Polycyclic aromatic hydrocarbon-degrading species isolated from Hawaiian soils: *Mycobacterium crocinum* sp. nov., *Mycobacterium pallens* sp. nov., *Mycobacterium rutilum* sp. nov., *Mycobacterium rufum* sp. nov. and *Mycobacterium aromaticivorans* sp. nov.

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Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants. In this study, both pristine and contaminated soils were sampled as a source of PAH-degrading organisms. Nine strains isolated from these soils were identified as rapidly growing members of the genus *Mycobacterium* through basic phenotypic characteristics and through sequence similarity of three genes. Because the sequence similarity of the 16S rRNA gene is relatively high among members of this genus, additional conserved genes encoding the β subunit of RNA polymerase (*rpoB*) and a heat-shock protein (*hsp65*) were sequenced. Several analyses were completed to differentiate the strains from one another and to determine their species-level taxonomy, including fatty acid methyl ester analysis, biochemical tests and substrate-utilization profiling. A phylogenetic tree incorporating sequences for all three genes was constructed with the isolates and their close described relatives. Results for biochemical tests, substrate-utilization tests and DNA sequencing were compared with those of the phylogenetically similar organisms to establish the isolated strains as representatives of novel species with characteristics unlike those of previously described species of *Mycobacterium*. Finally, DNA–DNA hybridization was performed between strains and their close relatives to confirm their position within novel species. Our results demonstrated that the isolates represent five novel species, which were named *Mycobacterium crocinum* sp. nov. (type strain czh-42^T = ATCC BAA-1373^T = CIP 109262^T; reference strains czh-1A = ATCC BAA-1370 = CIP 109266 and czh-3 = ATCC BAA-1371 = CIP 109267), *Mycobacterium pallens* sp. nov. (type strain czh-8^T = ATCC BAA-1372^T = CIP 109268^T), *Mycobacterium rutilum* sp. nov. (type strain czh-117^T = ATCC BAA-1375^T = CIP 109271^T; reference strains czh-107 = ATCC BAA-1374 = CIP 109270 and czh-132 = ATCC BAA-1376 = CIP 109272), *Mycobacterium rufum* sp. nov. (type strain JS14^T = ATCC BAA-1377^T = CIP 109273^T) and *Mycobacterium aromaticivorans* sp. nov. (type strain JS19b1^T = ATCC BAA-1378^T = CIP 109274^T).

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Abbreviations: FAME, fatty acid methyl ester; PAH, polycyclic aromatic hydrocarbon; RGM, rapidly growing mycobacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *rpoB* and *hsp65* sequences of the novel isolates are detailed in Supplementary Table S1.

Accession numbers of sequences used in tree construction, detailed Biolog GP2 results and single-gene trees are available as supplementary material with the online version of this paper.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) have received much attention as a class of carcinogenic and mutagenic organic pollutants that can be removed from contaminated environments through bioremediation. Microbial degradation of PAHs is considered the major avenue of removal from the environment (Cerniglia, 1992), and members of the genus *Mycobacterium* have been shown to be degraders of PAHs with larger number of aromatic rings, such as

fluoranthene (Bogan *et al.*, 2003; Lopez *et al.*, 2006), pyrene (Heitkamp *et al.*, 1988; Rehmann *et al.*, 1998) and benzo[*a*]pyrene (Moody *et al.*, 2004; Schneider *et al.*, 1996). Isolation of novel organisms remains interesting because of the potential for degradation of diverse substrates and/or for the presence of novel catabolic pathways.

Hawaii's delicate island ecosystem makes it necessary to investigate the means of removing persistent environmental pollutants. Activities that produce PAHs have long been occurring on the Hawaiian islands, ranging from natural causes such as volcanic activity to human industry and fossil fuel combustion. To avoid the introduction of foreign or genetically engineered micro-organisms into a delicate ecosystem, the removal of organic pollutants by indigenous species is highly desirable. For these reasons, there is interest in the discovery of native bacteria that are capable of degrading PAHs in Hawaiian soils. The diversity of the Hawaiian soil environment may also offer organisms with novel properties for use in biotechnology applications worldwide.

In this study, several bacteria belonging to the genus *Mycobacterium* were isolated in a screen for PAH-degrading organisms. For isolation, both contaminated and pristine soils were collected and enriched in a PAH-supplemented medium, yielding many bacterial isolates capable of degrading PAHs, including phenanthrene, fluoranthene, fluorene, pyrene and/or benzo[*a*]pyrene. Two of the isolates could degrade additional chemicals including organophosphorus pesticides (Seo *et al.*, 2007a). Because preliminary investigation of these organisms revealed interesting substrate ranges and characteristics unlike previously described *Mycobacterium* species, we characterized them using phenotypic, biochemical and genotypic methods. Although organisms belonging to other genera were also isolated [e.g. *Burkholderia* (Seo *et al.*, 2007b), *Arthrobacter* (Seo *et al.*, 2006) and *Stenotrophomonas* (Keum *et al.*, 2006)], the scope of this paper is limited to the identification and characterization of those belonging to the genus *Mycobacterium*.

