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

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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 (LL-A₂pm) have been described (13). The currently recognized LL-A₂pm-containing taxa include species of the genus *Nocardioides* (*Nocardioides simplex*, *Nocardioides albus*, *Nocardioides jensenii*, *Nocardioides fastidiosa*), *Terrabacter tumescens*, *Aeromicrobium erythreum*, and some propionibacteria (*Propionibacterium innocuum*). However, there is a considerable amount of evidence that LL-A₂pm-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 LL-A₂pm-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-A₂pm-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^T (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.

Biochemical 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 (5). Casein (1%, wt/vol) and gelatin (0.4%, wt/vol) hydrolysis tests were performed 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 Tweens (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 B₁₂, 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) 6% acrylamide–7 M urea gels at 55°C with an LKB

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

Characteristic	<i>N. plantarum</i>	<i>N. albus</i>	<i>N. luteus</i>	<i>N. fastidiosa</i>	<i>N. jensenii</i>	<i>N. simplex</i>	<i>T. tumescens</i>
Aerial mycelium	—	+	+	—	—	—	—
Utilization of the following compounds as sole carbon and energy sources:							
Azelaate	—	+	+	+	+	+	+
Cellobiose	+	+	+	+	—	—	+
Fructose	+	+	+	+	—	—	+
Histamine	—	V ^a	—	V	ND ^b	+	—
Malonate	—	+	+	—	—	+	+
Maltose	+	+	+	—	+	—	+
L-Proline	V	+	+	V	—	+	+
Phenol	—	—	+	—	ND	+	—
L-Rhamnose	+	+	—	—	+	—	V
Tetradecane	V	+	+	+	—	+	—
Thymine	—	—	—	—	+	+	+
Uracil	—	—	—	—	+	+	+
Suberate	—	+	+	+	+	+	V
Growth in glucose-mineral salts medium	—	+	+	—	+	+	—
Growth in glucose-mineral salts medium containing thiamine	+	—	—	—	—	—	+

^a V, variable.^b ND, not determined.

Macrophor 2010 sequencing unit (Pharmacia, Milton-Keynes, United Kingdom) operated at 50 W per gel. The sequences generated were aligned with sequences of other actinomycetes obtained from the GenBank (EMBL) data library, and similarity values were determined by using the Wisconsin Molecular Biology Package (6). Nucleotide substitution rates (K_{nuc} values) were calculated, and an unrooted phylogenetic tree was produced by using the algorithm of Fitch and Margoliash (8, 9).

Nucleotide sequence accession number. The 16S rRNA gene sequence determined in this study has been deposited in the GenBank data library under accession number X69973.

RESULTS AND DISCUSSION

The cells of the two LL-A₂pm-containing strains which we studied were gram-positive, nonmotile, irregular, short rods. Coccoid forms were also formed. Unlike *N. albus* and *Nocardioides luteus*, 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 azelaate and suberate, 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^T was amplified in vitro, and its nucleotide sequence was determined. The derived 16S rRNA primary structure is shown in Fig. 1. The sequence of a short fragment (approximately position 50 to position 500, which included diagnostic variable regions V1 through V3) of the 16S rRNA gene of strain Grainger J6, which belonged to the same phenotypic group (12) as Grainger J70^T, was also determined; this sequence was found to be identical to the sequence of strain Grainger J70^T, demonstrating that there is a close genotypic affinity between these two herbage isolates. These findings were confirmed by the results of chromosomal DNA-DNA pairing experiments, which revealed that there is 80% complementarity between strains Grainger J70^T and Grainger J6, indicating that these organisms are members of the same genomic species. We observed significantly lower levels of DNA relatedness between strain Grainger J70^T and

TTCTACGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGCGTCTTGCAAGTCGAGCGGTAAGGCACCTTCGGTGTACACGAGCGGCGAACGGGTGA
GTAACACGTGAGTAATCTGCCCTTCACTCTGGGATAAGCACTGGAAAGGGTGTCTAATACCGGATACGACGCACTCACGCATGTGATGTGTGGAACT
TTTGTGGGTGGGGATGTGCTCGCGCCTATCAGCTTGTGGTGGGGTAATGGCCTACCAAGGCTTCGACGGGTAGCCGGCTGAGAGGGTGACCGGCCA
CACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAAGCAGTGGGGAATATTGGACAATGGGCGGAAGCCTGTATCCAGCAACCGCCGCTGAGGGATG
ACGGCCTTCGGGTTGTAAACCTCTTTAGTATCGACGAAGCGNNGCTGTGNGTGGTGACGGTATAGGTACAGAAGAAGCACCGGCCAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGGTGCAGCGTTGTCCGGAATTATTGGGCGTAAAGGCTCGTAGGTGGTTTGTGCGCTCGGGAGTGAAACACTGGGCTTAACC
GAGTGCTTGTCTTCGATACGGGAGACTTGAGGCATTGAGGGGAGAACGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTG
CGAAGGCGGTTCTCTGGCAATGTTCTGACGCTGAGGAGCGAAAGTGTGGGGAGCGAACAGGATTAGATACCTTGTAGTCCACACCGTAAACGTTGGGC
GCTAGGTGTGGGCGNATTCACGGGTTCCGTGCCGAGCTAACGCATTAAAGCGCCCGCCTGGGGAGTACGGCGCAAGGCTAAACTCAAAGGAATTG
ACGGGGGCCCGCACAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATACACCTGCCGCTCAGAGATGGGG
CTTCTTTTGNNGGTGTACAGGTGGTGCATGGCTGTCTGCTAGCTCGTGTCTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTCGTTCTATGTT
GCCAGCACGTCTTTCGGGATGGTGGGACTCATAGGAGACTGCCGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCTTATGTCC
AGGCTTCACGCATGCTACATGGCCGTTACAAAGGCTGCGATCCCGTGAAGGGGAGCGAATCCCAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCA
ACTGACCCCATGAAGTCGGAGTCGCTAGTNATCCGAGATCAGCAACGCTGCGGTGAATACGTTCCCGGCCTTGACACACCGNNGTCANGTCACGAA
AGTCGGCAACACCGAAGCNAGTGGCCTAACCACTTGTGTGGGGGAGCTGTGCAAGGTGGGCTGGCGATTGGGACGAAGTCGGTAACAAGGTAGCCG
TACCGGAAGGTG

FIG. 1. Derived 16S rRNA gene sequence of *N. plantarum* Grainger J70^T.

TABLE 2. Levels of sequence similarity for a 1,266-nucleotide region of 16S rRNAs of *Nocardioide*s species and other related taxa

Species	Accession no. ^a	% Sequence similarity											
		<i>Aeromicrobium erythreum</i>	<i>Arthrobacter globiformis</i>	<i>Micrococcus luteus</i>	<i>Mycobacterium bovis</i>	<i>Nocardioide</i> s <i>albus</i>	<i>Nocardioide</i> s <i>fastidiosa</i>	<i>Nocardioide</i> s <i>jensenii</i>	<i>Nocardioide</i> s <i>luteus</i>	<i>Nocardioide</i> s <i>plantarum</i>	<i>Nocardioide</i> s <i>simplex</i>	<i>Streptomyces lividans</i>	<i>Streptovercillium cinnamomeum</i>
<i>Aeromicrobium erythreum</i>	M37200												
<i>Arthrobacter globiformis</i>	M23411	89.2											
<i>Micrococcus luteus</i>	M38242	89.3	95.2										
<i>Mycobacterium bovis</i>	X55589	88.4	86.8	87.4									
<i>Nocardioide</i> s <i>albus</i>	X53189	91.3	87.7	88.1	87.3								
<i>Nocardioide</i> s <i>fastidiosa</i>	X53189	98.0	88.9	88.9	88.5	91.8							
<i>Nocardioide</i> s <i>jensenii</i>	X53214	93.4	88.2	88.7	88.7	94.7	93.7						
<i>Nocardioide</i> s <i>luteus</i>	X53212	91.2	87.6	88.1	87.4	98.8	91.8	94.2					
<i>Nocardioide</i> s <i>plantarum</i>	X69973	91.8	86.7	87.8	87.6	92.2	92.3	92.4	92.2				
<i>Nocardioide</i> s <i>simplex</i>	X53213	92.3	89.7	89.5	89.6	95.7	92.7	94.4	95.4	93.5			
<i>Streptomyces lividans</i>	Y00484	89.6	88.8	89.8	86.8	88.1	89.4	89.4	87.7	87.9	88.6		
<i>Streptovercillium cinnamomeum</i>	X53165	89.7	88.7	89.2	87.4	86.7	88.5	88.5	86.5	88.3	88.8	95.8	
<i>Terrabacter tumescens</i>	X53215	89.2	92.1	92.3	89.3	89.3	88.7	89.3	89.4	89.0	90.0	89.4	88.4

^a EMBL-GenBank nucleotide sequence accession numbers.

other *Nocardioide*s spp. (*N. albus* NCIMB 11454^T, 22%; *N. jensenii* NCIMB 9770^T, 27%; *N. luteus* NCIMB 11455^T, 21%; *N. simplex* NCIMB 8929^T, 29%).

The 16S rRNA sequence of strain Grainger J70^T 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 LL-A₂pm-containing coryneform bacterium exhibited an affinity for the "*Nocardioide*s 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 *Nocardioide*s branch and related species. The highest levels of sequence similarity were observed with *Nocardioide*s species (2, 3) and the recently described organism *A. erythreum* (15). A significantly lower level of relatedness was observed with the LL-A₂pm-containing coryneform bacterium *T. tumescens* (2). A tree depicting the position of the previously undescribed bacterium within the *Nocardioide*s branch is shown in Fig. 2.

