on the basis of its morphological and physiological characteristics. In the present study,

Published online ahead of print on 27 February 2004 as DOI 10.1099/ijs.0.02812-0.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain CPW301^T (=KCTC 12096^T = IAM 15024^T) is AB094401.

A phylogenetic tree and DNA–DNA relatedness data are available as supplementary material in IJSEM Online.

phylogenetic analysis on the basis of 16S rDNA sequences was performed and DNA–DNA relatedness and some important phenotypic characteristics were examined to determine the precise taxonomic position of this strain. On the basis of the results obtained in this study, it is proposed that strain CPW301^T should be placed in the genus *Herbaspirillum* as the type strain of a novel species, namely *Herbaspirillum chlorophenolicum* sp. nov.

Strain CPW301^T was originally isolated from soil sediment that was collected in a stream near an industrial region (Cheongju, Korea) (Bae *et al.*, 1996) and deposited in the Korean Collection for Type Cultures as KCTC 12096^T (=IAM 15024^T). *H. seropedicae* IAM 14977^T, *H. rubrisubalbicans* IAM 14976^T and *H. frisingense* IAM 14974^T were obtained from the IAM Culture Collection, Institute of Molecular and Cellular Bioresources, University of Tokyo, Tokyo, Japan. Flagellation and morphological characteristics were determined by transmission electron microscopy after staining cells negatively with 1 % (w/v) phosphotungstic acid. Gram-staining and catalase and oxidase tests were performed according to procedures outlined by Smibert & Krieg (1981). Substrate utilization and some physiological characteristics were determined with API 20 NE, ID 32 GN and API 50 CHB kits (bioMérieux).

Phenol- and 4-chlorophenol-degradation ability was assessed in a 500 ml Erlenmeyer flask that contained 50 ml mineral salts medium (Bae *et al.*, 1996) with 0.5 mM phenol or 0.5 mM 4-chlorophenol as the sole carbon source in a shaking incubator at 30 °C. Nitrogen-fixing ability was determined by growth in 50 ml nitrogen-free medium (DSMZ medium no. 3) in a 500 ml Erlenmeyer flask. The medium contained [(l distilled water)⁻¹]: 5.0 g glucose; 5.0 g mannitol; 0.1 g CaCl₂·2H₂O; 0.1 g MgSO₄·7H₂O; 5.0 mg Na₂MoO₄·2H₂O; 0.9 g K₂HPO₄; 0.1 g KH₂PO₄; 0.01 g FeSO₄·7H₂O; 5.0 g CaCO₃; and 1 ml trace element mixture. The trace element mixture (SL-6, DSMZ medium no. 27) contained [(l distilled water)⁻¹]: 0.1 g ZnSO₄·7H₂O; 0.03 g MnCl₂·4H₂O; 0.3 g H₃BO₃; 0.2 g CoCl₂·6H₂O; 0.01 g CuCl₂·2H₂O; and 0.02 g NiCl₂·6H₂O. Growth was determined after 2 weeks at 30 °C in a shaking incubator.

Ubiquinones were extracted from cells that had been grown on nutrient broth (Difco) and were analysed as described by Komagata & Suzuki (1987), using reverse-phase HPLC. Cellular fatty acids were analysed in strains CPW301^T, *H. frisingense* IAM 14974^T, *H. rubrisubalbicans* IAM 14976^T and *H. seropedicae* IAM 14977^T, grown on trypticase soy agar (Difco) for 1–2 days. Cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification system (MIDI). Fatty acids were analysed by GC (Hewlett Packard 6890) and identified by the Microbial Identification software package (Sasser, 1990).

Chromosomal DNA was extracted from cells that had been grown on a nutrient agar plate (Difco) as described by Ausubel *et al.* (1995). RNA in the DNA solution was

removed by incubation with a mixture of ribonucleases A and T1 (each at 20 U ml⁻¹) at 30 °C for 1 h. Chromosomal DNA G + C content was analysed as described by Mesbah *et al.* (1989), using reverse-phase HPLC. DNA–DNA relatedness was determined as described by Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and micro-dilution wells.

