***Janibacter terrae* sp. nov., a bacterium isolated from soil around a wastewater treatment plant**

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A bacterial strain, CS12^T, which was isolated from soil around a wastewater treatment plant, was subjected to a polyphasic taxonomic study using phenotypic characterizations and genetic methods. The cell wall of strain CS12^T contains meso-diaminopimelic acid as the diamino acid but no arabinose and galactose. The predominant menaquinone is MK-8(H₄). Mycolic acids are absent. Strain CS12^T has a cellular fatty acid profile containing saturated, unsaturated, branched and 10-methyl fatty acids. The major fatty acids are iso-C_{16:0}, C_{18:1} ω9c and anteiso-C_{17:0}. The G+C content is 69 mol%. A phylogenetic tree based on 16S rDNA sequences showed that strain CS12^T forms an evolutionary lineage within the radiation enclosing the members of the family *Intrasporangiaceae* and, in particular, a coherent cluster with *Janibacter limosus* DSM 11140^T. The level of 16S rDNA similarity between strain CS12^T and *J. limosus* DSM 11140^T is 98.7%. The phenotypic characteristics and DNA–DNA relatedness data indicate that strain CS12^T should be distinguished from *J. limosus* DSM 11140^T. Therefore, on the basis of the data presented, a new species of the genus *Janibacter*, *Janibacter terrae*, is proposed. The type strain of the new species is strain CS12^T (= KCCM 80001^T = JCM 10705^T).

Keywords: *Janibacter terrae* sp. nov., actinomycete, polyphasic taxonomy

INTRODUCTION

The genus *Janibacter* was recently proposed by Martin *et al.* (1997) with one species, *Janibacter limosus*. This species was isolated from a 1-year-old sludge sample collected from a wastewater treatment plant. The genus *Janibacter* has been placed in the family *Intrasporangiaceae*, together with the genera *Intrasporangium*, *Terrabacter*, *Terracoccus* and *Sanguibacter* (Stackebrandt *et al.*, 1997; Prauser *et al.*, 1997). Since there is currently only one validly described species in the genus *Janibacter*, the finding of new species may be very meaningful from the point of view of biological diversity of this genus. Recently, we have isolated many strains that are important for the bioremediation of toxic aromatic compounds from a wastewater treatment plant in Korea and some of these strains have been found to be new actinomycete species (Cho *et al.*, 1998; Yoon *et al.*, 1999). We have also isolated

many strains from soil around a wastewater treatment plant. Among them, one isolate (strain CS12^T) was considered to be a *Janibacter*-like strain from the results of 16S rDNA sequence comparisons. Accordingly, strain CS12^T has been subjected to a polyphasic characterization to investigate the possibility that it is a second species of the genus *Janibacter*. Here, we describe the morphological, phenotypic, phylogenetic and genomic characteristics of this strain. On the basis of the results described, strain CS12^T is considered to be a new species of the genus *Janibacter*, for which we propose the name *Janibacter terrae* sp. nov.

METHODS

Bacterial strain and culture conditions. Strain CS12^T was isolated from a soil sample taken from around a wastewater treatment plant in Korea by dilution plating on trypticase soy agar (BBL). For investigating its morphological and physiological characteristics, strain CS12^T was grown at 28 °C on solid or in liquid R medium (Martin *et al.*, 1997). Cell mass for analyses of its cell wall, menaquinones, mycolic acids and polar lipids was obtained from growth in liquid R

The GenBank accession number for the 16S rDNA sequence of strain CS12^T is AF176948.

Fig. 1. Phylogenetic tree showing the positions of strain CS12^T and some other related taxa based on 16S rDNA sequences. Scale bar represents 0.01 substitution per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points.

medium. Biomass of strain CS12^T and *J. limosus* DSM 11140^T for DNA extraction was also obtained from growth in liquid R medium. The strains were cultivated at 28 °C on a horizontal shaker at 150 r.p.m. and the broth cultures were checked for purity microscopically before they were harvested by centrifugation. Strain CS12^T and *J. limosus* DSM 11140^T were also grown at 28 °C for 7 d on trypticase soy

agar (BBL), nutrient agar and solid R medium for fatty acid methyl ester (FAME) analysis.

