A novel actinomycete, strain WS-26<sup>T</sup>, was isolated from beach sand on the coast of Jeju Island, Republic of Korea, and subjected to a polyphasic taxonomic characterization. The organism produced reddish orange-coloured substrate mycelium and white aerial mycelium, both of which fragmented into rod-shaped elements. A neighbour-joining tree, based on 16S rRNA gene sequence studies, revealed that the isolate formed a distinct branch at the base of a cluster that included Nocardia carnea, N. flavorosea, N. pigrifrangens, N. sienata and N. testacea. This branching pattern was also found in a tree constructed using the maximum-likelihood method, but was not supported by the maximum-parsimony method. Levels of 16S rRNA gene sequence similarity between the isolate and its phylogenetic neighbours ranged from 96·5 to 97·8%. Chemotaxonomic properties, such as the principal amino acid of peptidoglycan, predominant menaquinone and polar lipids, supported its assignment to the genus Nocardia. On the basis of phenotypic and phylogenetic data, the organism was different from Nocardia species with validly published names. The name Nocardia harenae sp. nov. is proposed, with WS-26<sup>T</sup> (=KCCM 42317<sup>T</sup> = NRRL B-24459<sup>T</sup>) as the type strain.

Morphological characteristics were investigated by light microscopy. Cells were incubated on oatmeal agar for 14 days at 30°C. A coverslip with growing cells attached was observed directly using a light microscope. The degree of growth and the level of pigmentation were investigated on ISP 2, ISP 3 and ISP 4 agar media (Shirling & Gottlieb, 1966), and tryptic soy broth (Difco) supplemented with agar (TSBA) after incubation at 30°C for 14 days. Strain WS-26<sup>T</sup> showed good growth on ISP 2 medium and TSBA, but only moderate growth on ISP 3 and ISP 4 media. Abundant, branched substrate mycelium, which was reddish orange in colour, was produced on all of the media tested and fragmented into irregular rod-shaped elements. The intensity of pigmentation was stronger on the nutrient-rich media, namely ISP 2 medium and TSBA. Diffusible pigments were not produced on most of the media tested. White, fragmenting aerial mycelium was produced to a moderate level on ISP 3 and ISP 4 media, but was sparse on ISP 2 medium and TSBA.

Genomic DNA was extracted and purified as described previously (Lee, 2006). The 16S rRNA gene of strain WS-26<sup>T</sup> was amplified by PCR as described previously (Lee et al., 2000a) and purified using the Wizard PCR Prep DNA purification system (Promega). The resultant 16S rRNA gene was sequenced directly using an ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 3730xl; Applied Biosystems). An almost complete 16S rRNA gene sequence
of strain WS-26$^T$ was determined in this study and comprised a continuous stretch of 1397 nt. The sequence was subjected to a BLAST search (http://www.ncbi.nlm.nih.gov/), which indicated that the organism was a member of the family Nocardiaceae. Assignment to this family was also supported by the presence of a set of family-specific signature nucleotides in the 16S rRNA gene sequence (Stackebrandt et al., 1997).

CLUSTAL_X (Thompson et al., 1997) was used to align the sequence with available reference sequences retrieved from GenBank. Multiple alignments were optimized manually according to 16S rRNA secondary structure. Phylogenetic analyses were carried out using three treeing algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A total of 1257 unambiguous aligned positions present in all strains between nt 91 and 1392 (nucleotide numbering according to Escherichia coli positions; Brosius et al., 1978) was used for phylogenetic analyses. A phylogenetic tree was reconstructed by the neighbour-joining method from evolutionary distances calculated by the method of Jukes & Cantor (1969). Tsukamurella paurometabola DSM 20162$^T$ (GenBank accession no. X80628) was used as an outgroup. The reliability of the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985).

