Transfer of *Tsukamurella wratislaviensis* Goodfellow *et al.* 1995 to the genus *Rhodococcus* as *Rhodococcus wratislaviensis* comb. nov.

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A polyphasic study was undertaken to clarify the taxonomic position of the type strain (N805^T) of *Tsukamurella wratislaviensis*. This organism showed a combination of phenotypic properties, notably chemotaxonomic markers, consistent with its classification in the genus *Rhodococcus*. Comparative 16S rDNA sequencing studies indicated that strain 805^T falls into the *Rhodococcus erythropolis* subclade, where it forms a monophyletic group with the type strains of *Rhodococcus opacus* and *Rhodococcus percolatus*. The close relationship between these strains was underpinned by the results of mycolic acid analyses. However, strain N805^T was distinguished from the *R. opacus* and *R. percolatus* strains in DNA–DNA pairing experiments and by using a range of phenotypic properties. In light of these studies, it is clear that strain N805^T is misclassified in the genus *Tsukamurella*. It is, therefore, proposed that *Tsukamurella wratislaviensis* Goodfellow *et al.* 1995 be transferred to the genus *Rhodococcus* as *Rhodococcus wratislaviensis* comb. nov.

Keywords: *Rhodococcus wratislaviensis*, comb. nov., polyphasic taxonomy, 16S rDNA sequencing

INTRODUCTION

The application of chemotaxonomic, numerical-phenetic and molecular-systematic procedures led to a radical reappraisal of the taxonomy of mycolic acid-containing actinomycetes (Goodfellow *et al.*, 1998a, 1999). It is evident from such studies that members of this group have many phenotypic properties in common, form a distinct phyletic line in the 16S rDNA tree and can be assigned to the genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Skermania*, *Tsukamurella* and *Williamsia* using a combination of chemical and morphological markers. All of these taxa, together with the genus *Turicella*, are classified in the suborder *Corynebacterineae* Stackebrandt *et al.* 1997. This improved

classification was instrumental in highlighting the anomalous position of taxa such as *Nocardia amarae* and *Nocardia pinensis*, species subsequently reclassified as *Gordonia amarae* Klatte *et al.* 1994a and *Skermania piniformis* Chun *et al.* 1997, respectively. In the present study, a case is made for the transfer of *Tsukamurella wratislaviensis* Goodfellow *et al.* 1995 to the genus *Rhodococcus* as *Rhodococcus wratislaviensis* comb. nov.

METHODS

Source, maintenance and cultivation of strains. The tested strains, *Rhodococcus opacus* DSM 43205^T, *Rhodococcus percolatus* DSM 44240^T and *Tsukamurella wratislaviensis* N805^T (from R. E. Gordon, IMRU 878; D. M. Powelson, J-17), were maintained on glucose/yeast extract agar (Gordon & Mihm, 1962) at 4 °C and as glycerol suspensions (20%, v/v) at –20 °C. Biomass for some of the chemical investigations [detection of predominant isoprenoid quinones, major whole-organism sugars and diaminopimelic acid (A₂pm) isomers] and for the molecular-systematic studies was grown in shake flasks of modified Sauton's broth (Jones,

Abbreviations: 7AMC, 7-amino-4-methylcoumarin; A₂pm, diaminopimelic acid; FAME, fatty acid methyl ester; MAME, mycolic acid ester; 4MU, 4-methylumbelliferone.

The GenBank accession number for the 16S rDNA sequence of *Rhodococcus wratislaviensis* N805^T is Z37138.

1949) for 7 days at 30 °C. Cells for chemotaxonomic analyses were washed twice in distilled water, whereas those for molecular-biological studies were washed in NaCl/EDTA buffer (0.1 M EDTA, pH 8.0, 0.1 M NaCl).

DNA extraction and PCR amplification of 16S rDNA. Chromosomal DNA was isolated from *T. wratislaviensis* N805^T using a procedure (Chun & Goodfellow, 1995) modified slightly from that of Pitcher *et al.* (1989). The 16S rDNA was enzymically amplified by employing two universal primers, p27f and p1525r (Lane, 1991), and then purified further by using an Ultrafree-MC filter unit (pore size, 0.45 µm) (Millipore) and the protocol recommended by the manufacturer. The PCR procedure that we used has been described previously (Chun & Goodfellow, 1995).