METHODS

Strains and media. The type strains of four related bacteria, *Mycobacterium aichiense* ATCC 27280^T, *M. chubuense* ATCC 27278^T, *M. rhodesiae* ATCC 27024^T (Tsukamura *et al.*, 1981) and *M. chlorophenolicum* ATCC 49826^T (Häggblom *et al.*, 1994), were obtained from the American Type Culture Collection (Manassas, VA, USA) for comparison with isolated organisms. All isolates from this study grew on Middlebrook 7H9 with glycerol and ADC enrichment, Middlebrook 7H10 solid medium with OADC enrichment, tryptic soy broth or agar (TSB or TSA), Luria–Bertani (LB) broth and standard mineral base (SMB) (Stanier *et al.*, 1966) supplemented with an appropriate carbon source such as glucose, mannitol or PAHs. Liquid SMB medium contained (l⁻¹) 50 ml freshly prepared phosphate buffer (1 M Na₂HPO₄ titrated with 1 M KH₂PO₄ to pH 6.8), 10 ml 10% ammonium sulfate and 20 ml concentrated mineral base. Concentrated mineral base contained

(l⁻¹) 10 g nitrilotriacetic acid, 7.3 g KOH, 14.45 g MgSO₄ (anhydrous), 3.35 g CaCl₂·2H₂O, 9.25 mg (NH₄)₆Mo₇O₂₄·4H₂O and 50 ml metals 44 solution. Metals 44 solution contained (l⁻¹) 2.5 g EDTA (free acid), 10.95 g ZnSO₄·7H₂O, 5 g FeSO₄·7H₂O, 1.54 g MnSO₄·H₂O, 392 mg CuSO₄·5H₂O, 250 mg Co(NO₃)₂·6H₂O and 177 mg Na₂B₄O₇·10H₂O. LB/glycerol medium was used for preservation of strains at -80 °C.

Isolation of PAH-degrading organisms. The soils from which bacterial strains were isolated included non-contaminated soil from central Oahu (site 1; strains czh-1A, czh-3, czh-42^T and czh-8^T), non-contaminated soil from an urban park in Honolulu (site 2; strains czh-107, czh-132 and czh-117^T) and contaminated soils from a former oil-gasification company site in Hilo (site 3; strains JS14^T and JS19b1^T). The isolation of two strains included in this study, JS14^T and JS19b1^T, has been described previously (Seo *et al.*, 2007a). For the other seven organisms, isolation is detailed in this work. Pyrene was delivered to sterile flasks in acetone and then the solvent was evaporated sterily. For enrichment, 1 g soil was mixed with 50 ml mineral medium and added to flasks containing 50 µg pyrene. When growth occurred as indicated by turbidity in the medium, 1 ml cell suspension was transferred to new medium. Transfers were completed three times, after which cell suspensions were plated in serial dilutions on plates prepared according to the following method. SMB 2% agar medium was sterilized and poured into Petri plates (approx. 10 ml in a 10 cm plate). Molten SMB agar (7 ml of 1.7% agar) to be used as an overlayer was poured into glass test tubes, which were capped, autoclaved and then placed into a 65 °C water bath. PAHs were dissolved in acetone as stock solutions (pyrene at 25 mg ml⁻¹, benzo[*a*]pyrene at 10 mg ml⁻¹, fluoranthene at 37.5 mg ml⁻¹ and phenanthrene at 50 mg ml⁻¹). Working in a laminar flow hood, 100 µl of the appropriate stock solution was added to the molten SMB agar in the glass test tubes and the mixture was vortexed briefly. The PAH-spiked agar was then poured over the solidified SMB agar base. The plates were left open under the hood for 1 h in the dark and then closed and transferred to a dark incubator (set at 28 °C) for 24 h to evaporate any remaining solvent. The amount of PAH added was optimized at a concentration high enough to form an opaque layer, but low enough to avoid flocculation of the chemical in the medium. Final amounts of pyrene, benzo[*a*]pyrene, fluoranthene and phenanthrene per plate were 2.5, 1.0, 3.75 and 5 mg, respectively. From the serially plated enrichment dilutions, colonies that produced clearing zones around single colonies on solid media were purified on fresh PAH-containing plates, and strains that produced clearing zones over successive transfers were preserved at -80 °C.

Substrate utilization and biochemical characterization. Citrate utilization was tested using Difco medium according to the manufacturer's instructions. For substrate oxidation tests using the Biolog system, cells were grown on TSA and resuspended in sterile GN-GP inoculation fluid, and the turbidity was adjusted to an OD₆₀₀ of 0.7. GN-GP inoculation fluid contained (l⁻¹) 0.1 g Phytigel, 0.4 g NaCl and 0.3 g Pluronic F-68. Three to five drops of guanidine isothiocyanate were added to each suspension to prevent false-positive results due to exopolysaccharide (EPS) degradation. Biolog GP2 plates were inoculated with 150 µl cell suspension, incubated at 28 °C for 24 h and interpreted by visual comparison of substrate oxidation with that of the water control.