Morphological and physiological tests. The morphology of cells was examined by light microscopy and transmission electron microscopy (TEM). Presence or absence of flagella was examined by TEM using cells from exponentially growing cultures. These were negatively stained with 1%

(w/v) phosphotungstic acid, and after air drying, grids were examined with a model CM-20 transmission electron microscope (Philips). Catalase activity was determined by bubble formation in a 3% hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% *p*-aminodimethylaniline oxalate. DNase activity was determined as described previously (Cowan & Steel, 1965) with DNase test agar (Difco). Nitrate reduction, indole production, methyl red and Voges–Proskauer reactions, hydrogen sulfide production and hydrolysis of aesculin were tested as described previously (Lanyi, 1987). Hydrolyses of casein, gelatin, hypoxanthine, starch, Tween 80, tyrosine and xanthine, and production of urease were also determined as described previously (Cowan & Steel, 1965). Hydrolysis of arbutin was determined according to the method of Kurup & Fink (1975). Acid production from carbohydrates was detected by the method of Hugh & Leifson (1953). Tolerance of NaCl and growth at various temperatures were measured on R medium and brain heart infusion (BHI) medium.

Isolation of DNA. Chromosomal DNA was isolated and purified according to the method of Yoon *et al.* (1996), with the exception that ribonuclease T1 was used together with ribonuclease A.

Chemotaxonomic characterization. The isomer type of the diamino acid of the cell wall was analysed by the method of Komagata & Suzuki (1987) and wall sugars were determined by the method of Saddler *et al.* (1991). Menaquinones were analysed as described by Komagata & Suzuki (1987). Presence or absence of mycolic acids was determined using the methods of Minnikin *et al.* (1975). Polar lipids were extracted using the procedures of Minnikin *et al.* (1984) and identified by two-dimensional TLC and by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). Fatty acids were extracted and analysed following the instructions of the Microbial Identification System (MIDI).

Determination of G+C content. G+C content was determined by the HPLC method of Tamaoka & Komagata (1984).

DNA–DNA hybridization. DNA–DNA hybridization to determine genomic relatedness was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells.

16S rDNA sequencing and phylogenetic analysis. 16S rDNA was amplified by PCR using the two universal primers described previously (Yoon *et al.*, 1998). The PCR product was purified with a QIAquick PCR purification kit (Qiagen) and the 16S rDNA sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The purified sequencing reaction mixtures were automatically electrophoresed using an Applied Biosystems model 310 automatic DNA sequencer. 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 analyses. Evolutionary distance matrices were calculated using the algorithm of Jukes & Cantor (1969) with the DNADIST program within the PHYLIP package (Felsenstein, 1993). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with the NEIGHBOR program of the same package. The stability of relationships was assessed by a bootstrap analysis of 1000 data sets by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package.

Nucleotide sequence accession numbers. GenBank/EMBL accession numbers for reference 16S rDNA gene sequences used in the phylogenetic analysis are shown in Fig. 1.

RESULTS

Morphological and physiological characteristics

Strain CS12^T is a Gram-positive, non-acid-fast, non-spore-forming and non-motile bacterium. Its cells are cocci, which are 0.6–1.1 µm in diameter after 24 h culture in liquid R medium at 30 °C and occur singly, in pairs, in short chains or often in irregular clumps. Cells were always cocci in all growth stages, both in liquid and on solid R media. Colonies on R and nutrient agar were similar to those of *J. limosus* DSM 11140^T in colour and morphology. Colonies of strain CS12^T were cream-coloured on R agar and pale-cream-coloured on nutrient agar. Colonies of *J. limosus* DSM 11140^T on nutrient agar were described as white by Martin *et al.* (1997), but it is more appropriate to describe these as being pale-cream-coloured. Colonies of strain CS12^T were circular, opaque, glistening and convex on R and nutrient agar. The growth of strain CS12^T on trypticase soy agar was poor relative to *J. limosus* DSM 11140^T on the same medium. Neither substrate mycelia nor primary mycelia were seen.

Strain CS12^T grew optimally at pH 7.0–8.0, and growth was inhibited or slow below pH 5.0 and above pH 10. It grew optimally at 28–30 °C. Growth did not occur at 40 °C on solid R medium or on solid or in liquid BHI media, but occurred weakly at 40 °C in liquid R medium. Both strain CS12^T and *J. limosus* DSM 11140^T grew in the presence of 8% (w/v) NaCl in liquid R and BHI media, but strain CS12^T did not grow in liquid R or BHI media containing 10% NaCl. No growth occurred under anaerobic conditions. Physiological properties of strain CS12^T, together with those of *J. limosus* DSM 11140^T, which were simultaneously determined, are given in Table 1.