Isolation and cloning of amplified 16S rDNA. Purified 16S rDNA fragments were ligated into the pGEM-T vector (Promega) by following the manufacturer's instructions. Ligated plasmids were then transformed into *Escherichia coli* JM 109 and transformants were selected on the basis of the results of the blue/white screening procedure (Sambrook *et al.*, 1989).

Sequencing of 16S rDNA. Plasmids containing the 16S rDNA fragment were isolated by using the standard purification method recommended by the manufacturer (Promega). Purified plasmids were sequenced by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Sequencing gel electrophoresis was carried out and nucleotide sequences were obtained automatically by using an Applied Biosystems model 373A DNA sequencer and software provided by the manufacturer. The oligonucleotide primers used in the sequencing reaction have been described previously (Chun & Goodfellow, 1995).

Phylogenetic analysis. The nucleotide sequence of the tested strain was aligned manually, using the program PHYDIT (Chun, 1995), against corresponding 16S rDNA sequences of representatives of all of the genera currently classified in the suborder *Corynebacterineae*; these latter sequences were retrieved from the GenBank database. Evolutionary trees were inferred by using four treeing algorithms, namely, the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) methods. *Mycobacterium tuberculosis* ATCC 27294^T (accession number X52917) was used as the outgroup. The resultant tree topologies were evaluated by carrying out bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings using the SEQBOOT and CONSENSE programs in the PHYLIP package (Felsenstein, 1993).

DNA–DNA hybridization. DNA was extracted from the type strains of *R. opacus*, *R. percolatus* and *T. wratislaviensis* by chromatography on hydroxyapatite following the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) using the modification of Huß *et al.* (1983) and a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed using the program TRANSFER.BAS (Jahnke, 1992).

Analyses of A₂pm isomers, sugars, menaquinones and mycolic acids. The diagnostic isomer of A₂pm and predominant whole-organism sugars of strain N805^T were determined using standard procedures (Staneck & Roberts, 1974; Saddler *et al.*, 1991). Menaquinones were extracted from freeze-dried biomass (50 mg) by using the small-scale

procedure of Minnikin *et al.* (1984). Purified menaquinones were separated by HPLC, as described by Chun & Goodfellow (1995). Mycolic acids were extracted from strain N805^T and appropriate marker cultures and examined using the TLC procedure described by Minnikin *et al.* (1975).

Extraction and detailed analyses of fatty acids and mycolic acids. Fatty acid methyl esters (FAMES) were extracted from wet cells of the three type strains, as described by Miller (1982). Half (0.3 ml) of each extract was passed through a silica gel column (Analytichem Bond Elut; Varian) to trap the mycolic acids; the eluants were then used for gas chromatographic analyses of FAMES. Trimethylsilylated derivatives of the mycolic acids were prepared by mixing the untreated halves of the extracts with 0.1 ml of a solution containing *n*-methyl-*n*-(trimethylsilyl)-heptafluorobutyramide and trimethylchlorosilane (10:1, v/v; Macherey & Nagel). The FAME preparations and the silylated derivatives of the mycolic acid esters (MAMES) were analysed by capillary gas chromatography using a Hewlett Packard model 5898A gas chromatograph run by Microbial Identification software (Microbial ID). Microbial Identification System conditions were used for the FAME analyses. The trimethylsilylated derivatives of the MAMES were analysed by high-temperature gas chromatography with a model HP 5790A gas chromatograph (Hewlett Packard) equipped with a flame-ionization detector and a 12 m type HT5 column (part no. 051385; SGE), using hydrogen as the carrier gas at a flow rate of 30 ml min⁻¹. The oven temperature was increased from 210 to 400 °C at a rate of 10 °C min⁻¹ and the final temperature was maintained for 10 min. Peaks of the derivatives were identified by comparing their retention times with those of known standard mycolic acids.