Substrates that were oxidized differentially by closely related isolates and described organisms were tested as growth sources. Substrate stock solutions were prepared in SMB mineral medium at 10% (w/v) and filter-sterilized into sterile tubes. Test tubes were prepared with 5 ml sterile SMB, to which stock solution was added to achieve a final concentration of 1% substrate. Cells were grown in 10 ml TSB medium over 4 days, pelleted at 3600 g, washed with 5 ml SMB twice and then adjusted to an OD₆₀₀ of 0.3 in SMB medium. Inocula

(20 µl) were added to substrate-containing test tubes and incubated over 10 days at 28 °C. Turbidity of the medium compared with the control (no substrate) at 2, 4 or 7 days was recorded as positive growth on the substrate. If 10 days or more were required for growth on the substrate compared with the control, a partial positive was recorded. For this test, the isolates were divided into two groups based on 16S rRNA gene, *rpoB* and *hsp65* sequences: those related more closely to *M. aichiense* and *M. rhodesiae* were isolates czh-1A, czh-3, czh-42^T, czh-8^T and JS19b1^T, and those closer to *M. rhodesiae*, *M. chubuense* and/or *M. chlorophenolicum* included czh-107, czh-117^T, czh-132 and JS14^T. The group containing strains czh-1A, czh-3, czh-42^T, czh-8^T and JS19b1^T were tested for growth on dextrin, L-arabinose, D-fructose, D-mannitol, D-mannose, D-ribose, trehalose, D-xylose, succinic acid, glycerol and D-glucose. The other group including czh-107, czh-117^T, czh-132 and JS14^T were tested for growth on D-glucose, D-fructose, D-mannitol, D-sorbitol, trehalose, xylitol and D-xylose.

Use of PAHs (phenanthrene, fluoranthene, pyrene and benzo[a]pyrene) as sole carbon sources was determined by clearing-zone formation on solid medium as described above. Where clearing zones were observed, degradation was confirmed by combining strains with 100 p.p.m. PAH in liquid SMB medium and incubating for 7 days, followed by extraction and HPLC analysis of parent compounds. PAH removal in inoculated samples was compared with that of abiotic controls incubated for the same period.

For total cellular fatty acid methyl ester (FAME) analysis, isolates were grown on TSA and sent to Microbial ID (Newark, DE, USA). Biochemical capacities of novel isolates and of purchased organisms were determined using API Coryne strips (bioMérieux) according to the manufacturer's instructions.

DNA–DNA hybridization. For DNA–DNA hybridization, isolates were grown in 300 ml TSB over 4–6 days, depending on the speed of growth. Cells were pelleted at 4300 g at 4 °C, washed once with 5 ml SMB medium, pelleted again and then weighed to determine the wet biomass. Two to three grams of wet biomass were sent to the DSMZ (Braunschweig, Germany) for determination of whole DNA relatedness. Each isolate was hybridized with its nearest described neighbour(s) and with other isolates that had the same nearest relatives to determine whether novel species were represented among them.

Sequencing and phylogenetic analysis. Sequencing of three genes was used as the basis for assigning isolates to a genus and to determine close specific relatives. DNA was extracted from all isolates using the MoBio UltraClean Soil DNA Isolation kit. Genes that have been used to characterize *Mycobacterium* species were amplified, including the genes encoding the 16S rRNA, the β subunit of RNA polymerase (*rpoB*) and a heat-shock protein (*hsp65*). Primers used in this study to amplify the 16S rRNA gene were 27F and 1492R (Lane, 1991). Amplification and sequencing of the *rpoB* and *hsp65* genes were accomplished using primer sets MF/TBBrpOB2 and Tb11/Tb112, respectively (Devulder *et al.*, 2005). PCRs were set up with 10–50 ng template DNA, 10 pmol each primer and 27 µl Invitrogen Platinum PCR Supermix in a 30 µl reaction. Products were sequenced in both the forward and reverse direction and, in the case of the 16S rRNA gene, the middle section was sequenced using primers 1078R (5'-CCCAACATCTCAGACACGAG) and 530R (Sacchi *et al.*, 2002) at the Greenwood Facility for Biotechnology (University of Hawaii at Manoa). Full-length sequences were assembled using Vector NTI Advance 9.0 software (Invitrogen). Sequences were compared to the non-redundant BLASTN (Altschul *et al.*, 1997) database to identify the organisms as members of the genus *Mycobacterium* and to determine their specific taxonomic designation. Sequences for all three genes were downloaded from the NCBI database for all available rapidly growing *Mycobacterium* species.

For phylogenetic analysis, sequences were trimmed so that they started and ended at the same nucleotide position and then sequences

for the three genes were concatenated. Sequence alignments were performed using CLUSTAL W v. 2 software (Larkin *et al.*, 2007). The PHYLIP software package (version 3.63; Felsenstein, 2005) was used to calculate evolutionary distances using Kimura's two-parameter substitution model (DNADIST) with bootstrapping of 100 replicates (SEQBOOT). Programs NEIGHBOR and CONSENSE were used to create neighbour-joining trees. TreeView 1.6.6 (Page, 1996) and NJPlot (Perrière & Gouy, 1996) programs were used to view trees, define the outgroup (*Nocardia abscessus* DSM 44432^T) and root the tree. Details of sequences included in the trees are given in Supplementary Table S1, available in IJSEM Online.

RESULTS AND DISCUSSION

Phenotypic and biochemical characterization

Isolates were classified as members of *Mycobacterium* based on 16S rRNA gene, *rpoB* and *hsp65* BLAST sequence similarity with members of that genus. Additionally, all isolates had the following characteristics in common with members of the genus *Mycobacterium*: they were non-motile, catalase-positive, rod-shaped cells that stained weakly Gram-positive. All isolates are rapidly growing mycobacteria (RGM), producing single colonies within 7 days of plating.