Two pairs of primer systems, Fdb261–Fdb260 (Stoltzfus *et al.*, 1997) and PolF–PolR (Poly *et al.*, 2001), were used to amplify the *nifD* and *nifH* genes, respectively. PCR was run for 35 cycles with a DNA thermal cycler (model 2400; Perkin-Elmer). The following thermal profiles were used for PCR: denaturation at 94 °C for 45 s, primer annealing at 50 °C for 45 s and extension at 72 °C for 45 s. The final cycle included an extension for 7 min. PCR products were separated by electrophoresis on an agarose gel (0.8 %, w/v) and stained with ethidium bromide. Band size was determined by comparison with a 100 bp ladder standard (Bio-Rad).

16S rDNA was amplified from chromosomal DNA of strain CPW301^T by using a universal eubacterial primer set, 9F [5′-GAGTTTGATCCTGGCTCAG-3′; positions 9–27 (*Escherichia coli* 16S rDNA numbering)] and 1512R [5′-ACGG(H)TACCTTGTTACGACTT-3′; positions 1512–1492] (Weisburg *et al.*, 1991). The PCR product, purified with a GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences), was sequenced with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an automatic DNA sequencer (model 310; Applied Biosystems). Primers used for full sequencing were 9F, 1512R, 341F [5′-CCTACGGGAGGCAGCAG-3′; positions 341–357 (*E. coli* 16S rDNA numbering)], 519F [5′-CAGCA-GCCGCGGTAATAC-3′; positions 519–536], 907F [5′-AA-ACTCAAAGGAATTGACGG-3′; positions 907–926], 536R [5′-GTATTACCGCGGCTGCTG-3′; positions 536–519] and 1100R [5′-GGGTTGCGCTCGTTG-3′; positions 1114–1110]. The partial 16S rDNA sequences were combined in the program BioEdit (Hall, 1999). 16S rDNA sequences of related taxa were obtained from GenBank. Multiple alignments were performed by using CLUSTAL_X (Thompson *et al.*, 1997). Gaps were edited in BioEdit (Hall, 1999). Evolutionary distances were calculated by using the Kimura two-parameter model (Kimura, 1983). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) in the program MEGA2 (Kumar *et al.*, 2001). A neighbour-joining bootstrap method was used to obtain confidence levels for the neighbour-joining analysis, with a bootstrap dataset of 1000 (Felsenstein, 1985).

Strain CPW301^T is an aerobic, Gram-negative, motile and slightly curved rod (0.7 µm in diameter and 2.3 µm long) with one unipolar flagellum. Colonies that form on nutrient agar plates (Difco) are smooth, circular, non-glossy, brownish and convex. The strain gave positive results for catalase, oxidase and urease. Nitrate was not reduced to nitrite. The strain grew well at 30 °C and pH 6–8, but did not grow well at 45 °C. These morphological and physiological

characteristics are summarized in Table 1, along with data for *Herbaspirillum* species with validly published names. *nifD* and *nifH* are genes that encode key enzymes in

nitrogen-fixing pathways in various micro-organisms (Yong, 1992). An attempt was made to detect these genes in strain CPW301^T by a PCR amplification approach using

Table 1. Comparison of characteristics of strain CPW301^T, *Herbaspirillum* species and *C. testosteroni*

Taxa: 1, *H. chlorophenolicum* CPW301^T; 2, *H. seropedicae* IAM 14977^T; 3, *H. rubrisubalbicans* IAM 14976^T; 4, *H. frisingense* IAM 14974^T; 5, *H. lusitanum* LMG 21760^T; 6, *C. testosteroni* ATCC 11996^T. Characteristic results for *H. seropedicae* IAM 14977^T, *H. rubrisubalbicans* IAM 14976^T, *H. frisingense* IAM 14974^T, *H. lusitanum* LMG 21760^T and *C. testosteroni* ATCC 11996^T are from Baldani *et al.* (1986, 1996), Kirchhof *et al.* (2001), Valverde *et al.* (2003) and Chang *et al.* (2002). The following characteristics were determined in this study: detection of *nifD* and *nifH*, growth on phenol and 4-chlorophenol and fatty acid composition. +, Positive; –, negative; ND, not determined; TR, trace (<0.5 %).