Physiological tests. Carbon-source utilization and qualitative enzyme tests were carried out on the three type strains in standard microtitration plates (F-form; Greiner) according to Kämpfer *et al.* (1990), as modified by Klatte *et al.* (1994b).

RESULTS AND DISCUSSION

A complete 16S rDNA nucleotide sequence was obtained for *T. wratislaviensis* strain N805^T (1496 nt between positions 28 and 1524 using the *E. coli* numbering system; Brosius *et al.*, 1978). Comparison of the sequence with corresponding 16S rDNA sequences of representatives of the genera classified in the suborder *Corynebacterineae* showed clearly that this organism falls within the subline encompassed by the genus *Rhodococcus* (data not shown), a result that substantiates earlier informal assignments of strain N805^T to this genus (Goodfellow *et al.*, 1998a, 1999; Kattar *et al.*, 2001). The transfer of strain N805^T to this genus is also supported by key phenotypic properties highlighted in this and an earlier study (Goodfellow *et al.*, 1991). Thus, the organism is an aerobic, Gram-positive, slightly acid–alcohol-fast, non-motile actinomycete that contains *meso*-A₂pm, arabinose and galactose in whole-organism hydrolysates (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970), predominant amounts of dihydrogenated menaquinones with eight isoprene units [not unsaturated menaquinones with nine isoprene units (MK-9) as reported previously by Goodfellow *et al.* (1991)] and

Fig. 1. Unrooted neighbour-joining tree based on nearly complete 16S rDNA sequences showing relationships between *Tsukamurella wratislaviensis* N805^T and representatives of the suborder *Corynebacterineae*, including the type strains of all of the validly described species of the genus *Rhodococcus*. The final dataset includes 1312 unambiguously aligned sites. Numbers at the nodes indicate percentages of bootstrap support based on neighbour-joining analyses of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 0.01 nucleotide substitutions per nucleotide position.

mycolic acids that co-migrated with those of the type strain of *Rhodococcus rhodochrous* to give a single spot on the chromatogram. These lipid data serve to distinguish strain N805^T from members of the genus *Tsukamurella* and the other validly described genera classified in the suborder *Corynebacterineae*, apart from *Rhodococcus* (Goodfellow *et al.*, 1998a, 1999). In contrast to rhodococci, tsukamurellae contain MK-9 as the predominant isoprenologue and give two mycolic acid spots when whole-organism methanolysates are examined by TLC (Yassin *et al.*, 1995, 1996, 1997).

It is evident from Fig. 1 that strain N805^T belongs to the *Rhodococcus erythropolis* subclade, one of four monophyletic subclades recognized within the 16S rDNA phylogenetic line occupied by members of the genera *Nocardia* and *Rhodococcus* (Rainey *et al.*, 1995; McMin *et al.*, 2000). Strain N805^T also contains nucleotide signatures characteristic of members of the *R. erythropolis* subclade (Goodfellow *et al.*, 1998a). It is also clear from Fig. 1 that the organism forms a monophyletic group with *R. opacus* and *R. percolatus*, a relationship that is supported by all four treeing

algorithms and by a bootstrap value of 81 %. Strain N805^T shares 16S rDNA nucleotide sequence similarity values of 99.6 and 99.0 % with the type strains of *R. opacus* and *R. percolatus*, values which correspond respectively to 6 and 14 nucleotide differences.