Growth media included LB, TSB, Middlebrook 7H9 with ADC enrichment/glycerol and SMB mineral medium with a carbon source. All isolates grew well in Middlebrook 7H9 medium, except for strain JS14^T, which preferred TSB for growth. Strains czh-8^T and JS14^T grew optimally at 28 °C, whereas czh-1A, czh-3 and czh-107 grew better at 37 °C. Other strains grew equally well at both temperatures. All strains preferred shaking to still incubation.

Oxidation of substrates was determined using the Biolog system with GP2 plates. Oxidation by strain czh-107 could not be determined because of excessive non-specific reactions produced, so substrate oxidation information cannot be shown here. Strains czh-117^T and czh-132 had similar substrate oxidation reactions. Results of substrate oxidation by isolates are listed in Supplementary Table S2. Substrate growth results for isolates and test organisms are detailed in Table 1. Utilization of PAHs by novel isolates is given in Table 2.

FAME profiles were determined for eight of the new isolates (Table 3). These profiles were used to support taxonomic groupings and to determine what ranges of FAME profiles were common within the new groupings.

DNA–DNA hybridization

Results for hybridizations are shown in Table 4. Based on the current recommended limit for species designation of 70 % relatedness of whole DNA, the eight isolates tested were found to comprise five groups with relatively low levels of DNA relatedness to described *Mycobacterium* species. Strains czh-1A, czh-3 and czh-42^T had whole DNA relatedness values between 82 and 100 % and can be

Table 1. Phenotypic characteristics of the novel *Mycobacterium* isolates and closely related organisms

Reference strains: 1, *M. aichiense* ATCC 27280^T; 2, *M. chlorophenicum* ATCC 49826^T; 3, *M. chubuense* ATCC 27278^T; 4, *M. rhodesiae* ATCC 27024^T. Data were obtained in this study unless indicated. NT, Not tested; d, strain-dependent reaction; (+), partial positive reaction. All strains variably stained Gram-positive and were non-motile. All strains reacted positively for catalase and negatively for β -glucuronidase, β -galactosidase, *N*-acetyl- β -glucosaminidase and gelatin hydrolysis. All tested strains utilized glucose and none utilized citrate.

Characteristic	czh-1A	czh-3	czh-42 ^T	czh-8 ^T	czh-107	czh-117 ^T	JS14 ^T	JS19b1 ^T	1	2	3	4
Cell morphology*	LTR	LTR	LTR	MR	MLTR	MLTR	MLTR	CLR	SR ^{a†}	RC ^b	C ^a	SR ^a
Colony morphology‡	M Ro Ra Mu St	S Ro Fl Dr St	S Ro Fl Dr St	M Ir Fl Dr Un	M Ro Ra Mu Tu	M Ro Ra Sd Sw	L Ro Ra Dr St	Mu NS	Smooth ^a	Slightly mucoid ^b	Smooth ^a	Smooth ^a
Colony colour§	YO	YO	YO	PO	DO	DO	O	Y	Y	O	O	Y
Pigmentation in dark	+	+	+	—	—	—	+	+	+	+	+	+
Growth at 45 °C	—	—	—	—	+	+	NT	NT	— ^a	— ^b	— ^a	— ^a
Growth in 2.5/5.0 % NaCl	+/-	+/-	+/-	-/-	+/+	+/+	-/-	+/+	+/+	+/+	+/-	+/-
Nitrate (nitrite) reduction	+	+	+	+	- (-)	- (-)	+	- (-)	- (-)	- (-)	+ ^a	- ^a
Pyrrolidonyl arylamidase	—	—	—	—	+	+	—	—	—	—	NT	NT
Alkaline phosphatase	+	+	+	+	—	—	—	+	+	+	NT	NT
α -Glucosidase	—	—	—	(+)	(+)	+	+	+	+	+	NT	NT
Aesculin hydrolysis	—	—	—	—	—	—	(+)	—	—	(+)	NT	NT
Urease	—	—	—	—	+	+	+	—	-	—	+ ^a	+ ^a
Utilized substrates												
D-Fructose	—	+	+	+	+	—	+	+	+	+	+	+
D-Mannitol	—	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	—	(+)	—	(+)	—	+	+	+	+	+	(+)
D-Xylose	—	—	—	—	+	+	+	—	—	+	+	+
L-Arabinose	—	—	—	—	NT	NT	NT	—	—	— ^b	— ^a	—
Dextrin	—	—	—	—	NT	NT	NT	—	—	NT	NT	—
Glycerol	—	—	—	—	NT	NT	NT	—	+	— ^b	NT	—
D-Mannose	—	+	—	(+)	NT	NT	NT	+	(+)	d ^b	+ ^a	+
D-Ribose	—	—	—	—	NT	NT	NT	—	—	d ^b	NT	—
Succinic acid	(+)	+	+	+	NT	NT	NT	+	+	NT	NT	+
D-Sorbitol	NT	NT	NT	NT	+	+	+	NT	— ^a	+	+	—
D-Xylitol	NT	NT	NT	NT	+	+	+	NT	NT	+	+	—

*C, Cocci; CLR, coccoid to long rods; LTR, long thin rods; MLTR, medium to long thin rods; MR, medium rods; RC, rods to cocci; SR, short rods.

†Data taken from: *a*, Tsukamura *et al.* (1981); *b*, Häggblom *et al.* (1994).