The 16S rRNA gene sequence was compared with those of the 59 type strains of Nocardia species with validly published names at the time of writing. A neighbour-joining tree (Fig. 1), based on 16S rRNA gene sequence studies, showed that the organism formed the deepest branch outside the Nocardia carnea cluster, which included Nocardia carnea, N. flavorosea, N. pigrifrangens, N. sienata and N. testacea, with a bootstrap value of 41%. This phylogenetic placement was also found in the tree constructed using the maximum-likelihood method, although strain WS-26$^T$ formed a cluster with Nocardia transvalensis when the maximum-parsimony method was employed (not shown). Levels of 16S rRNA gene sequence similarity between strain WS-26$^T$ and members of the genus Nocardia ranged from 94.6 to 97.9%. The organism showed high 16S rRNA gene sequence similarity values to closely associated neighbours, namely N. carnea DSM 43398$^T$ (97.8%), Nocardia cyriacigeorgica DSM 44484$^T$ (97.8%), N. flavorosea JCM 3332$^T$ (97.7%), N. pigrifrangens JCM 11884$^T$ (96.5%), N. sienata IFM 10088$^T$ (97.7%) and N. testacea IFM 0937$^T$ (97.8%). Among loosely associated members, Nocardia abscessus DSM 44432$^T$, Nocardia asiatica IFM 0245$^T$, Nocardia brasiliensis ATCC 19296$^T$ and N. transvalensis DSM 43405$^T$ (sequence similarity values of 97.7–97.9%) were equidistantly related to strain WS-26$^T$, representing slightly higher values than those between the isolate and its phylogenetic neighbours. The unstable topology of the isolate in the tree (Fig. 1), as well as the somewhat low sequence similarities to the type strains of the genus Nocardia, indicated that strain WS-26$^T$ merits classification as a novel genomic species without the need to perform DNA–DNA hybridization experiments.

The following chemotaxonomic analyses were performed as indicated: analysis of mycolic acids (Minnikin et al., 1980), phospholipids (Minnikin et al., 1977), lipoquinones (Kroppenstedt, 1985), the isomer of diaminopimelic acid (Staneck & Roberts, 1974) and the acyl type (Uchida & Aida, 1984) of the peptidoglycan and the whole-cell sugar composition (Saddler et al., 1991). Cellular fatty acids were extracted from biomass obtained in trypticase soy broth (Difco) incubated at 30°C for 3 days. Fatty acid methyl esters were prepared by alkaline methanolysis (Minnikin, 1988) and analysed using a gas chromatograph (model 6850; Agilent) as described previously (Lee et al., 2000b). The G+C content of the DNA was determined by HPLC as described previously (Mesbah et al., 1989). The results of chemical analyses, particularly the predominant lipoquione, revealed that the organism could be assigned to the genus Nocardia (Collins et al., 1977). Whole-cell hydrolysates contained arabinose and galactose as characteristic sugars and meso-diaminopimelic acid as the principal diamino acid (type IV cell wall; Lechevalier & Lechevalier, 1970). The glycan moiety of the murein was N-acetylated. The predominant lipoquione was a tetrahydrogenated menaquione with eight isoprene units in which the two terminal isoprene moieties were cyclized [MK-8(H4, 8-cyclo)]. This type of lipoquione is found in all species of the genus Nocardia (Collins et al., 1977). Mycolic acids were present, with an Rf value of 0.47 on TLC. The phospholipids detected were phosphatidylethanolamine, phosphatidylinositol and diphasphatidylglycerol (phospholipid type II pattern; Lechevalier et al., 1977). Cellular fatty acid profiles were characterized by the predominance of hexadecanoic (C16:0; 25.3%), tubulostearic (10-methyl-C18:0; 16.7%) and cis-9-octadecenoic (cis9-C18:1; 12.7%) acids, followed by 16-methylheptadecanoic (i-C18:0; 8.4%) and octadecanoic (C18:0; 6.3%) acids and 2-hydroxytetradecanoic (2-OH-C14:0; 5.2%) acids. Minor amounts of other saturated, iso- and anteiso-branched fatty acids were also detected in the whole-cell methanolysates.

The following physiological tests were performed using previously described methods (Lee, 2006): production of hydrogen sulfide, nitrate reduction, gelatin liquefaction, hydrolysis of casein, aesculin and starch and degradation of adylene, hypoxanthine, D,L-tyrosine and xanthine. For hydrolysis and decomposition tests, strain WS-26$^T$ was grown on YE-SW agar as the basal medium. Gram staining and oxidase tests were carried out using the method of MacFaddin (1980). Catalase activity was determined with a 3% (v/v) hydrogen peroxide solution. Urease activity was determined by a colour change in Bacto urea broth (Difco). NaCl tolerance was tested by growth on yeast extract/malt extract agar (ISP 2 medium) supplemented with NaCl at final concentrations of 0–9% (w/v). Growth was tested at temperatures of 4, 10, 20, 30, 37 and 45°C and at pH 4.1–10.1 (at intervals of 1.0) using YE-SW agar as the basal medium. Utilization of carbohydrates, alcohols and organic acids as sole carbon and energy sources was tested using ISP 9 medium (Shirling & Gottlieb, 1966) containing...
filter-sterilized carbon sources at final concentrations of 1% (w/v) for carbohydrates and alcohols and 0·1% (w/v) for organic acids. The results of morphological, cultural and physiological tests are given in the species description and in Table 1. Strain WS-26T utilized a limited range of carbohydrates and organic acids as the sole carbon source. Interestingly, strain WS-26T showed growth over a broad pH range of 4·1–10·1. The ability to grow at pH 4·1 may be a key characteristic for identification of strain WS-26T, as most nocardial strains do not grow under acidic conditions.

