Janibacter melonis sp. nov., isolated from abnormally spoiled oriental melon in Korea

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Two Gram-positive bacterial strains, CM2104^T and CM2110, isolated from the inner part of abnormally spoiled oriental melon (*Cucumis melo*) in Korea, were subjected to a polyphasic taxonomic study. The cell-wall peptidoglycan of strains CM2104^T and CM2110 contained *meso*-diaminopimelic acid as the diagnostic diamino acid. The predominant menaquinone was MK-8(H_4). The major fatty acids detected in the two strains were iso-$C_{16:0}$, $C_{17:1}\omega8c$ and $C_{18:1}\omega9c$ or $C_{17:0}$. The DNA G+C content of the two strains was 73 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that the strains formed a coherent cluster with a clade comprising two *Janibacter* species, *Janibacter limosus* and *Janibacter terrae*. Strains CM2104^T and CM2110 exhibited a 16S rRNA gene sequence similarity value of 99·7 % and a mean DNA–DNA relatedness level of 89 %. Strains CM2104^T and CM2110 showed 16S rRNA gene sequence similarity levels of 97·8–98·4 % to the type strains of *J. limosus* and *J. terrae*. DNA–DNA relatedness between strains CM2104^T and CM2110 and the type strains of these two *Janibacter* species was 7–11 %. On the basis of the phenotypic and phylogenetic data and genomic distinctiveness, strains CM2104^T and CM2110 should be placed within the genus *Janibacter* as members of a novel species, for which the name *Janibacter melonis* sp. nov. is proposed. The type strain is CM2104^T (=KCTC 9987^T=DSM 16063^T=JCM 12321^T).

The genus *Janibacter* was proposed by Martin *et al.* (1997) with a single species, *Janibacter limosus*. Subsequently, two further species, *Janibacter terrae* (Yoon *et al.*, 2000) and *Janibacter brevis* (Imamura *et al.*, 2000), have been described. However, *J. brevis* has recently been recognized as a later heterotypic synonym of *J. terrae* (Lang *et al.*, 2003). All *Janibacter* species have been isolated from environmentally polluted samples. In this study, we describe two strains, CM2104^T and CM2110, which were isolated from the inner part of abnormally spoiled oriental melon (*Cucumis melo*) in Korea. These isolates were considered to be *Janibacter*-like strains based on 16S rRNA gene sequence comparison. Accordingly, the aim of the present study was to determine the exact taxonomic positions of strains CM2104^T and CM2110 with a combination of phenotypic typing, detailed phylogenetic analysis based on 16S rRNA gene sequence analysis and genomic relatedness.

Abnormally spoiled oriental melon collected from a cultivation field was used as the source for isolation of endophytic bacteria. Strains CM2104^T and CM2110 were isolated by a standard dilution plating technique on nutrient agar (NA; Difco) at 30 °C. The strains were cultivated on rich (R) medium (Martin *et al.*, 1997) or brain heart infusion (BHI) medium at 30 °C to investigate their morphological and physiological characteristics. Cell mass for analyses of the cell wall, menaquinones and polar lipids and for DNA extraction was obtained after cultivation for 2 days in liquid R medium at 30 °C. Reference strains included *J. limosus* DSM 11140^T and *J. terrae* KCCM 80001^T. For fatty acid methyl ester (FAME) analysis, cell mass of strains CM2104^T and CM2110 was obtained from agar plates after cultivation for 7 days at 30 °C on solid R medium and trypticase soy agar (TSA; Difco). Cell morphology was

Published online ahead of print on 17 September 2004 as DOI 10.1099/ijs.0.63167-0.