Chemotaxonomic characteristics and DNA base composition

Strain CS12^T contained *meso*-diaminopimelic acid as the diamino acid in the cell wall. The sugars arabinose and galactose were not detected, although ribose and galactose were present. The predominant isoprenoid quinone was MK-8(H₄) and the cellular phospholipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. Mycolic acids were absent. Strain CS12^T had a cellular fatty acid profile containing saturated, unsaturated, branched and 10-methyl fatty acids and lacked hydroxy fatty acids (Table 2). The fatty acid profile of strain CS12^T was different from that of *J. limosus* DSM 11140^T in the composition of some fatty acids. In particular, C_{17:0}, one of the major fatty acids in *J. limosus* DSM 11140^T, was a minor component in strain CS12^T, and anteiso-C_{17:0}, a minor component in *J. limosus* DSM 11140^T,

Table 1. Morphological and physiological characteristics of strain CS12^T and *J. limosus* DSM 11140^T

+, Positive reaction; –, negative reaction; W, weakly positive reaction. Both strains were positive for catalase, decomposition of gelatin, nitrate reduction, production of H₂S and growth in the presence of 2, 4, 6 and 8% NaCl. Both strains were negative for decomposition of arbutin, aesculin, hypoxanthine, starch, urea and xanthine, production of indole, Voges–Proskauer and methyl red tests, and acid production from L-arabinose, D-cellobiose, D-fructose, D-galactose, glycerol, inulin, lactose, maltose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, starch, trehalose and D-xylose.

Characteristic	Strain CS12 ^T	<i>J. limosus</i> DSM 11140 ^T
Colour of colonies	Cream or pale cream	Cream or pale cream
Morphology	Cocci	Cocci, rods
Motility	–	–
Gram staining	+	+
Spore formation	–	–
Acid production from D-glucose	–	+
Decomposition of:*		
Casein	++	+
Tween 80	++	+
Tyrosine	++	+
Oxidase	W	–
DNase	+	W
Growth in the presence of:		
10% NaCl (R medium)	–	+
10% NaCl (BHI medium)	–	–
Growth on BHI medium at:		
28 °C	+	+
37 °C	+	+
40 °C	–	–
Growth on R medium at:		
10 °C	+	+
28 °C	+	+
37 °C	+	–
40 °C	+	–

* ++, Positive reaction stronger than that of *J. limosus* DSM 11140^T.

was present in large amounts in strain CS12^T. In addition, some fatty acids were characteristically detected in only strain CS12^T or *J. limosus* DSM 11140^T (Table 2). The genomic DNA G + C content of strain CS12^T was 69 mol%.

Phylogenetic analysis based on 16S rDNA sequence

An almost complete 16S rDNA sequence of 1480 nucleotides (approx. 96% of the *Escherichia coli* sequence) was determined for strain CS12^T. Strain CS12^T has highest 16S rDNA similarity with the genera *Janibacter*, *Terrabacter*, *Terracoccus*, *Intrasporangium* and *Sanguibacter*, all members of the family *Intrasporangiaceae* (Stackebrandt *et al.*, 1997; Martin *et al.*, 1997; Prauser *et al.*, 1997). Accordingly, its 16S rDNA sequence was compared with known 16S rDNA sequences of members of the family *Intrasporangiaceae* and other actinomycetes. The resulting phylogenetic tree showed that strain CS12^T forms a

lineage within the radiation enclosing the members of the family *Intrasporangiaceae* and, especially, a coherent cluster with *J. limosus* DSM 11140^T (Fig. 1). The relationship between strain CS12^T and *J. limosus* DSM 11140^T is supported by a bootstrap resampling value of 100%. The level of 16S rDNA sequence similarity between strain CS12^T and *J. limosus* DSM 11140^T was 98.7%, whilst strain CS12^T had levels of 16S rDNA similarity of 94.3–96.2% with the members of the genera *Terrabacter*, *Terracoccus*, *Intrasporangium* and *Sanguibacter*.

DNA–DNA relatedness

DNA–DNA hybridization was performed to determine the genomic relatedness between strain CS12^T and *J. limosus* DSM 11140^T. These species exhibited two independent levels of DNA–DNA relatedness of 22.3% and 23.7%, respectively, when each of their DNAs was separately used as labelled DNA probe.

