Smaragdicoccus niigatensis gen. nov., sp. nov., a novel member of the suborder *Corynebacterineae*

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A polyphasic taxonomic approach was applied to determine the taxonomic position of a hydrocarbon-degrading actinomycete, strain Hou_blue^T, which was isolated from soil samples collected from an oil spring in Niigata, Japan. The results of 16S rRNA and *gyrB* gene sequence comparisons indicated that strain Hou_blue^T represented a novel lineage in the suborder *Corynebacterineae*. Colonies were malachite green-like in colour on 1/10 trypticase soy agar and the cell morphology was coccoid in all growth phases. The cell-wall diamino acid and sugar indicated chemotype IV and variation A1γ. The sugars of the peptidoglycan were glycolated. The polar lipids were composed of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and some unspecified glycolipids. The organism contained two novel cyclic forms of menaquinone, smaragdiquinone A-8(H₄, ω-cycl) and smaragdiquinone B-8(H₄, dicycl). The major fatty acids were *cis*-9-18 : 1 (34.46 %) and 16 : 0 (25.1 %). Small amounts of 10-methyl-branched fatty acids were also present (10-methyl-17 : 0, 0.17 %), but not tuberculostearic acid (10-methyl-18 : 0), which has been shown to be present in all nocardiae. Gas-chromatographic analysis of the mycolic acid revealed a carbon-chain length of C₄₃–C₄₉. The DNA G + C content was 63.7 mol%. On the basis of phenotypic and phylogenetic distinctness, the organism is proposed to represent a novel genus and species, *Smaragdicoccus niigatensis* gen. nov., sp. nov., with the type strain Hou_blue^T (= MBIC 06267^T = DSM 44881^T).

Strain Hou_blue^T was isolated from an enrichment culture containing petroleum-contaminated soil, obtained from the ground around a spurt of petroleum at Nishiyama-cho in Niigata, Japan, by using hexadecane as the sole carbon source. On W-medium (Peng *et al.*, 2003) malachite green-like, round colonies of 1–3 mm diameter were obtained after incubation for 7 days in a hexadecane-saturated atmosphere at 25 °C. Subcultivation was done on 1/10 trypticase soy agar (TSA; Difco) at 30 °C for 7 days. On this medium, strain Hou_blue^T was able to grow at 4–37 °C, but not at 45 °C. Growth at 30 °C was also observed on

International Streptomyces Project (ISP) medium 2 (Daigo), medium 6 and TSA.

Gram-staining was performed as described by Gerhardt *et al.* (1994). Cell morphology was observed under a Nikon phase-contrast microscope at ×1000, with cells grown for 14 days at 30 °C on 1/10 TSA. The size of the cells was determined by using a scanning electron microscope with specimens that were fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer containing several drops of 4 % osmium tetroxide for 1 h at room temperature. Suspensions were transferred to the surface of a polylysine-coated glass plate, and dehydrated in ethanol and *t*-butyl alcohol at room temperature. Preparations were sputter-coated with Pt/Pd on aluminium mounts and observed with a Hitachi S2500 scanning electron microscope (see Supplementary Fig. S1 in IJSEM Online). The 16S rRNA and *gyrB* genes were analysed as described by Katsuta *et al.* (2005). Sequences were aligned by using CLUSTAL_X (Thompson *et al.*, 1997) based on aligned sequences supplied by RDP-II release 9 (Cole *et al.*,

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strain Hou_blue^T are AB243007 and AB243008, respectively.

A scanning electron micrograph of cells of strain Hou_blue^T, a phylogenetic tree based on *gyrB* gene sequences and structures of the novel quinones present in strain Hou_blue^T are available as supplementary figures in IJSEM Online.

2005). A neighbour-joining phylogenetic tree based on genetic distances calculated using the Kimura two-parameter model was constructed with MEGA version 3.1 (Kumar *et al.*, 2004). PHYL (version 2.4.4; Guindon & Gascuel, 2003) was used to construct a maximum-likelihood tree. The phylogenetic relationship based on the GTR substitution model was analysed, and a tree was built up from the neighbour-joining tree as the starter tree. The robustness of the topology was evaluated by using the maximum-likelihood method with bootstrap analysis based on 100 replications (Fig. 1). The 16S rRNA gene sequence of strain Hou_blue^T was a continuous stretch of 1478 bp. Sequence similarity calculations after a BLAST search against GenBank indicated that the closest relatives of strain Hou_blue^T were *Nocardia africana* (95.0 %), *Nocardia araoensis* (94.5 %),

Nocardia arthritidis (94.8 %), *Nocardia beijingensis* (94.7 %), *Nocardia elegans* (94.9 %), *Nocardia takedensis* (94.5 %) and *Nocardia paucivorans* (94.5 %). Twelve of 16 signature nucleotides of the 16S rRNA gene in *Nocardiaceae* (Stackebrandt *et al.*, 1997) were identified in the 16S rRNA gene sequence of strain Hou_blue^T. The *gyrB* gene-based phylogenetic tree is available as Supplementary Fig. S2 in IJSEM Online.