16S rDNA genes can be conserved to such an extent that information in nucleotide sequences may not be sufficiently discriminatory to distinguish between recently diverged species (Fox *et al.*, 1992; Goodfellow *et al.*, 1997). In contrast, DNA–DNA relatedness studies are usually effective in separating such closely related species, as exemplified by comparisons of DNA–DNA pairing and 16S rDNA sequencing data derived from representatives of validly described *Tsukamurella* species (Yassin *et al.*, 1995, 1996, 1997; Goodfellow *et al.*, 1998b; Kattar *et al.*, 2001). The two most closely related *Rhodococcus* species, *R. opacus* and *R. percolatus*, share a 16S rDNA nucleotide sequence similarity value of 99.3 %, which corresponds to 11 differences out of 1406 nucleotide positions (data from the present study) and a DNA–DNA relatedness value of 71 % (Briglia *et al.*, 1996). In the present investigation, the DNA–DNA relatedness values found between *T. wratislaviensis* strain N805^T and the type strains of *R. opacus* and *R. percolatus* were respectively 69 and 67 %. The minimum level of DNA–DNA relatedness between strains required to define a species is recommended as 70 % (Wayne *et al.*, 1987), though there is strong evidence that genomic homology above 80 % equates to species-level relatedness in the genus *Rhodococcus* (Sukhoom, 1999). In this context, it is interesting that Briglia *et al.* (1996) recognized that *R. opacus* and *R. percolatus* were readily distinguished using phenotypic properties, even though they reported that the type strains of these species shared a DNA–DNA similarity value above the 70 % cut-off point.

The type strains of *R. opacus*, *R. percolatus* and *T. wratislaviensis* were rich in straight-chain saturated and unsaturated fatty acids, though quantitative differences between the strains were found for some components. The organisms contained pentadecanoic acid (C_{15:0}, 10–13 % of total fatty acid composition), heptadecanoic acid (C_{17:0}, 6.5–7.0 %), palmitic acid (C_{16:0}, 23–26 %), palmitoleic acid (C_{16:1}, 0.1–13 %), heptadecenoic acid (C_{17:1}, 13–16 %), octadecenoic acid (9–10 %) and tuberculostearic acid (10-methyl branched C_{18:0}, 1–8 %). All three type strains synthesized a homologous series of mycolic acids ranging from C₄₆ to C₅₄ with the major homologues at C₅₁ and C₅₂. In sharp contrast, strain N805^T can be readily distinguished from the type strains of *R. opacus* and *R. percolatus*, as well as from other representatives of the *R. erythropolis* subclade, by using a combination of carbon-source utilization and enzymic tests (Table 1). Additional phenotypic tests have been recommended for the separation of *R. opacus* and *R. percolatus* both from one another and from representatives of other *Rhodococcus* species (Briglia *et al.*, 1996). It is also interesting that both these taxa and strain N805^T can

be distinguished from the remaining validly described species assigned to the *R. erythropolis* subclade by the overall size of their mycolic acids (Table 1).

It is clear from the present study that *T. wratislaviensis* N805^T has properties consistent with its assignment to the genus *Rhodococcus*. The taxon is very closely related to *R. opacus* and *R. percolatus* but can be distinguished from these species by using a combination of genotypic and phenotypic data. It is proposed, therefore, that *T. wratislaviensis* be transferred to the genus *Rhodococcus* as *Rhodococcus wratislaviensis* comb. nov.

Description of *Rhodococcus wratislaviensis* comb. nov.

Basonym: *Tsukamurella wratislaviensis* Goodfellow *et al.* 1995.

Rhodococcus wratislaviensis (wra.tis.la.vi.en'sis. M.L. n. *wratislavia* Wroclaw, Poland; M.L. adj. *wratislaviensis* pertaining to Wroclaw).

The description is based upon information taken from the present study and from Goodfellow *et al.* (1991). Strictly aerobic, Gram-positive, acid–alcohol-fast actinomycete that forms straight to slightly curved rods and white to creamy, irregular colonies that are easy to emulsify. Cleaves a range of conjugated substrates based on the fluorophores 7-amino-4-methylcoumarin (7AMC) and 4-methylumbelliferone (4MU), namely benzyloxycarbonyl-L-alanine-L-alanine-L-phenylalanine 7AMC, benzyloxycarbonyl-L-arginine-L-arginine 7AMC, benzyloxycarbonyl-L-arginine 7AMC, benzyloxycarbonyl-L-phenylalanine-L-arginine 7AMC (endopeptidases); β-alanine 7AMC, D-alanine 7AMC, succinyl-benzylcysteine 7AMC, isoleucine 7AMC, L-lysine 7AMC, L-methionine 7AMC, L-serine 7AMC, L-tyrosine 7AMC, L-valine 7AMC (exopeptidases); 4MU 2-acetamido-2-deoxy-β-D-glucopyranoside, 4MU α-L-arabinopyranoside, 4MU β-D-cellobioside, 4MU β-D-glucopyranoside (glycosidases); 4MU bis-phosphate (pH 5.2), 4MU phosphate (pH 5.2 and 9.0), 4MU pyrophosphate, 4MU sulphate (inorganic esters); 4MU elaidate, 4MU heptanoate, 4MU laurate, 4MU nonoate, 4MU oleate, 4MU palmitate and 4MU stearate (organic esters).