Table 2. Cellular fatty acid profiles of strain CS12^T and *J. limosus* DSM 11140^T on trypticase soy agar (TSA), nutrient agar (NA) and R agar

Values are percentages of total cellular fatty acids.

Fatty acid	Strain CS12 ^T			<i>J. limosus</i> DSM 11140 ^T		
	TSA	NA	R agar	TSA	NA	R agar
Saturated fatty acids						
C _{14:0}	0.1		0.2	0.2	0.1	0.3
C _{15:0}	0.5	0.1	0.8	1.9	0.4	1.2
C _{16:0}	0.8	0.7	3.0	2.5	5.1	7.5
C _{17:0}	0.8	0.3	1.4	16.3	5.9	14.2
C _{18:0}	0.4	0.6	1.0	2.8	5.9	7.3
C _{19:0}				0.7	0.5	0.9
Unsaturated fatty acids						
C _{15:1} ω6c	0.3		0.2	0.2		
C _{17:1} ω8c	11.0	6.3	7.1	28.7	11.9	17.5
C _{18:1} ω7c	0.8	2.1	1.1	0.5	1.5	1.0
C _{18:1} ω9c	12.8	18.6	13.3	9.5	39.2	22.6
C _{19:1} iso I*	0.3	0.3	0.3			
Branched fatty acids						
iso-C _{12:0}	0.1		0.2	0.1		0.1
iso-C _{13:0}	0.1		0.1			
ante-C _{13:0}	0.1	0.1	0.2			
iso-C _{14:0}	1.1	0.6	1.1	0.9		0.3
iso-C _{15:0}	4.6	4.1	9.0	0.6	0.2	0.5
ante-C _{15:0}	1.1	1.3	2.6			
iso-C _{16:1} H*	1.3	1.1	0.4	0.1		
iso-C _{16:0}	31.9	27.0	22.8	19.1	11.6	13.1
iso-C _{17:0}	6.2	4.9	5.8	0.9	1.2	1.3
iso-C _{17:1} ω9c	4.7	5.2	2.9			
ante-C _{17:1} ω9c	1.1	1.2	1.1			
ante-C _{17:0}	9.1	12.3	14.6	0.5	0.9	1.1
iso-C _{18:0}	0.7	0.5	0.4	1.8	1.8	1.5
10-Methyl fatty acids						
C _{16:0}				0.3	0.7	0.4
C _{17:0}	4.4	3.5	1.1	5.5	2.9	1.7
C _{18:0}	0.3	2.8	0.2	1.2	4.8	1.1
Summed features†						
3	4.1	6.6	8.8	1.9	2.5	4.0
6	1.8		0.5	3.8	2.9	2.5

* The double bond positions indicated by upper-case letters are unknown.

† Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 3 contained one or more of following fatty acids: iso-C_{15:0} 2-OH and/or C_{16:1} ω7c. Summed feature 6 contained one or more of following fatty acids: C_{19:1} ω9c and/or C_{19:1} ω11c.

DISCUSSION

Phylogenetic inference based on 16S rDNA sequence comparison shows that strain CS12^T is the nearest phylogenetic neighbour of *J. limosus* DSM 11140^T and both are closely related to the genera *Intrasporangium*, *Sanguibacter*, *Terrabacter* and *Terracoccus*, all members of the family *Intrasporangiaceae* (Stackebrandt *et al.*, 1997; Prauser *et al.*, 1997) (Fig. 1). These data suggest that strain CS12^T may be a member of the

genus *Janibacter*. Comparative chemotaxonomic analyses confirm that strain CS12^T belongs to the genus *Janibacter*. Strain CS12^T is clearly differentiated from members of the genera *Intrasporangium*, *Sanguibacter*, *Terrabacter* and *Terracoccus* by the type of diamino acid in the cell wall and by the profile of cellular fatty acids (Martin *et al.*, 1997). Strain CS12^T contains *meso*-diaminopimelic acid as the diamino acid in the cell wall, whereas the genera *Intrasporangium*, *Terrabacter* and *Terracoccus* all have *LL*-diaminopimelic