‡Size: L, large; M, medium; S, small. Colony shape: Fl, flat; Ir, irregular; Ra, raised; Ro, round. Characteristics: Dr, dry; Mu, mucoid; Sd, semi-dry. Border: St, smooth; Sw, smooth white; Tu, transparent undulated; Un, undulated. NS, No single colonies were formed on any media tested.

§DO, Dark orange; O, orange; PO, pale orange; Y, yellow; YO, yellow–orange.

||Data reported by this study that contradict data of Tsukamura *et al.* (1981), possibly because of differences in test methods.

Table 2. PAH utilization by novel *Mycobacterium* isolates

All strains utilized phenanthrene. Isolate JS19b1^T also utilized fluorene (Seo *et al.*, 2007a). NT, Not tested.

PAH	czh-1A	czh-3	czh-42 ^T	czh-8 ^T	czh-107	czh-117 ^T	czh-132	JS14 ^T	JS19b1 ^T
Fluoranthene	+	+	+	+	—	—	+	+	—
Pyrene	—	+	+	+	+	+	+	—	—
Benzo[a]pyrene	NT	—	NT	NT	+*	—	—	NT	—

*Degraded less than 10 % of the supplied benzo[a]pyrene at 50 p.p.m. over 28 days.

Table 3. Whole-cell FAME profiles of *Mycobacterium* isolates

Values are percentages of total fatty acids. Values in bold are referred to in the text. ND, Not detected. Other FAMES that were detected but which constituted less than 1 % in all strains are not reported. The fatty acid profile of strain czh-132 was not determined.

FAME	czh-1A	czh-3	czh-42 ^T	czh-8 ^T	czh-107	czh-117 ^T	JS14 ^T	JS19b1 ^T
10:0	1.85	1.88	2.19	4.69	3.98	2.72	0.29	1.87
12:0	0.95	0.88	1.08	1.30	0.16	0.12	0.48	0.73
14:0	5.97	5.71	5.69	6.50	2.00	2.05	5.27	6.95
15:0	0.20	2.33	0.81	1.86	0.22	0.77	1.47	1.16
16:1 ω 9c	0.73	0.74	0.74	0.73	1.36	1.82	1.60	0.59
16:1 ω 7c + 16:1 ω 6c	10.93	9.68	10.29	9.59	11.53	10.36	9.40	12.20
16:0	25.55	24.84	24.89	20.84	24.04	25.62	26.24	23.17
17:1 ω 7c	15.75	16.44	16.18	21.05	12.79	13.42	8.44	15.85
17:0	0.52	1.21	0.53	0.60	0.26	0.71	1.94	0.51
10-Methyl 17:0	0.44	0.83	0.42	0.98	0.10	0.36	ND	0.22
18:1 ω 9c	22.59	24.45	22.44	18.24	28.68	27.04	36.72	29.69
18:1 ω 7c	0.62	1.37	0.65	1.58	ND	1.19	0.89	ND
18:0	1.48	1.31	1.51	0.65	2.40	1.55	3.14	1.32
10-Methyl 18:0	9.32	5.33	10.05	7.60	10.24	11.10	1.40	3.02
20:0	0.83	0.73	0.92	0.81	0.41	0.24	0.82	0.84

Table 4. DNA–DNA hybridization between the novel isolates and reference strains

Results are percentages of whole DNA relatedness as a mean of two replicate measurements. Relative standard deviations (RSD) ranged between 0.1 and 8.3 % and the mean RSD was 3.6 %. ND, Not done. Strain czh-132 was not included in DNA–DNA hybridization experiments.

Strain	czh-1A	czh-3	czh-42 ^T	czh-8 ^T	JS19b1 ^T
czh-3	81.7				
czh-42 ^T	99.9	99.1			
czh-8 ^T	67.2	69.2	74.4		
JS19b1 ^T	36.7	39.4	28.5	33.2	
<i>M. aichiense</i> ATCC 27280 ^T	32.2	47.6	36.3	26.9	ND
<i>M. rhodesiae</i> ATCC 27024 ^T	33.1	12.4	51.3	26.1	58.3
Strain	czh-107	czh-117 ^T	JS14 ^T		
czh-117 ^T	94.2				
JS14 ^T	32.5	36.8			
<i>M. rhodesiae</i> ATCC 27024 ^T	19.2	19.9	ND		
<i>M. chlorophenolicum</i> ATCC 49826 ^T	ND	ND	32.4		
<i>M. chubuense</i> ATCC 27278 ^T	ND	ND	34.4

Fig. 1. Phylogenetic tree constructed from concatenated sequences (*hsp65*, 16S rRNA gene and *rpoB*) for the novel *Mycobacterium* isolates and most other RGM. Sequence alignments and tree construction are described in Methods. *Nocardia abscessus* DSM 44432^T was defined as the outgroup and used to root the tree. All downloaded sequences for nearest *Mycobacterium* neighbours were obtained from the NCBI database associated with the publication by Devulder *et al.* (2005); accession numbers are detailed in Supplementary Table S1. Bar, 1 change per 100 nucleotide positions.

considered members of the same species. Similarly, strain czh-107 and czh-117^T were closely related and together comprise one species. Comparisons of whole DNA relatedness between organisms that were considered taxonomically diverse species will be discussed below.