Abbreviations: FAME, fatty acid methyl ester; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CM2104^T and CM2110 are AY522568 and AY522569, respectively.

examined by light microscopy (Nikon E600) and transmission electron microscopy (TEM). The presence of flagella was examined by TEM using cells from exponentially growing cultures. Growth at various temperatures and various NaCl concentrations was measured in liquid R and BHI media. Growth under anaerobic conditions was determined in an anaerobic chamber with anaerobically prepared R agar. Oxidase activity was determined by using 1 % (w/v) *p*-aminodimethylaniline oxalate. Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Hydrolysis of aesculin, H_2S production, methyl-red and Voges–Proskauer reactions, and nitrate reduction were determined as described by Lanyi (1987). Hydrolysis of casein, gelatin, hypoxanthine, starch, Tween 80, tyrosine, urea and xanthine was determined as described by Cowan & Steel (1965). Utilization of various substrates as sole carbon and energy sources was determined as described by Gordon & Mihm (1957). Acid production from carbohydrates was determined according to Hugh & Leifson (1953). Other physiological tests were performed with the API 20E system (bioMérieux).

The isomer type of the diamino acid in the cell-wall peptidoglycan was analysed using TLC according to the method described by Komagata & Suzuki (1987). Menaquinones were analysed as described by Komagata & Suzuki (1987), using reversed-phase HPLC. For quantitative analysis of the cellular fatty acid composition, a loop of cell mass was harvested and FAMEs were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Polar lipids were extracted using the procedures described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), with the exception that ribonuclease T1 was used together with ribonuclease A. The DNA G+C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

16S rRNA genes were amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene was performed as described by Yoon *et al.* (2003). Alignment of sequences was carried out with CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5′ and 3′ ends of the alignment were omitted from further analysis. Phylogenetic trees were inferred using three tree-making algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods in the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were calculated with the algorithm of Jukes & Cantor (1969) with the DNADIST program. The stability of relationships was assessed by a bootstrap analysis based on 1000 resamplings of the neighbour-joining dataset using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. Of the values obtained, the highest and lowest values for each sample were excluded; DNA–DNA relatedness values are the mean of the remaining three values.

Morphological, cultural, physiological and biochemical characteristics of strains CM2104^T and CM2110 are given in the species description (see below) and in Table 1, together with those of *J. limosus* and *J. terrae*. Strains CM2104^T and CM2110 contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell wall. The predominant isoprenoid quinone was tetrahydrogenated menaquinone with eight isoprene units [MK-8(H_4)]. The major fatty acids of strains CM2104^T and CM2110 were iso-$C_{16:0}$, $C_{17:1}\omega8c$ and $C_{18:1}\omega9c$ or $C_{17:0}$ (Table 2). There were differences in the proportions of some fatty acids when the two strains were grown on solid R medium and TSA (Table 2). These cellular fatty acid profiles were similar to those of *J. limosus* DSM 11140^T and *J. terrae* KCCM 80001^T, although there were differences in the proportions of some fatty acids. The cellular polar lipids found in the two strains were phosphatidylglycerol, diphosphatidylglycerol and phosphatidylinositol. The DNA G+C content of strains CM2104^T and CM2110 was 73 mol%.

Almost complete 16S rRNA gene sequences of strains CM2104^T and CM2110 comprised 1479 nt, corresponding to positions 28 and 1524 of the *Escherichia coli* 16S rRNA gene sequence. The 16S rRNA gene sequences of strains CM2104^T and CM2110 differed in four positions (0·3 % difference). Sequence comparison revealed that the two strains had the highest 16S rRNA gene sequence similarity to members of the family *Intrasporangiaceae*, particularly to the genus *Janibacter*. The sequences of strains CM2104^T and CM2110 contained 32 signature nucleotides associated with the family *Intrasporangiaceae* (Stackebrandt & Schumann, 2000). The two sequences contained 28 of 29 signature nucleotides defined for the genus *Janibacter* as described by Maszenan *et al.* (2000); the two strains had the matching base pair G–C at positions 140–223 instead of G–T or A–T as given by Maszenan *et al.* (2000). In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strains CM2104^T and CM2110 formed a coherent cluster, which was supported by a bootstrap resampling value of 100 %, and joined the phylogenetic clade comprising *J. limosus* and *J. terrae* (Fig. 1). This tree topology was also found in the tree generated with the maximum-parsimony algorithm (data not shown). Strains CM2104^T and CM2110 exhibited 16S rRNA gene sequence similarity levels of 97·8–98·4 % to *J. limosus* DSM 11140^T and *J. terrae* KCCM 80001^T, 96·9–98·0 % to *Knoellia sinensis* DSM 12331^T and *Knoellia subterranea* DSM 12332^T and less than 97·0 %

Table 1. Phenotypic characteristics of *J. limosus*, *J. terrae* and *Janibacter melonis* sp. nov.