Utilizes glucose, mannose, sucrose, trehalose, meso-inositol, D-mannitol (all at 1 %, w/v), amyl alcohol, androsterone, betaine, butan-1-ol, butan-1,3-diol, sodium butyrate, citraconic acid, ethanol, sodium fumarate, gluconic acid, *n*- and *p*-hydroxybenzoic acid, DL-norleucine, D-mandelic acid, paraffin, L-phenylalanine, sodium pimelate, L-proline, sodium pyruvate, sodium sebacate, spermine, testosterone and vanillic acid (all at 0.1 % w/v or v/v) as sole carbon sources but not adonitol, cellobiose, dulcitol, galactose, raffinose (all at 1 %, w/v), anthranilic acid, sodium malate, methanol, sodium tartrate or vanillin (all at 0.1 %, w/v or v/v). D-Alanine, L-alanine and L-asparagine are used as sole carbon and nitrogen sources but acetamide, benzamide and L-threonine (all at 0.1 %, w/v) are not.

Table 1. Chemical and physiological tests that distinguish between species belonging to the *R. erythropolis* subclade

Taxa are listed as: 1, *R. erythropolis* (13 strains analysed); 2, *Rhodococcus fascians* (7 strains); 3, *Rhodococcus globerulus* (2 strains); 4, *R. opacus* (5 strains); 5, *R. percolatus* DSM 44240^T; 6, *R. wratislaviensis* N805^T. For *R. erythropolis*, *R. fascians* and *R. globerulus*, the number of strains giving a positive reaction is shown; these data were taken from Klatte *et al.* (1994b). For *R. opacus*, the number of positive strains and the reaction of the type strain are given. Information on *Rhodococcus marinonascens* is missing due to the inability of the type strain to grow on GYM agar (Klatte *et al.*, 1994b). The number of carbons in the mycolic acids of the type strain of *R. marinonascens* ranges from 34 to 50. Nearly all of the strains, including the type strains of *R. opacus*, *R. percolatus* and *R. wratislaviensis*, utilized citrate, gluconate, putrescine, quinate, D-sucrose, succinate and L-valine. pNP, p-Nitrophenyl.

Characteristic	1	2	3	4		5	6
				5 strains	DSM 43205 ^T		
Utilization of carbon compounds:							
Acetamide	11	0	0	0	—	+	—
L-Alanine	13	3	0	5	+	+	+
D-Arabitol	12	6	2	5	+	+	—
4-Aminobutyrate	7	1	1	3	+	+	—
L-Aspartate	0	0	0	5	+	+	+
Benzoate	4	2	1	3	+	+	+
Caprate	12	1	2	5	+	+	—
D-Galactose	11	5	0	5	+	+	—
Glutarate	4	3	0	0	—	—	—
D-Glucosaminic acid	1	5	0	5	+	+	+
<i>i</i> -Inositol	13	0	0	5	+	+	+
L-Leucine	11	1	2	5	+	+	+
<i>N</i> -Acetyl D-glucosamine	9	4	0	5	+	+	+
3-Hydroxybenzoate	0	0	1	3	+	+	+
4-Hydroxybenzoate	9	5	1	5	+	+	+
2-Hydroxyvalerate	11	3	0	4	+	+	+
2-Oxyglutarate	9	4	0	5	+	—	—
Phenylacetate	8	2	0	2	+	+	—
Pimelate	5	1	2	5	+	+	+
L-Proline	5	1	2	1	+	+	+
L-Rhamnose	0	0	0	0	—	—	+
D-Ribose	12	3	0	5	+	+	—
L-Serine	6	2	0	3	+	—	+
D-Turanose	0	1	0	5	+	+	+
Tyramine	2	0	0	5	+	+	—
Hydrolysis of chromogenic substrates:							
2-Deoxythymidine-5'-pNP phosphate	13	0	2	0	—	—	—
pNP phosphorylcholine	13	0	2	5	+	+	—
pNP β-D-xyloside	13	0	2	0	—	—	—
Overall size of mycolic acids (no. of carbons)	C ₃₀ –C ₄₄	C ₄₄ –C ₅₁	C ₃₀ –C ₄₂	C ₄₇ –C ₅₄	C ₄₆ –C ₅₄	C ₄₆ –C ₅₄	C ₄₆ –C ₅₄