Multigene phylogenetic analysis

Researchers have noted that the use of the 16S rRNA gene sequence to assign species designations is not appropriate for the genus *Mycobacterium* because of high interspecific similarity (Adekambi *et al.*, 2006; Devulder *et al.*, 2005; Kim *et al.*, 2005). Some groups have employed sequencing of other conserved genes, including *rpoB* (Adekambi *et al.*, 2006; Devulder *et al.*, 2005) and *hsp65* (Devulder *et al.*, 2005; Kim *et al.*, 2005), in place of or in addition to the 16S rRNA gene. In this study, a multigene approach was taken for analysis of phylogeny between the new isolates and other RGM. Partial nucleotide sequences of the 16S rRNA gene, *rpoB* and *hsp65* were determined for the novel isolates (Supplementary Table S1). Available sequences for described *Mycobacterium* species (Devulder *et al.*, 2005)

were downloaded for each gene. Phylogenetic trees were constructed for each gene, and one tree was prepared with the concatenated sequence of all three genes. Fig. 1 shows the positions of the novel isolates in a tree constructed with the concatenated multigene sequences of the novel isolates and other published RGM organisms. Single-gene trees are available as Supplementary Figs S1–S3.

Results from this study support the need for a multigene approach to species identification within the genus *Mycobacterium*. First, our results were consistent with previous claims that the accepted species cut-off of 97% 16S rRNA gene sequence similarity is too low for members of this genus. The sequence similarity of the 16S rRNA gene between isolates and their nearest described neighbours was higher than 97% in every case (98–99% over 1400 bp), although DNA–DNA hybridization results demonstrate that the isolates represent novel species. For example, the 16S rRNA gene sequence of czh-107 was 99% similar to that of *M. rhodesiae* CIP 106806^T, its highest match, but hybridization results showed them to be less than 20% related over the whole DNA. It has been shown previously that separate

species can have identical *hsp65* sequences, and *rpoB* sequences have better discriminatory power to resolve classification (e.g. *Mycobacterium farcinogenes* and *Mycobacterium senegalense*; Devulder *et al.*, 2005). In this study, the *rpoB* sequences of isolates of the same species (czh-107 and czh-117^T; 94 % whole DNA relatedness) were more divergent than those of distinct species *Mycobacterium chlorophenolicum* and *M. chubuense* or *M. farcinogenes* and *M. senegalense*. Thus, based on *rpoB* sequences, these isolates may appear to represent separate species; however, their close relationship was established correctly in the concatenated tree, as later confirmed by hybridization. In short, this study corroborates others in which sequencing of more than one gene was critical for the classification of some members of the genus *Mycobacterium*.

Differentiation of novel isolates from described species and each other

Species classification was based on phenotypic and genotypic comparisons with type strains of defined *Mycobacterium* species and similar comparisons between newly isolated strains. The isolates fell roughly into four groups based on the three gene sequences and phenotypic tests. Isolates czh-1A, czh-3, czh-42^T and czh-8^T were related to *M. aichiense* and *M. rhodesiae*. Strain JS19b1^T was related most closely to *M. rhodesiae*. Gene sequences of isolates czh-107, czh-117^T and czh-132 did not cluster closely with any organisms in the concatenated tree, but the 16S rRNA gene sequences were most similar to that of *M. rhodesiae* CIP 106806^T over 1400 bp. Isolate JS14^T was similar to *M. chlorophenolicum* and *M. chubuense*.

Tables 1–4, Supplementary Table S2 and Fig. 1 detail the similarities and differences between the new isolates and described species. Isolates czh-1A, czh-3 and czh-42^T differed from *M. aichiense* in colony morphology, salt tolerance (5 %), nitrate reduction, α -glucosidase activity and glycerol utilization. The isolates gave whole DNA relatedness values of 32–48 % with *M. aichiense* ATCC 27280^T. These three isolates were differentiated from *M. rhodesiae* in cellular morphology, colony morphology, nitrate reduction, urease activity and utilization of D-xylose, and whole DNA relatedness with *M. rhodesiae* ATCC 27024^T was 12–51 %. They differed from one another in percentages of the fatty acid 10-methyl 18:0 (tuberculostearic acid; TBSA), in substrate oxidation (dextrin, N-acetyl-D-glucosamine, L-arabinose, D-arabitol, D-fructose, D-mannitol, D-psicose, acetic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, pyruvic acid methyl ester, succinic acid monomethyl ester, pyruvic acid, succinic acid and glycerol) and in substrate utilization (D-fructose, D-mannose, D-mannitol, trehalose and pyrene). Cases for which partial positives were recorded in phenotypic tests were not counted as different from either positives or negatives, to provide a conservative estimate. Hybridization results showed that these isolates were 82–100 % related to one another.