Data for reference species were taken from Martin *et al.* (1997) (*J. limosus*) or Yoon *et al.* (2000) and Lang *et al.* (2003) (*J. terrae*). +, Positive; −, negative; W, weakly positive; ND, not determined. Data in parentheses are for the type strain. All species were positive for Gram-stain, catalase, hydrolysis of casein, starch and Tween 80, utilization of DL-malate, nitrate reduction and growth in the presence of 2, 4 and 6 % NaCl. All species were negative for spore formation, motility, hydrolysis of urea, production of indole, Voges–Proskauer and methyl red reactions, and acid production from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, D-trehalose and D-xylose.

Characteristic	*J. limosus*	*J. terrae*	*J. melonis*
Colour of colonies	Cream or pale cream	White, cream or yellowish	Cream
Morphology	Cocci, rods	Cocci or short rods	Cocci
Oxidase	−	− (W)	−
H_2S production	+	V (+)	−
Hydrolysis of:			
Aesculin	−	−	+
Gelatin	+	+	−
Utilization of:			
Acetate	V (+)	V (+)	+
Benzoate	−	V (+)	−
Citrate	+	− (ND)	+
Formate	+	V (ND)	+
Succinate	−	− (ND)	−
Growth in the presence of:			
8 % NaCl (R medium)	+	(+)	−
8 % NaCl (BHI medium)	+	+	V (−)
10 % NaCl (R medium)	+	(−)	−
10 % NaCl (BHI medium)	−	+	−
Growth on R medium at:			
37 °C	−	+	+
40 °C	−	+	−
Growth on BHI medium at:			
37 °C	+	(+)	+
40 °C	−	(−)	+

to other species (Fig. 1). The mean level of DNA–DNA relatedness between strains CM2104^T and CM2110 was 89 %, when each of their DNAs was separately used as labelled DNA probe. Strains CM2104^T and CM2110 exhibited levels of DNA–DNA relatedness of 7–10 % and 7–11 % to *J. limosus* DSM 11140^T and *J. terrae* KCCM 80001^T, respectively.

Strains CM2104^T and CM2110 are representatives of bacteria that cause abnormal spoilage in oriental melon, which results in great economic losses in melon cultivation. Detailed data regarding the abnormal spoilage caused by strains CM2104^T and CM2110 will be published elsewhere. It may be important to determine the exact taxonomic positions of strains CM2104^T and CM2110 for rapid identification and diagnosis of these isolates. The result of 16S rRNA gene sequence analysis showed that strains CM2104^T and CM2110 exhibited closest phylogenetic affiliation to the genus *Janibacter* (Fig. 1). Chemotaxonomic data obtained from the strains were also most similar to those of the genus *Janibacter* (Stackebrandt & Schumann, 2000; Yoon *et al.*, 2000; Groth *et al.*, 2002). The *meso*-diaminopimelic acid type clearly distinguished strains CM2104^T and CM2110 from members of the genus *Ornithinicoccus*, which contain L-ornithine, and the other genera of the *Intrasporangiaceae*, namely *Intrasporangium*, *Terrabacter* and *Terracoccus*, which contain LL-diaminopimelic acid (Groth *et al.*, 1999; Stackebrandt & Schumann, 2000). Differences in major fatty acid contents also distinguished strains CM2104^T and CM2110 from members of the genera *Ornithinicoccus*, *Intrasporangium*, *Terrabacter* and *Terracoccus*, which contain iso-$C_{15:0}$ as the major fatty acid, and the genus *Knoellia*, which contain unsaturated fatty acids (particularly $C_{17:1}$ and $C_{18:1}$) as minor components (Groth *et al.*, 1999, 2002; Maszenan *et al.*, 2000; Yoon *et al.*, 2000) (Table 2). In comparison with *Tetrasphaera*, strains CM2104^T and CM2110 differed in the amounts of anteiso-branched fatty acids, which were minor components in strains CM2104^T and CM2110 and comprised more than 32 % in the genus *Tetrasphaera* (Maszenan *et al.*, 2000; Hanada *et al.*, 2002). Accordingly, both phylogenetic and chemotaxonomic data indicate that strains CM2104^T and CM2110 are members of the genus *Janibacter*.