Characteristic	1	2	3	4	5	6
Cell size (µm)	0.7 × 2.3	0.6–0.7 × 1.5–5.0	0.6–0.7 × 1.4–1.8	0.5–0.7 × 1.4–1.8	0.5 × 1.6	ND
Flagellation	One; unipolar	One to three; bipolar	Several; bipolar	One to three; unipolar	One or two; unipolar	One to five
Optimal growth temperature (°C)	30	34	30	30–37	ND	ND
Optimal growth pH	6.0–7.0	5.3–8.0	6.7–6.8	6.0–7.0	5.0–8.0	ND
Detection of:						
<i>nifD</i>	–	+	+	+	+	ND
<i>nifH</i>	–	+	+	+	+	ND
Growth on:						
Nitrogen-free medium	–	+	+	+	+	ND
Mannose	–	+	+	–	+	ND
<i>N</i> -Acetylglucosamine	+	+	–	+	–	ND
<i>meso</i> -Inositol	–	+	–	–	–	+
Rhamnose	–	+	–	–	+	ND
Ribose	–	+	+	–	ND	–
Adonitol	–	+	+	ND	ND	ND
Glycerol	–	+	+	ND	ND	+
D-Lyxose	–	+	+	ND	ND	ND
Xylitol	–	+	+	ND	ND	ND
D-Xylose	–	+	+	ND	ND	–
Sorbitol	–	+	+	ND	ND	ND
Phenol	+	–	–	–	ND	+
4-Chlorophenol	+	–	–	–	ND	ND
DNA G+C content (mol%)	61.3	64–65	62–63	61–65	59.9	61.0–62.1
Major ubiquinone	Q-8	Q-8	Q-8	Q-8	ND	Q-8
Cellular fatty acids (%):						
C _{12:0}	3.70	1.04	3.24	0.70	ND	2.40
C _{14:0}	0.24	5.21	0.76	3.96	ND	1.00
C _{16:0}	33.73	22.76	26.31	27.44	ND	30.40
C _{18:0}	1.98	0.63	1.82	1.72	ND	TR
C _{18:1ω6c}	–	1.90	2.48	–	ND	–
C _{18:1ω7c}	13.43	13.62	22.07	23.75	ND	17.90
C _{12:0} 2-OH	2.65	1.77	0.40	1.13	ND	–
C _{10:0} 3-OH	1.33	1.82	1.37	1.37	ND	4.80
C _{12:0} 3-OH	3.27	4.18	3.14	3.33	ND	–
C _{14:0} 2-OH	0.07	1.61	2.01	1.14	ND	TR
C _{14:0} 2-OH	–	–	–	–	ND	2.00
cyclo-C _{17:0}	21.84	4.81	3.28	1.81	ND	3.00
cyclo-C _{19:0ω8c}	1.37	–	0.23	0.08	ND	0.90
Summed feature 3*	14.93	39.57	31.68	32.53	ND	33.10

*Summed feature 3 contains C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; these fatty acids could not be separated by GLC with the Microbial Identification system (MIDI).

two primer systems [Fdb261–Fdb260 for *nifD* (Stoltzfus *et al.*, 1997) and PolF–PolR for *nifH* (Poly *et al.*, 2001)], in order to determine whether the strain has the genetic capability to fix nitrogen. These genes, *nifD* (about 390 bp) and *nifH* (about 360 bp), were clearly amplified in nitrogen-fixing type strains, whereas they were not amplified in strain CPW301^T (Table 1). Strain CPW301^T did not grow in a nitrogen-free liquid medium, whereas *Herbaspirillum* species with validly published names grew well (Table 1).

Strain CPW301^T and all the type strains had Q-8 as the predominant ubiquinone. The major fatty acids of strain CPW301^T were C_{16:0} (33.7%), cyclo-C_{17:0} (21.8%), summed feature 3 (14.9%) and C_{18:1ω7c} (13.4%). Although the proportions are slightly different, these fatty acids are also observed in the type strains of *Herbaspirillum* species (Table 1). The DNA G + C content of strain CPW301^T was 61.3 mol%; this value is similar to those of the type strains of *Herbaspirillum* species (60–65%).

Almost all of the 16S rDNA sequence (1474 nt) of strain CPW301^T was determined in this study. Phylogenetic analysis of 16S rDNA sequences showed that strain CPW301^T fell in a cluster that comprised *Herbaspirillum* species (a phylogenetic tree is available as supplementary material in IJSEM Online). The 16S rDNA sequence similarities of strain CPW301^T to *H. frisingense* IAM 14974^T, *H. rubrisubalbicans* IAM 14976^T, *H. seropedicae* IAM 14977^T and *H. lusitanum* LMG 21710^T are 98.9, 98.7, 98.8 and 97.5%, respectively. Strain CPW301^T exhibited DNA–DNA relatedness levels of <15.7% to *H. frisingense* IAM 14974^T, *H. rubrisubalbicans* IAM 14976^T and *H. seropedicae* IAM 14977^T (hybridization data are available as supplementary material in IJSEM Online).

