Phylogenetic Analysis of a New LL-Diaminopimelic Acid-Containing Coryneform Bacterium from Herbage, Nocardioides plantarum sp. nov.

M. D. COLLINS,* 1 S. COCKCROFT, 2 AND SALLY WALLBANKS 1

Department of Microbiology, Institute of Food Research, Reading Laboratory, Reading RG6 2EF, 1 and Department of Microbiology, University of Reading, Reading RG6 2AF, 2 United Kingdom

The 16S rRNA gene sequence of a previously undescribed LL-diaminopimelic acid-containing coryneform bacterium isolated from herbage was determined in order to clarify the taxonomic position of this organism. A comparative sequence analysis revealed that the bacterium represents a new line of descent within the genus Nocardioides. On the basis of the results of a phylogenetic analysis and the phenotypic distinctiveness of the organism, a new species, Nocardioides plantarum, is proposed. The type strain is NCIMB 12834.

Relatively few species of coryneform bacteria which contain walls based on LL-diaminopimelic acid (L-L-A∞pm) have been described (13). The currently recognized L-L-A∞pm-containing taxa include species of the genus Nocardioides (Nocardioides simplex, Nocardioides albus, Nocardioides jenseni, Nocardioides fastidiosa), Terrabacter tumescens, Aeromicrobium erythreum, and some propionibacteria (Propionibacterium innocuum). However, there is a considerable amount of evidence that L-L-A∞pm-containing coryneform bacteria may be relatively common in nature (14) and that the vast majority of these organisms have not been described. During an investigation of the genetic diversity of L-L-A∞pm-containing coryneforms, we determined the almost complete 16S (small-subunit) rRNA gene sequence of a previously undescribed thiamine-requiring bacterium that was originally isolated by Grainger (12) from herbage. A comparison of this sequence with the sequences of other LL-A∞pm-containing coryneform and related taxa indicated that the herbage bacterium represents a new line of descent within the genus Nocardioides, for which the name Nocardioides plantarum sp. nov. is proposed.

MATERIALS AND METHODS

Cultures and cultivation. Strains Grainger J70 1 (T = type strain) and Grainger J6, which were originally isolated from herbage (12), were received from J. M. Grainger, University of Reading, Reading, United Kingdom. These organisms were routinely cultivated in EYPB broth and EYPB agar (see reference 4 for compositions of media) at 25°C and were maintained at 10°C.

Biocatalytic and physiological characterization. The ability to grow in the presence of various concentrations of NaCl was determined in EYPB broth containing 5, 7.5, and 10% (wt/vol) NaCl. Nitrate reduction was examined by using EYPB medium in which the mineral base contained 1 g of potassium nitrate per liter but no ammonium sulfate. Catalase and oxidase tests were performed with growth from 3-day cultures grown on EYPB agar, and the results were detected with Frazier’s reagent (10). Starch (0.2%, wt/vol) hydrolysis was examined by using the same medium and was detected with Lugol’s iodine solution. Hydrolysis of Tween 20 (1%, vol/vol) was determined in EYPB agar supplemented with 0.1 g of CaCl2 per liter. The incubation temperature in all cases was 25°C.

Carbon utilization tests were performed in basal medium containing (per liter of mineral base E [16]) 0.2 g of Bacto Yeast Extract (Difco, West Molesey, United Kingdom), 12 g of agar no. 1 (Oxoid, Basingstoke, United Kingdom), 2 μg of vitamin B 12, 10 mg of sodium glutamate, and 10 mg of methionine. Water-soluble substrates were filter sterilized, whereas insoluble substrates were autoclaved at 115°C for 5 min. With the exception of phenol (0.025 g/liter), thymine (5 g/liter), and uracil (5 g/liter), the substrates were tested at a concentration of 1 g/liter of basal medium. Plates were incubated at 25°C and were examined after 7, 10, and 21 days. A positive result was recorded when the growth on medium containing substrate was unequivocally greater than the growth on basal medium.

Menaquinone analysis. Menaquinones were extracted from dry cells, purified by thin-layer chromatography, and characterized as described previously (3).