Strains CM2104^T and CM2110 showed almost identical phenotypic characteristics, except growth in liquid BHI

Table 2. Percentage cellular fatty acid compositions of *J. limosus* DSM 11140^T, *J. terrae* KCCM 80001^T and strains CM2104^T and CM2110 collected from solid R medium and TSA

Data for *J. limosus* and *J. terrae* were taken from Yoon *et al.* (2000). Fatty acids that represent less than 0·5 % in all rows were omitted. −, Not detected.

Fatty acid	*J. limosus* DSM 11140^T		*J. terrae* KCCM 80001^T		Strain CM2104^T		Strain CM2110	
	R	TSA	R	TSA	R	TSA	R	TSA
Straight-chain								
$C_{15:0}$	1·2	1·9	0·8	0·5	0·6	12·1	0·5	7·4
$C_{16:0}$	7·5	2·5	3·0	0·8	2·9	2·4	2·1	2·8
$C_{17:0}$	14·2	16·3	1·4	0·8	5·9	13·4	6·3	13·5
$C_{18:0}$	7·3	2·8	1·0	0·4	3·1	1·5	2·3	1·7
$C_{19:0}$	0·9	0·7	−	−	0·6	0·5	0·4	0·5
Branched								
iso-$_{14:0}$	0·3	0·9	1·1	1·1	0·5	2·8	1·2	4·9
iso-$C_{15:0}$	0·5	0·6	9·0	4·6	5·3	5·1	8·5	5·3
anteiso-$C_{15:0}$	−	−	2·6	1·1	0·2	1·2	0·2	1·2
iso-$C_{16:0}$	13·1	19·1	22·8	31·9	20·6	16·7	23·8	18·7
iso-$C_{17:0}$	1·3	0·9	5·8	6·2	8·6	4·1	9·0	3·3
iso-$C_{17:1}\omega 9c$	−	−	2·9	4·7	0·5	−	0·7	−
anteiso-$C_{17:1}\omega 9c$	−	−	1·1	1·1	−	−	−	−
anteiso-$C_{17:0}$	1·1	0·5	14·6	9·1	3·0	2·2	1·6	2·2
iso-$C_{18:0}$	1·5	1·8	0·4	0·7	1·1	1·1	1·2	1·0
Unsaturated								
$C_{15:1}\omega 6c$	−	0·2	0·2	0·3	−	1·4	0·2	1·0
$C_{17:1}\omega 8c$	17·5	28·7	7·1	11·0	14·1	20·0	14·5	21·6
$C_{18:1}\omega 7c$	1·0	0·5	1·1	0·8	1·4	−	1·0	0·7
$C_{18:1}\omega 9c$	22·6	9·5	13·3	12·8	22·5	7·6	17·4	7·7
10-Methyl								
$C_{17:0}$	1·7	5·5	1·1	4·4	0·3	0·4	0·3	−
$C_{18:0}$	1·1	1·2	0·2	0·3	−	0·3	−	−
Summed features*								
3	4·0	1·9	8·8	4·1	5·9	2·1	4·3	2·4
6	2·5	3·8	0·5	1·8	3·1	3·7	3·6	2·8

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega 7c$. Summed feature 6 contained $C_{19:1}\omega 9c$ and/or $C_{19:1}\omega 11c$.