Phylogenetic analysis based on 16S rDNA sequences indicates clearly that strain CPW301^T is placed in the radiation of the cluster that contains *Herbaspirillum* species. The names of three species of *Herbaspirillum*, *H. seropedicae* (Baldani *et al.*, 1986), *H. rubrisubalbicans* (Baldani *et al.*, 1996) and *H. frisingense* (Kirchhof *et al.*, 2001), have been validly published and another species (*H. lusitanum*) has recently been proposed as a member of the genus *Herbaspirillum* by Valverde *et al.* (2003). Strain CPW301^T has similar morphological features to the type strains of these species and it also shares many physiological characteristics with them (Table 1). Furthermore, chemotaxonomic characteristics of CPW301^T determined in this study, e.g. ubiquinone, fatty acids and DNA G + C content, are well-matched to those of other *Herbaspirillum* species (Table 1). The phylogenetic, phenotypic and chemotaxonomic results described in this study support classification of strain CPW301^T in the genus *Herbaspirillum*.

It is worthwhile to compare the phenotypic characteristics of strain CPW301^T with those of the type strain of *C. testosteroni*, as our strain was originally classified in this species (Bae *et al.*, 1996). Our strain differs from *C. testosteroni* in meso-inositol and glycerol utilization and

fatty acid profile (Table 1). Thus, in addition to phylogenetic data, phenotypic characteristics support reclassification of strain CPW301^T in the genus *Herbaspirillum*.

Strain CPW301^T showed 98.7–98.9% 16S rDNA similarity to the type strains of *Herbaspirillum* species. Where 16S rDNA sequence similarity is >97%, DNA–DNA relatedness values play an important role in clarifying intergeneric relationships between species (Stackebrandt & Goebel, 1994). Strain CPW301^T exhibited <15.7% DNA–DNA relatedness to species of *Herbaspirillum* with validly published names. This level is low enough for strain CPW301^T to be classified in a novel *Herbaspirillum* species (Wayne *et al.*, 1987). Furthermore, strain CPW301^T can also be distinguished from *Herbaspirillum* species with validly published names with respect to some important physiological characteristics. For example, all the type strains of described *Herbaspirillum* species are nitrogen-fixing organisms (Baldani *et al.*, 1986, 1996; Kirchhof *et al.*, 2001), whereas strain CPW301^T did not grow in a nitrogen-free liquid medium and did not have the *nifD* or *nifH* genes, suggesting that it may not have a nitrogen-fixing ability. Substrate-utilization properties (Table 1) also differ between strain CPW301^T and the type strains of *Herbaspirillum* species. On the basis of the data and observations described above, strain CPW301^T should be assigned to the genus *Herbaspirillum* as the type strain of a novel species, for which the name *Herbaspirillum chlorophenolicum* sp. nov. is proposed.

Description of *Herbaspirillum chlorophenolicum* sp. nov.

Herbaspirillum chlorophenolicum (chlo.ro.phen.o'li.cum. Gr. n. adj. *chloros* greenish-yellow, containing chloride; N.L. adj. *phenolicus* relating to phenol; N.L. neut. adj. *chlorophenolicum* relating to chlorophenols).

Gram-negative, aerobic, motile, slightly curved rods, 0.7 µm in diameter and 2.3 µm long, after culture for 1 day on nutrient agar. Cells have one unipolar flagellum. Colonies grown on nutrient agar (Difco) for 1–2 days are smooth, circular, non-glossy, brownish and convex. Grows well at 30 °C and pH 6–8, but does not grow at 45 °C. Substrate utilization, enzyme production, acid production and other physiological characteristics are indicated in Table 1. Does not have *nifD* or *nifH* genes. Q-8 is the predominant ubiquinone. C_{16:0} and cyclo-C_{17:0} are the major cellular fatty acids. DNA G + C content of the type strain is 61.3 mol% (as determined by HPLC).

The type strain, CPW301^T (= KCTC 12096^T = IAM 15024^T), was isolated from soil sediment that was collected at a stream near an industrial region in Cheongju, Korea.

Acknowledgements

This work was supported by grants from the 21C Frontier Microbial Genomics and Applications Center Program, Ministry of Science and Technology, grant MG02-0101-001-2-2-0.

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