For analysis of cell-wall amino acids and sugars, cell walls were prepared from approximately 100 mg (dry weight) bacterial cells, as described by Schleifer & Kandler (1972). The amino acids in an acid hydrolysate of the cell walls were identified by using two-dimensional descending chromatography on cellulose TLC plates (Tokyo Kasei), following the

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences. The sequence of *Corynebacterium glutamicum* was used to root the tree. Numbers at branch points are bootstrap percentages based on 100 replications. Bar, 0.02 substitutions per nucleotide position.

method of Harper & Davis (1979), and by HPLC as their phenylthiocarbamoyl derivatives, with LC-10AD HPLC apparatus (Shimadzu) equipped with a Wakopak WS-PTC column (Wako Pure Chemical Industries, 1989). Whole-cell sugars were analysed using the method of Becker *et al.* (1964). The hydrolysate of whole cells of strain Hou_blue^T contained the sugars arabinose, galactose, glucose and fucose, whereas the hydrolysate of the cell wall contained *meso*-diaminopimelic acid, alanine, glutamic acid and glycine in a molar ratio of approximately 0.5:3:1:2. This combination of cell-wall diamino acid and sugar indicated chemotype IV *sensu* Lechevalier & Lechevalier (1970) and variation A1 γ of Schleifer & Kandler (1972). The murein acyl type was determined by using a modification of the colorimetric method of Uchida & Aida (1977). In contrast to the original procedure, the whole-cell hydrolysate was neutralized by being passed through an ion-exchange column (Analytichem Bond Elut SCX; Varian). As expected for a member of the family *Nocardiaceae*, the sugars of the peptidoglycan were glycolated. Polar lipids were extracted, examined by two-dimensional TLC and identified using published procedures (Minnikin *et al.*, 1977). The polar lipids were composed of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and some unspecified glycolipids. This pattern matched quite well with those reported by Minnikin *et al.* (1977) for members of the family *Nocardiaceae*. To determine the DNA base composition, DNA was extracted and purified by using a Genomic-tip and buffer set (Qiagen). Total DNA was digested with P1 nuclease using a GC kit (Yamasa Shoyu). The DNA G+C content was measured using HPLC (Tamaoka & Komagata, 1984).

Quinones were extracted with 5 ml chloroform/methanol (2:1, v/v) from 10 mg freeze-dried cells that were grown in 300 ml 1/10 trypticase soy broth (TSB). After centrifugation, the supernatant was evaporated to dryness and suspended in 10 μ l acetone. An aliquot of the acetone solution was analysed with an HPLC/PDA/APCI-MS/MS system using a C30 Develosil column (1.0 mm i.d. $\times$ 150 mm; Nomura Chemical). The crude extract was eluted at a rate of 0.1 ml min⁻¹ with methanol/2-propanol (8:2, v/v). Mass spectra were monitored in the mass range *m/z* 200–1200 with the LCQ Advantage system. The capillary temperature was set at 150 °C, the APCI vaporizer temperature was held at 400 °C, the capillary voltage was optimized to 23 V and the nitrogen gas flow was set to 28 (arbitrary units). HPLC peaks showing the characteristic UV spectra of naphthoquinones (Kroppenstedt, 1985) were identified by comparison with the MS data of authentic menaquinones (MK-7, 8, 9 and 10). Two major menaquinones were detected by using the HPLC/PDA/APCI-MS/MS system. One had the same patterns of UV and MS/MS as those of MK-8(H₄, ω -cycl), which is diagnostic for *Nocardia*, the other having a UV pattern of MK type with MS/MS data that have not been found previously. Several milligrams of these two quinones were obtained from a 10 l

liquid culture, and instrumental analyses using NMR and HRMS indicated the structures to be those of novel cyclic menaquinones, smaragdiquinone A-8(H₄, ω -cycl) and smaragdiquinone B-8(H₄, dicycl) (Adachi and others, unpublished data). Smaragdiquinones A and B are abbreviated to SQA and SQB. SQA-8(H₄, ω -cycl) is different from MK-8(H₄, ω -cycl) in the structure of the ω -ring (structures are available as Supplementary Fig. S3 in IJSEM Online).