Isolate czh-8^T had fewer common phenotypic properties with either the described organisms or isolates czh-1A,

czh-3 and czh-42^T. When compared with *M. aichiense*, czh-8^T differed in cellular morphology, colony morphology, pigmentation in the dark, colony colour, salt tolerance, nitrate reduction and substrate utilization (trehalose and glycerol). Whole DNA relatedness between czh-8^T and *M. aichiense* ATCC 27280^T was 27 %. Differences from *M. rhodesiae* included cellular morphology, colony morphology, pigmentation in the dark, colony colour, salt tolerance, nitrate reduction, urease activity and D-xylose utilization. Whole DNA relatedness with *M. rhodesiae* ATCC 27024^T was 26 %. Differences from isolates czh-1A, czh-3 and czh-42^T were apparent in cellular morphology, colony morphology, pigmentation in the dark, colony colour and salt tolerance. In the FAME analysis, the profile of czh-8^T looked quite different from those of the other three isolates (Table 3; see 10:0, 16:0, 17:1 ω 7c and 18:1 ω 9c). The phenotypic differences listed above suggest that czh-8^T is less related to the three isolates czh-1A, czh-3 and czh-42^T than they are to one another, and this separation was supported in the multigene phylogeny (Fig. 1). DNA–DNA hybridization results between isolate czh-8^T and isolates czh-1A, czh-3 and czh-42^T gave values of 67–74 % relatedness. This level of relatedness is at the limit of the definition of species at 70 % whole DNA relatedness, such that phenotypic characteristics play a large role in determining whether this organism should be assigned to the same species as the other three. In this case, the relatively large number of phenotypic differences listed above support its taxonomic designation within a separate species.

Isolate JS19b1^T was most closely related to *M. rhodesiae* in the multigene phylogeny. Phenotypic differences between this isolate and *M. rhodesiae* included cellular morphology, salt tolerance (5 %), urease activity and D-xylose utilization. DNA–DNA hybridization between JS19b1^T and *M. rhodesiae* ATCC 27024^T showed them to be 58 % related over whole DNA. Compared to the four other isolates (czh-1A, czh-3, czh-42^T and czh-8^T) closely related to *M. rhodesiae*, JS19b1^T had different reactions for cellular morphology, colony morphology, colony colour, salt tolerance (5 %), nitrate reduction, α -glucosidase activity, oxidized substrates (glycogen, L-rhamnose, D-sorbitol and L-alaninamide) and FAME profiles (Table 3; see 18:1 ω 9c, 18:1 ω 7c and TBSA). Whole DNA relatedness to these four other isolates ranged between 29 and 39 %. One additional notable characteristic of this isolate was its inability to grow as single colonies, whether streaked out or serially plated on the media tested, including TSA, LB and SMB/carbon source. It grew at dilutions that were high enough to form a lawn, but no growth appeared at lower levels.

Another group of isolates, czh-107, czh-117^T and czh-132, were most closely related to *M. rhodesiae* CIP 106806^T in their 16S rRNA gene sequence. Their relationship to any described organism was not supported by high bootstrap values in the multigene phylogeny such that, in this case, the choice for species comparison was made based on the highest BLAST match with the 16S rRNA gene sequence over

1400 bp. These strains were differentiated from *M. rhodesiae* in several phenotypic characteristics, including cellular morphology, colony morphology, colony colour, pigmentation in the dark, growth temperatures, salt tolerance (5 %) and substrate utilization (D-sorbitol and xylitol). DNA–DNA hybridization results showed that isolates czh-107 and czh-117^T were about 20 % related to *M. rhodesiae* ATCC 27024^T over the whole DNA. There were no phenotypic differences between czh-117^T and czh-132, and few between them and czh-107, including only D-fructose utilization and some FAME percentages. The similar reactions of these isolates in the phenotypic tests performed, their close grouping within the multigene phylogeny and their DNA relatedness of 94 % indicate that they are strains within the same species.

The last group included only one isolate, JS14^T, which was most closely related to *M. chlorophenolicum* and *M. chubuense*. Strain JS14^T could be differentiated from *M. chlorophenolicum* on the basis of cellular morphology, salt tolerance (2.5 and 5 %), nitrate reduction, alkaline phosphatase and urease activities and from *M. chubuense* in cellular morphology, growth temperature and salt tolerance (2.5 %). Whole DNA relatedness with *M. chlorophenolicum* ATCC 49826^T was 32 % and with *M. chubuense* ATCC 27278^T was 34 %.

Description of *Mycobacterium crocinum* sp. nov.

Mycobacterium crocinum (cro'ci.num. L. neut. adj. *crocinum* saffron-coloured, pertaining to the colony pigmentation of known strains).

Thin, long, finger-like rods that show weak uptake of Gram stain. Forms colonies within 7 days that are small to medium-sized, round, raised or flat, mucoid or dry, with a smooth border and yellow–orange– (amber-) pigmented in light or dark. Grows at 28 and 37 °C. Growth media include LB, TSB, Middlebrook 7H9/glycerol/ADC (preferred) and SMB plus a carbon source. Tolerates moderate (2.5 %) but not high (5.0 %) salinity. Non-motile aerobe. Reduces nitrate. Positive for catalase and alkaline phosphatase and negative for β -glucuronidase, β -galactosidase, *N*-acetyl- β -glucosaminidase, gelatin hydrolysis, pyrrolidonyl arylamidase, α -glucosidase, aesculin hydrolysis and urease activities. Oxidation and utilization of substrates and the composition of whole-cell fatty acids are listed in Tables 1, 2 and 3 and Supplementary Table S2.

The type strain, czh-42^T (=ATCC BAA-1373^T =CIP 109262^T), and reference strains czh-1A (=ATCC BAA-1370 =CIP 109266) and czh-3 (=ATCC BAA-1371 =CIP 109267) were isolated from non-contaminated soil from Wahiawa, HI, USA.

Description of *Mycobacterium pallens* sp. nov.