Grows at 10 and 40 °C, but not at 45 °C, and at pH 4.5 and 9.0, but not at pH 4.0. Grows in the presence of crystal violet (0.001%, w/v), sodium chloride (5%, w/v), gentamicin sulphate (16 µg ml⁻¹), neomycin sulphate (9 µg ml⁻¹), novobiocin (2 µg ml⁻¹), oleandomycin phosphate (32 µg ml⁻¹), penicillin (32 µg ml⁻¹), polymyxin B sulphate (32 µg ml⁻¹), rifampicin (4 µg ml⁻¹), streptomycin sulphate (4 µg ml⁻¹), tobramycin sulphate (8 µg ml⁻¹) and vancomycin hydrochloride (2 µg ml⁻¹). Growth does not occur in the presence of phenol (0.001%, w/v), phenyl ethanol (0.3%, v/v), sodium azide (0.001%, w/v), sodium chloride (7%, w/v), chloramphenicol (32 µg ml⁻¹),

chlortetracycline hydrochloride (4 µg ml⁻¹), rifampicin (8 µg ml⁻¹) or vancomycin hydrochloride (4 µg ml⁻¹). Additional phenotypic properties of the type strain are given in Table 1. The fatty acid pattern consists mainly of C_{15:0} (12.9%), C_{16:1}*cis*9 (5.1%), C_{16:1}*cis*10 (8.5%), C_{16:0} (22.6%), C_{17:1}*cis*9 (12.6%), C_{17:0} (6.5%), 10-methyl C_{17:0} (6.3%), C_{18:1}*cis*9 (10.4%) and 10-methyl C_{18:0} (6.1%). The organism also contains mycolic acids with 46–54 carbons with up to three double bonds; C_{14:0}, C_{15:0} and C_{16:0} fatty acids are released on pyrolysis gas chromatography of the methyl mycolates. The G + C content of the DNA is 66–68 mol%.

Strains classified as *Tsukamurella wratislaviensis* (cluster 5) in a polyphasic taxonomic study of the genera *Gordonia* and *Tsukamurella* (Goodfellow *et al.*, 1991) have phenotypic properties consistent with their assignment to *Rhodococcus wratislaviensis*. Representatives of this taxospecies, with the exception of strain N805^T, were found to share DNA–DNA relatedness values of over 91 % with reference DNA from *Tsukamurella wratislaviensis* strain N800. In contrast, strain N805^T was erroneously reported to share a DNA homology value with the reference strain of 41 % (Goodfellow *et al.*, 1991); the recalculated DNA relatedness value between these strains is 80 %, thereby underscoring the congruence between the phenotypic and genotypic data. Several of the additional *Rhodococcus wratislaviensis* strains were isolated from soil, suggesting that this may be the primary habitat for members of this species.

The type strain is strain N805^T (= DSM 44107^T = NCIMB 13082^T).

ACKNOWLEDGEMENTS

We are indebted to Professor R. E. Gordon for the gift of strains. J.C. is grateful to the British Council (Seoul, Republic of Korea) for financial support and for an Overseas Research Studentship Award.

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