The type strains of *N. abscessus*, *N. asiatica*, *N. brasiliensis* and *N. transvalensis*, which showed high sequence similarity values of 97·8–97·9%, albeit with loosely associated relationships in the neighbour-joining tree (Fig. 1), could be differentiated from the isolate on the basis of cultural and physiological features, as well as the origin of isolation (Lechevalier, 1989; Chun et al., 1998; Yassin et al., 2000, 2003; Kageyama et al., 2004a, b; Wang et al., 2004). The phenotypic properties that differentiated the isolate from its phylogenetic relatives are given in Table 1. In particular, strain WS-26T was readily distinguished from *N. transvalensis*, which formed a phylogenetic cluster with the isolate in the tree constructed using the maximum-parsimony method (data not shown), by pigmentation of the substrate mycelium (Lechevalier, 1989), in the utilization of D-xylose, meso-erythritol, D-sorbitol and acetate, in the decomposition of hypoxanthine and in nitrate reduction (Table 1). The results of polyphasic taxonomic characterization strongly suggest that the organism be classified as a novel species of the genus *Nocardia*. The name *Nocardia harenae* sp. nov. is proposed, with WS-26T as the type strain.
Form a well-developed and branched substrate mycelium that fragments into irregular rod-shaped elements. The colour of the substrate mycelium is reddish orange. White aerial mycelium is produced to a moderate level on ISP 3 (referring to the isolation of the type strain from beach sand). Description of *Nocardia harenae* sp. nov.

*Nocardia harenae* (ha.re.nae. L. gen. n. harenae of sand, referring to the isolation of the type strain from beach sand).

Forms a well-developed and branched substrate mycelium that fragments into irregular rod-shaped elements. The colour of the substrate mycelium is reddish orange. White aerial mycelium is produced to a moderate level on ISP 3 (referring to the isolation of the type strain from beach sand). The type strain is strain WS-26T (=KCCM 42317^T^ = NRRL B-24459^T^), isolated from beach sand on the coast of Jeju Island, Republic of Korea.

Acknowledgements

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References


Table 1. Phenotypic characteristics that distinguish strain WS-26^T^ from its phylogenetic relatives in the genus *Nocardia*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
<th>Strain 4</th>
<th>Strain 5</th>
<th>Strain 6</th>
<th>Strain 7</th>
<th>Strain 8</th>
<th>Strain 9</th>
<th>Strain 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colours of mycelium</td>
<td>Reddish orange</td>
<td>Yellow</td>
<td>Orange to tan</td>
<td>Cream to peach</td>
<td>ND</td>
<td>Orange</td>
<td>Orange</td>
<td>Pale yellow</td>
<td>Orange to brick</td>
<td>Pale tan to cream</td>
</tr>
<tr>
<td>Utilization of:</td>
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<td>Acetate</td>
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<td>+</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Adonitol</td>
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<td>D-Glucose</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>L-Rhamnose</td>
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<td>D-Sorbitol</td>
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<tr>
<td>D-Xylose</td>
<td>+</td>
<td>ND</td>
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<td>–</td>
<td>–</td>
<td>ND</td>
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<td>Decomposition of:</td>
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<td>Casein</td>
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<td>Hypoxanthine</td>
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<td>Urea</td>
<td>+</td>
<td>V</td>
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<td>Nitrate reduction</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ND</td>
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<td>Growth at 45 °C</td>
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<td>Beach sand</td>
<td>Clinical samples</td>
<td>Clinical samples</td>
<td>Soil and air</td>
<td>Clinical samples</td>
<td>Soil</td>
<td>Contaminated agar plate</td>
<td>Clinical samples</td>
<td>Clinical samples</td>
<td>Clinical samples</td>
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</tbody>
</table>

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Nocardia harenae sp. nov.