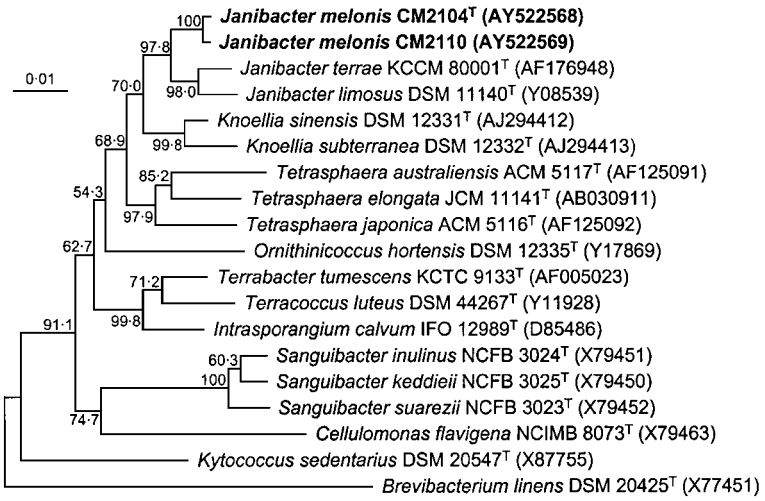

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strains CM2104^T and CM2110, *Janibacter* species and some other related taxa based on 16S rRNA gene sequences. Bar, 0·01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at the branching points.

medium and hydrolysis of casein (Table 1). The two strains were also phylogenetically similar, showing four nucleotide differences (0·3 % difference) in their 16S rRNA gene sequences (Fig. 1). DNA–DNA relatedness indicates that strains CM2104^T and CM2110 are members of the same genomic species (Wayne *et al.*, 1987). Strains CM2104^T and CM2110 are differentiated from other *Janibacter* species by several physiological characteristics, such as temperature for growth, NaCl tolerance and hydrolysis of certain substrates (Table 1). DNA–DNA relatedness values between strains CM2104^T and CM2110 and the type strains of two *Janibacter* species were far below the threshold value (70 %) suggested for species delineation in current bacterial systematics (Wayne *et al.*, 1987). Genomic distinctiveness, together with differential phenotypic properties and 16S rRNA gene sequence similarity data, justify a taxonomic discrimination of strains CM2104^T and CM2110 from the two accepted *Janibacter* species. Therefore, on the basis of the data presented, strains CM2104^T and CM2110 should be placed in the genus *Janibacter* as members of a novel species, for which the name *Janibacter melonis* sp. nov. is proposed.

Description of *Janibacter melonis* sp. nov.

Janibacter melonis [me.lo′nis. L. gen. n. *melonis* of melon (*Cucumis melo*), referring to the fruit from which the organisms were isolated].

Cells are aerobic, non-spore-forming, non-motile cocci, 0·8–1·0 μm in diameter. Gram-positive. Non-acid-fast. Colonies are smooth, circular, convex, glistening, cream-coloured and 1·5–3·0 mm in diameter after 7 days incubation on solid R medium; those of the type strain wrinkle as cultures age. Neither substrate nor primary mycelium is formed. Optimal temperature for growth is 30 °C. Grows occurs at 10 °C but not at 4 °C. Grows occurs at 40 °C in liquid BHI medium but not in liquid R medium. Optimal pH for growth is around 7·0; growth occurs at pH 5·5 but not at pH 5·0. Tweens 20, 40 and 60 and tyrosine are hydrolysed. Hypoxanthine and xanthine are not hydrolysed. Acid is not produced from adonitol, *myo*-inositol, D-melezitose, melibiose or sucrose. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid as the diagnostic diamino acid. The predominant menaquinone is MK-8(H_4). The major fatty acids are iso-$C_{16:0}$, $C_{17:1}\omega8c$ and $C_{18:1}\omega9c$ or $C_{17:0}$. The cellular polar lipids are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylinositol. The DNA G+C content is 73 mol% (determined by HPLC). Other characteristics are given in Table 1.

The type strain, CM2104^T (=KCTC 9987^T=DSM 16063^T =JCM 12321^T), was isolated from the inner part of abnormally spoiled oriental melon in Korea.

Acknowledgements

J.-H. Y. and H. B. L. contributed equally to this work. This work was supported by the 21C Frontier programme of Microbial Genomics and Applications (grant MG02-0401-001-1-0-0) from the Ministry of Science and Technology (MOST) of the Republic of Korea.

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