For analysis of the fatty acid and mycolic acid composition, strain Hou_blue^T was grown on TSA for 4 days at 28 °C. For other chemotaxonomic analyses, cells were grown in TSB for 4 days at 28 °C on a rotary shaker, harvested by centrifugation and washed twice with distilled water and lyophilized. Fatty acid methyl esters were obtained by saponification, methylation and extraction, using the method of Miller (1982) with minor modifications (Kuykendall *et al.*, 1988), from 40 mg cells that had been scraped from Petri dishes. The fatty acid methyl ester mixture was separated by using the Sherlock Microbial Identification System (MIS; Microbial ID) consisting of a 5980 gas chromatograph fitted with a 5 % phenylmethyl silicone capillary column (0.2 mm $\times$ 25 m), a flame-ionization detector, a 7673A automatic sampler and a Kayak XA computer (Hewlett Packard). The peaks were integrated automatically and the fatty acids present and their content (%) were calculated using the MIS software. The gas chromatographic parameters were as follows: carrier gas, ultra-high-purity hydrogen; column head pressure, 60 kPa; injection volume, 2 μ l; column split ratio, 100:1; septum purge, 5 ml min⁻¹; column temperature, 170–270 °C at 5 °C min⁻¹; injection port temperature, 250 °C; and detector temperature, 300 °C. The fatty acid pattern of strain Hou_blue^T did not match any of the patterns found previously for members of the genus *Nocardia*. Interestingly, only very small amounts of 10-methyl-branched fatty acids were present in strain Hou_blue^T (10-methyl-17:0, 0.17 %) and, unlike all other nocardiae, tuberculostearic acid (10-methyl-18:0) was not present. In addition, a significant amount of iso-16:0 (8.12 %) was synthesized by strain Hou_blue^T; in contrast, this fatty acid, if present at all, has only been found previously in trace amounts among nocardiae and all other members of the suborder *Corynebacterineae*. Half of the fatty acid methyl ester extract (0.3 ml) was mixed with 0.1 ml *N*-methyl-*N*-(trimethylsilyl)-heptafluorobutyramide (MSHFBA) and trimethylchlorosilane (TMCS) [10:1 (v/v); Macherey and Nagel], yielding trimethylsilylated derivatives (TMS-MAME) of the -OH group of mycolic acid methyl ester (MAME) at room temperature (Klatte *et al.*, 1994). A TMS-MAME analysis was carried out using a 5890A gas chromatograph (Hewlett Packard) equipped with a flame-ionization detector and a 7673A automatic sampler (Hewlett Packard). Chromatographic data were sampled and processed by using a Vectra Series 4 computer (Hewlett Packard) with MIS version 3.3 software package (Microbial ID). The TMS-MAME analysis was performed using a 12 m HT5 column (SGE) of 0.32 mm i.d. and 0.1 μ m film

thickness using H₂ as the carrier gas and a split ratio of 50 : 1. The oven temperature was increased from 210 to 400 °C, at 10 °C min⁻¹, the final temperature being held for 10 min. The MIS apparatus was calibrated with a series of saturated TMS-MAMEs derived from *Corynebacterium bovis* DSM 20582^T, *Rhodococcus erythropolis* DSM 43066^T, *Rhodococcus rhodnii* DSM 43336^T and *Gordonia sputi* DSM 44019. The chain length of the mycolic acids of strain Hou_blue^T was shorter (C₄₃–C₄₉) than that found in other nocardiae (C₅₀–C₆₂), the pattern being very complex, i.e. highly unsaturated mycolic acids. It was therefore difficult to identify the numbers of the double bond. A detailed comparison of the chemotaxonomic data of strain Hou_blue^T with those of other mycolic acid-containing genera is given in Table 1. Results of phenotypic characterization by using Biolog and API ZYM assays are given in the species description.

The results of 16S rRNA and *gyrB* gene sequence analyses, the cell wall and peptidoglycan characterization and the polar lipid analysis indicated that strain Hou_blue^T belonged to the family *Nocardiaceae*. The profiles of quinones, fatty acids and mycolic acids indicated that strain Hou_blue^T should be classified as representing a novel genus and species of the family *Nocardiaceae*, for which the name *Smaragdicoccus niigatensis* gen. nov., sp. nov. is proposed.

Description of *Smaragdicoccus* gen. nov.