DNA studies. Chromosomal DNA was isolated from wet packed cells as described by Pitcher et al. (17). DNA base composition was estimated by thermal denaturation in standard saline citrate as described by Garvie (11), using DNA from Escherichia coli K-12 (51.5 mol% G + C) as the standard. Chromosomal hybridization was performed as described by Farrow et al. (7).

Determination of 16S rRNA gene sequences. The 16S rRNA gene was amplified by a PCR in which universal primers pA (positions 8 to 28; E. coli numbering) and pH* (positions 1542 to 1522) (1) were used. Approximately 2 μg of chromosomal DNA was amplified in a total volume of 100 μl containing 1 U of Taq polymerase (Advanced Biotechnologies, Ltd., West Hampstead, United Kingdom). The reaction involved 36 cycles of denaturation at 92°C for 2 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1.5 min. The DNA was extracted with chloroform and purified with a Magic DNA clean-up system (Promega Corp.) according to the manufacturer’s instructions. A qualitative analysis of the DNA fragments was performed by agarose gel electrophoresis. The sequence of the amplified product was determined with α-35S-labeled dATP and a Sequenase version 2.0 sequencing kit (Cambridge Bioscience, Cambridge, United Kingdom). The reaction products were separated on 55-cm wedge-shaped (0.2- to 0.6-mm) 8% acrylamide–7 M urea gels at 55°C with an LKB...
Utilization of the following compounds as sole carbon and energy sources:

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N. plantarum</th>
<th>N. albus</th>
<th>N. lacteus</th>
<th>N. fastidiosa</th>
<th>N. jensenii</th>
<th>N. simplex</th>
<th>T. tumescens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerial mycelium</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

\[ \text{Utilization of the following compounds as sole carbon and energy sources:} \]

- Azelate
- Fructose
- Histamine
- Malonate
- Maltose
- L-Proline
- Phenol
- L-Rhamnose
- Tetradecane
- Thymine
- Ureil
- Suberate
- Growth in glucose-mineral salts medium
- Growth in glucose-mineral salts medium containing thiamine

Nucleotide sequence accession number. The 16S rRNA gene sequence of N. plantarum sp. nov. from other Nocardioides species and T. tumescens

**RESULTS AND DISCUSSION**

The cells of the two \( \text{L}_{2}\text{Apm} \)-containing strains which we studied were gram-positive, nonmotile, irregular, short rods. Coccoid forms were also formed. Unlike N. albus and Nocardioides lacteus, neither substrate mycelium nor primary mycelium was produced. The two strains were similar to Nocardioides spp. and T. tumescens in being obligately aerobic, but they more closely resembled the latter taxon in requiring thiamine for growth in glucose-mineral salts medium. A wide range of organic compounds were utilized as sole carbon and energy sources by the herbage isolates (see species description below). Both strains were readily distinguished from previously described Nocardioides spp. because they did not utilize azelate and substrate, while the inability of these strains to utilize thymine and uracil distinguished them from T. tumescens. Other characteristics that are useful for differentiating the previously undescribed strains from Nocardioides spp. and T. tumescens are shown in Table 1.

The 16S rRNA gene of strain Grainger J70\(^2\) was amplified in vitro, and its nucleotide sequence was determined. The derived 16S rRNA primary structure is shown in Fig. 1. The sequence determined in this study has been deposited in the GenBank data library under accession number X69973.

**TABLE 1. Characteristics useful for differentiating \( N. \) plantarum sp. nov. from other Nocardioides species and \( T. \) tumescens**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N. plantarum</th>
<th>N. albus</th>
<th>N. lacteus</th>
<th>N. fastidiosa</th>
<th>N. jensenii</th>
<th>N. simplex</th>
<th>T. tumescens</th>
</tr>
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</table>

- N. plantarum
- N. albus
- N. lacteus
- N. fastidiosa
- N. jensenii
- N. simplex
- T. tumescens

\[ * \text{V, variable.} \] 
\[ \text{ND, not determined.} \]
TABLE 2. Levels of sequence similarity for a 1,266-nucleotide region of 16S rRNAs of Nocardioides species and other related taxa