Mycobacterium pallens (pal'lens. L. neut. adj. *pallens* pale yellow, pertaining to the colony pigmentation of the type strain).

Medium rods that show weak uptake of Gram stain. Forms colonies within 7 days that are medium-sized, flat and dry with an undulated border and which turn pale orange once exposed to light. Grows at 28 (preferred) and 37 °C. Growth media include LB, TSB, Middlebrook 7H9/glycerol/ADC (preferred) and SMB plus a carbon source. Does not tolerate salinity (2.5 or 5 %). Non-motile aerobe. Reduces nitrate. Positive for catalase, alkaline phosphatase and α -glucosidase (partial positive) and negative for β -glucuronidase, β -galactosidase, *N*-acetyl- β -glucosaminidase, gelatin hydrolysis, pyrrolidonyl arylamidase, aesculin hydrolysis and urease activities. Oxidation and utilization of substrates and the composition of whole-cell fatty acids are listed in Tables 1, 2 and 3 and Supplementary Table S2.

The type strain, czh-8^T (=ATCC BAA-1372^T =CIP 109268^T), was isolated from non-contaminated soil from Wahiawa, HI, USA.

Description of *Mycobacterium aromaticivorans* sp. nov.

Mycobacterium aromaticivorans [a.ro.ma.ti'ci.vo'rans. L. adj. *aromaticus* aromatic, fragrant; L. part. adj. *vorans* devouring; N.L. part. adj. *aromaticivorans* devouring aromatic (compounds)].

Pleomorphic, with coccoid and long rod shapes, showing weak uptake of Gram stain. Rapidly growing, but does not form single colonies on any media tested, only growing on solid media at dilutions turbid enough to support a lawn of growth. Mucoid and yellow-pigmented in light or dark. Grows at 28 and 37 °C. Growth media include LB, TSB, Middlebrook 7H9/glycerol/ADC (preferred) and SMB plus a carbon source. Tolerates moderate to high salinity (2.5–5.0 %). Non-motile aerobe. Does not reduce nitrate or nitrite. Positive for catalase, alkaline phosphatase and α -glucosidase and negative for β -glucuronidase, β -galactosidase, *N*-acetyl- β -glucosaminidase, gelatin hydrolysis, pyrrolidonyl arylamidase, aesculin hydrolysis and urease activities. Oxidation and utilization of substrates and the composition of whole-cell fatty acids are listed in Tables 1, 2 and 3 and Supplementary Table S2.

The type strain, JS19b1^T (=ATCC BAA-1378^T =CIP 109274^T), was isolated from PAH-contaminated soil of a former oil-gasification company site in Hilo, HI, USA.

Description of *Mycobacterium rutilum* sp. nov.

Mycobacterium rutilum (ru.ti'lum. L. neut. adj. *rutilum* rust-coloured, pertaining to the colony pigmentation of known strains).

Medium to long, thin rods that show weak uptake of Gram stain. Forms colonies within 7 days that are medium-sized, round, raised or flat, mucoid or dry with an undulated transparent border. Colonies are unpigmented until exposed to light, under which they turn deep orange. Grows at 28, 37 (preferred) and 45 °C. Growth media include LB, TSB

(preferred), Middlebrook 7H9/glycerol/ADC (preferred) and SMB plus a carbon source. Tolerates moderate to high salinity (2.5 to 5.0%). Non-motile aerobe. Does not reduce nitrate or nitrite. Positive for catalase, pyrrolidonyl arylamidase, α -glucosidase and urease activities and negative for β -glucuronidase, β -galactosidase, *N*-acetyl- β -glucosaminidase and alkaline phosphatase activities and gelatin and aesculin hydrolysis. Oxidation and utilization of substrates and the composition of whole-cell fatty acids are listed in Tables 1, 2 and 3 and Supplementary Table S2.

The type strain, czh-117^T (=ATCC BAA-1375^T =CIP 109271^T), and reference strains czh-107 (=ATCC BAA-1374 =CIP 109270) and czh-132 (=ATCC BAA-1376 =CIP 109272) originate from soil from an urban park in Honolulu, HI, USA.

Description of *Mycobacterium rufum* sp. nov.

Mycobacterium rufum (ru'fum. L. neut. adj. *rufum* ruddy or red, pertaining to the colony pigmentation of the type strain).

Medium to long rods that show weak uptake of Gram stain. Forms colonies within 7 days that are large, round, raised, dry with a smooth border and orange pigmented in light or dark. Grows well at 28 °C. Growth media include LB, TSB (preferred), Middlebrook 7H9/glycerol/ADC and SMB plus a carbon source. Non-motile aerobe. Does not tolerate salinity (2.5–5.0%). Reduces nitrate. Displays positive reactions in tests for catalase, α -glucosidase, aesculin hydrolysis and urease and negative reactions for β -glucuronidase, β -galactosidase, *N*-acetyl- β -glucosaminidase, gelatin hydrolysis, alkaline phosphatase and pyrrolidonyl arylamidase. Oxidation and utilization of substrates and the composition of whole-cell fatty acids are listed in Tables 1, 2 and 3 and Supplementary Table S2.

The type strain, JS14^T (=ATCC BAA-1377^T =CIP 109273^T), was isolated from PAH-contaminated soil of a former oil-gasification company site in Hilo, HI, USA.

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