The 16S rRNA sequence analysis clearly showed that the

FIG. 2. Phylogenetic network showing the position of *N. plantarum* within the genus *Nocardioide*s. Noc., *Nocardioide*s.

previously undescribed LL-A₂pm-containing coryneform bacterium isolated from herbage is phylogenetically a member of the genus *Nocardioide*s. Its 16S rRNA evolutionary distances from other *Nocardioide*s 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 suberate, distinguishes the herbage bacterium from all other *Nocardioide*s species. Therefore, we propose that the LL-A₂pm-containing coryneform bacterium should be classified in the genus *Nocardioide*s as *Nocardioide*s *plantarum* sp. nov.

Description of *Nocardioide*s *plantarum* sp. nov. *Nocardioide*s *plantarum* (plan. tar' um. M. L. gen. pl. n. *plantarum*, of plants). Cells are gram-positive, short rods; coccoid 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 cellobiose, 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, suberate, crotonate, pantoate, thymine, and uracil are not utilized. Some strains utilize L-proline and tetradecane. 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 LL-A₂pm 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.

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REFERENCES

1. Boddingtonhaus, B., J. Wolters, W. Heikens, and E. C. Böttger. 1990. Phylogenetic analysis and identification of six different serovars of *Mycobacterium intracellulare* at the molecular level. *FEMS Microbiol. Lett.* **70**:197–204.
2. Collins, M. D., M. Dorsch, and E. Stackebrandt. 1989. Transfer of *Pimelobacter tumescens* to *Terrabacter* gen. nov. as *Terrabacter tumescens* comb. nov. and *Pimelobacter jensenii* to *Nocardioides* as *Nocardioides jensenii* comb. nov. *Int. J. Syst. Bacteriol.* **39**:1–6.
3. Collins, M. D., and E. Stackebrandt. 1989. Molecular taxonomic studies on some LL-diaminopimelic acid-containing coryneforms from herbage: description of *Nocardioides fastidiosa* sp. nov. *FEMS Microbiol. Lett.* **57**:289–294.
4. Cure, G. L., and R. M. Kieddie. 1973. Methods for the morphological examination of aerobic coryneform bacteria. *Soc. Appl. Bacteriol. Tech. Ser.* **7**:123–135.
5. Davis, G. H. G. 1955. The classification of lactobacilli from the human mouth. *J. Gen. Microbiol.* **13**:481–493.
6. Devereux, J., P. Haeberli, and D. Smithies. 1984. A comprehensive set of sequence programs for the VAX. *Nucleic Acids Res.* **12**:387–395.
7. Farrow, J. A. E., D. Jones, B. A. Phillips, and M. D. Collins. 1983. Taxonomic studies on some group D streptococci. *J. Gen. Microbiol.* **129**:1423–1432.
8. Felsenstein, J. 1982. Numerical methods for inferring evolutionary trees. *Q. Rev. Biol.* **57**:379–404.
9. Fitch, W. M., and E. Margoliash. 1967. Construction of phylogenetic trees: a method based on mutation distances as estimated from cytochrome *c* sequences is of general applicability. *Science* **155**:279–284.
10. Frazier, W. C. 1926. A method for detecting changes in gelatin due to bacteria. *J. Infect. Dis.* **39**:302.
11. Garvie, E. I. 1978. *Streptococcus raffinolactis* Orla-Jensen and Hansen, a group N streptococcus found in raw milk. *Int. J. Syst. Bacteriol.* **28**:190–193.
12. Grainger, J. M. 1963. Studies on coryneform bacteria from soil and herbage. Ph.D. thesis. University of Reading, Reading, United Kingdom.
13. Kieddie, R. M., and G. L. Cure. 1977. The cell wall composition and distribution of free mycolic acids in named strains of coryneform bacteria and in isolates from various natural sources. *J. Appl. Bacteriol.* **42**:229–252.
14. Kieddie, R. M., B. G. S. Leask, and J. M. Grainger. 1966. A comparison of coryneform bacteria from soil and herbage: cell wall composition and nutrition. *J. Appl. Bacteriol.* **29**:17–43.
15. Miller, E. S., C. R. Woese, and S. Brenner. 1991. Description of the erythromycin-producing bacterium *Arthrobacter* sp. strain NRRL B-3381 as *Aeromicrobium erythreum* gen. nov., sp. nov. *Int. J. Syst. Bacteriol.* **41**:363–368.
16. Owens, J. D., and R. M. Kieddie. 1969. The nitrogen nutrition of soil and herbage coryneform bacteria. *J. Appl. Bacteriol.* **32**:338–347.
17. Pitcher, D. G., N. A. Saunders, and R. J. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* **8**:151–156.