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<th>Species</th>
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<th>Aeromicrobium erythreum</th>
<th>Arthrobacter globiformis</th>
<th>Micrococcus luteus</th>
<th>Mycobacterium bovis</th>
<th>Nocardioides albus</th>
<th>Nocardioides fastidiosa</th>
<th>Nocardioides jenensis</th>
<th>Nocardioides luteus</th>
<th>Nocardioides plantarum</th>
<th>Nocardioides simplex</th>
<th>Strepoverticillium cinnamoneum</th>
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* EMBL-GenBank nucleotide sequence accession numbers.

other Nocardioides spp. (N. albus NCIMB 11454T, 22%; N. jenensis NCIMB 9770T, 27%; N. luteus NCIMB 11455T, 21%; N. simplex NCIMB 8929T, 29%).

The 16S rRNA sequence of strain Grainger J70T was aligned and compared with the 16S rRNA sequences of other high-G+C-content actinomycetes available from the GenBank data library. Sequence similarity calculations revealed that the previously undescribed 16S-A,pm-containing coryneform bacterium exhibited an affinity for the "Nocardioides" branch of nonsporulating actinomycetes. Table 2 shows the similarity values for a region that is approximately 1,266 nucleotides long (from positions 60 to position 1346; E. coli numbering system) for the new sequence and the sequences of members of the Nocardioides branch and related species. The highest levels of sequence similarity were observed with Nocardioides species (2, 3) and the recently described organism A. erythreum (15). A significantly lower level of relatedness was observed with the 16S-A,pm-containing coryneform bacterium T. tumescens (2). A tree depicting the position of the previously undescribed bacterium within the Nocardioides branch is shown in Fig. 2.

The 16S rRNA sequence analysis clearly showed that the previously undescribed 16S-A,pm-containing coryneform bacterium isolated from herbage is phylogenetically a member of the genus Nocardioides. Its 16S rRNA evolutionary distances from other Nocardioides species were approximately 6.5 to 8%, values which are great enough to warrant description as a distinct species in the genus. The proposal to place this organism in a new species is supported by its phenotypic distinctiveness. This organism requires thiamine as the only growth factor in glucose-mineral salts media (12). This requirement, together with the inability to utilize azelate and substrates, distinguishes the herbage bacterium from all other Nocardioides species. Therefore, we propose that the 16S-A,pm-containing coryneform bacterium should be classified in the genus Nocardioides as Nocardioides plantarum sp. nov.

Description of Nocardioides plantarum sp. nov. Nocardioides plantarum (plan. tar' um. M. L. gen. pl. n. plantarum, of plants). Cells are gram-positive, short rods; coccolid forms also occur. Neither substrate mycelium nor primary mycelium is formed. Colonies are circular, entire, convex, smooth, shiny, and nonpigmented. Cells are nonmotile. Growth occurs at 5 and 30°C, but not at 37°C or in the presence of 5% NaCl. Strictly aerobic. Thiamine is required for growth. A wide range of organic compounds are utilized as sole or principal carbon and energy sources for growth; these compounds include cellubiose, fructose, glycerol, glucose, maltose, melezitose, l-rhamnose, sucrose, trehalose, d-xylose, isobutyrate, propionate, fumarate, and succinate. l-Arabinose, d-galactose, histidine, histamine, malonate, phenol, azelate, substrate, crotone, pantate, thymine, and uracil are not utilized. Some strains utilize l-proline and tetracade. Casein, Tween 40, Tween 60, Tween 80, and gelatin are hydrolyzed. Cellulose, chitin, urea, starch, and hippurate are not hydrolyzed. Nitrate is not reduced. The cell wall murein contains l-A,pm as the diamino acid. MK-8(H4) is the main menaquinone component; a minor amount of MK-7(H4) is also produced. The cellular fatty acids are composed of saturated, unsaturated, and iso-, anteiso-, and 10-methyl-branched types. Small amounts of 2-hydroxy fatty acids are also synthesized. The G+C content of the DNA is 69 mol% (as determined by the thermal denaturation method). Isolated from herbage. The type strain is NCIMB 12834 (= Grainger J70).
Description of the type strain. The description of the type strain corresponds to that of the species given above, except that L-proline and tetradecane are not utilized.

ACKNOWLEDGMENTS

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REFERENCES