acid (Prauser *et al.*, 1997), and the genus *Sanguibacter* has L-lysine (Fernandez-Garayzabal *et al.*, 1995). Differences in major fatty acids are also valuable in distinguishing strain CS12^T from the other four genera. Both the genera *Intrasporangium* and *Terrabacter* have iso-C_{15:0} (Prauser *et al.*, 1997), the genus *Terracoccus* has iso-C_{15:0} and anteiso-C_{15:0} (Prauser *et al.*, 1997) and the genus *Sanguibacter* has anteiso-C_{15:0} and C_{16:0} (Fernandez-Garayzabal *et al.*, 1995; Martin *et al.*, 1997). Although strain CS12^T and *J. limosus* DSM 11140^T show a few differences in their fatty acid compositions, both contain iso-C_{16:0} as a major fatty acid (Table 2). The predominant menaquinones found in the genera *Intrasporangium* and *Sanguibacter* are different from that of strain CS12^T. The genera *Intrasporangium* and *Sanguibacter* have MK-8 and MK-9(H₄), respectively, whilst strain CS12^T has MK-8(H₄). However, the predominant menaquinones of *Terrabacter* and *Terracoccus* are the same as that of strain CS12^T (Martin *et al.*, 1997; Prauser *et al.*, 1997). Polar lipid profiles also distinguish strain CS12^T and the genus *Janibacter* from the genera *Intrasporangium*, *Terrabacter* and *Terracoccus* (Martin *et al.*, 1997; Prauser *et al.*, 1997). The genus *Intrasporangium* is also morphologically distinctive in having hyphae, a property not found in strain CS12^T (Kalakoutskii *et al.*, 1967; Schumann *et al.*, 1997). Accordingly, the morphological, chemotaxonomic and phylogenetic data all indicate that strain CS12^T is a member of the genus *Janibacter*.

Strain CS12^T is similar to *J. limosus* DSM 11140^T in its morphological and in most of its physiological characteristics. However, there are some differences between strain CS12^T and *J. limosus* DSM 11140^T, including those in their ability to degrade some substrates, their tolerance of 10% NaCl and their growth at 37 °C and 40 °C (Table 1). It is noteworthy that the fatty acid profile of strain CS12^T also differs from that of *J. limosus* DSM 11140^T in the composition of some fatty acids (Table 2). The 16S rDNA sequences of strain CS12^T and *J. limosus* DSM 11140^T have 19 bp sequence differences in the region compared. Levels of DNA–DNA relatedness provide decisive evidence that strain CS12^T and *J. limosus* DSM 11140^T are members of different genomic species (Wayne *et al.*, 1987). Therefore, differences in some phenotypic characteristics and genetic distinctiveness together indicate that strain CS12^T is a new species of the genus *Janibacter*.

On the basis of the data described above, strain CS12^T should be placed in the genus *Janibacter* as a new species, for which the name *Janibacter terrae* sp. nov. is proposed.

Description of *Janibacter terrae* sp. nov.

Janibacter terrae (ter'rae. M.L. gen. n. *terrae* of the earth).

Cells are non-spore-forming and non-motile cocci, which occur singly, in pairs, in short chains or often in irregular clumps. Gram-positive and non-acid-fast.

Colonies are circular, opaque, glistening and convex, and cream-coloured on R agar and pale-cream-coloured on nutrient agar. Neither substrate mycelia nor primary mycelia are seen. The optimal growth temperature is 28–30 °C. Growth does not occur at 40 °C on solid R medium or on solid or in liquid BHI media, but occurred weakly at 40 °C in liquid R medium. The optimal pH for growth is 7.0–8.0, and growth is inhibited below pH 5.0 and above pH 10. Growth occurs in the presence of 8% NaCl in liquid R and BHI media. No growth occurs under anaerobic conditions. Catalase- and DNase-positive. Urease-negative. Oxidase is weakly positive only on R agar. Casein, gelatin, Tween 80 and tyrosine are hydrolysed. Arbutin, aesculin, hypoxanthine, starch and xanthine are not hydrolysed. Nitrate is reduced to nitrite. H₂S is produced. Indole is not produced. Voges–Proskauer and methyl red reactions are negative. No acid is produced from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inulin, lactose, maltose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, starch, trehalose or D-xylose. The cell wall contains *meso*-daminopimelic acid. The predominant menaquinone is MK-8(H₄). The major fatty acids are iso-C_{16:0}, C_{18:1} ω9*c* and anteiso-C_{17:0} or C_{17:1} ω8*c*. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. The G + C content is 69 mol% (determined by HPLC). Isolated from soil around a wastewater treatment plant, Korea. The type strain is CS12^T (= KCCM 80001^T = JCM 10705^T).

ACKNOWLEDGEMENTS

This work was supported by grants HS2321 and HS2701 from the Ministry of Science and Technology (MOST) of the Republic of Korea.

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