Smaragdicoccus [Sma.rag.di.coc'cus. L. n. *smaragdus* malachite; N.L. masc. n. *coccus* (from Gr. masc. n. *kokkos*), grain; N.L. masc. n. *Smaragdicoccus* malachite(-coloured) coccus].

Gram-positive, non-spore-forming cocci. The type of cell-wall diamino acid and sugar is chemotype IV and variation A1γ. Sugars of the peptidoglycan are glycolated. Polar lipids are composed of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and some unspecified glycolipids. Carbon-chain length of mycolic acid is C₄₃–C₄₉. Major fatty acids are straight-chain saturated and unsaturated fatty acids, whereas 10-methyl-branched fatty acids are present in trace amounts or absent. Two menaquinone species, SQA-8(H₄, ω-cycl) and SQB-8(H₄, dicycl), are present. 16S rRNA and *gyrB* gene sequence analyses indicate that the genus is a member of the family *Nocardiaceae*. The type species is *Smaragdicoccus niigatensis*.

Description of *Smaragdicoccus niigatensis* sp. nov.

Smaragdicoccus niigatensis (ni.i.ga.ten'sis. N.L. masc. adj. *niigatensis*, pertaining to the Niigata Prefecture of Japan, the source of the soil from which the organism was isolated).

Table 1. Chemotaxonomic markers of *Smaragdicoccus* gen. nov. and other genera of the suborder *Corynebacterineae*

Taxa: 1, strain Hou_blue^T; 2, *Nocardia*; 3, *Skermania*; 4, *Rhodococcus*; 5, *Gordonia*; 6, *Williamsia*; 7, *Mycobacterium*; 8, *Tsukamurella*; 9, *Segniliparus*; 10, *Turicella*; 11, *Dietzia*; 12, *Corynebacterium*; 13, *Corynebacterium amycolatum*. Chemotaxonomic characteristics summarized by Kämpfer *et al.* (1999) were updated. The whole-cell lysate of all organisms contained meso-diaminopimelic acid. +, Present; –, absent; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9*	10	11	12	13
Acyl type†	G	G	G	G	G	G	G	G	ND	ND	A	A	ND
Major menaquinone	SQA-8(H ₄ , ω-cycl), SQB-8(H ₄ , dicycl)	MK-8 (H ₄ , ω-cycl)	MK-8 (H ₄ , ω-cycl)	MK-8 (H ₂)	MK-9 (H ₂)	MK-9 (H ₂)	MK-9 (H ₂)	MK-9 (H ₂)	ND	MK-10, MK-11	MK-8 (H ₂)	MK-8 (H ₂)	MK-9
PE‡	+	+	+	+	+	+	+	+	ND	ND	–	–§	ND
Fatty acid composition	S, U	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, ¶	S, U
Mycolate size (no. of carbons)#	43–49	50–62	58–64	34–54	54–66	50–56	70–90	64–78	ND	–	34–38	22–36	–
DNA G+C content (mol%)	63.7	64–72	68	63–73	63–69	64–65	70–72	67–68	68–72	65–72	73	51–67	ND

*Data from Butler *et al.* (2005).

†A, Acetylated muramic acid; G, glycolated muramic acid.

‡Phosphatidylethanolamine.

§Present in *C. bovis* and *Corynebacterium urealyticum* (Kämpfer *et al.*, 1999).

||S, Saturated fatty acid; T, tuberculostearic acid; U, unsaturated fatty acid.

¶Tuberculostearic acid present in *Corynebacterium ammonii*, *C. bovis*, *Corynebacterium minutissimum*, *C. urealyticum* and *Corynebacterium variabile* (Kämpfer *et al.*, 1999).

#Number of carbon atoms in the mycolic acid molecule, range of homologous series of mycolic acids.

Cells are coccoid without branching ($0.86 \times 0.86 \mu\text{m}$). Utilizes the following carbon sources after incubation for 14 days at 30°C : D-fructose, D-glucose, sodium n-butyrate and hexadecane. After prolonged incubation (1 month) growth occurs with sucrose. Better growth is observed on W-medium containing 0.1 than 1% of these carbon sources. The following carbon sources are not used for growth: L-arabinose, *myo*-inositol, D-mannitol, L-rhamnose, raffinose and D-xylose. Positive for activities of esterase (C4), esterase lipase (C8), lipase (C4), leucine arylamidase and naphthol-AS-BI-phosphohydrolase (API ZYM).

The DNA G + C content of the type strain is 63.7 mol%. The type strain is Hou_blue^T (= MBIC 06267^T = DSM 44881^T), which was isolated from soil samples from an oil spring in Niigata, Japan.

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