Four novel *Arthrobacter* species isolated from filtration substrate

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Four Gram-positive, non-motile, non-spore-forming bacterial strains, LC4^T, LC6^T, LC10^T and LC13^T, were isolated from a filtration substrate made from trass, a volcanic rock, and their taxonomic positions were investigated by a polyphasic taxonomic approach. The novel strains grew over a temperature range of 5–40 °C, at pH values of 6–11 and in the presence of 3–7 % (w/v) NaCl. A phylogenetic tree based on 16S rRNA gene sequences showed the novel strains formed a distinct evolutionary lineage within the genus *Arthrobacter*. Chemotaxonomic analyses demonstrated that the major menaquinone was MK-9(H₂), a menaquinone typical of the *Arthrobacter globiformis* group. The major fatty acid was anteiso-C_{15:0} and the major amino acid present in the cell-wall peptidoglycan was L-lysine. These observations supported the affiliation of the novel strains to the genus *Arthrobacter*. On the basis of their morphological, physiological and genotypic characteristics, the new isolates are considered to represent four novel species of the genus *Arthrobacter*, for which the names *Arthrobacter niigatensis* sp. nov. (type strain LC4^T=IAM 15382^T=CCTCC AB 206012^T), *Arthrobacter alkaliphilus* sp. nov. (type strain LC6^T=IAM 15383^T=CCTCC AB 206013^T), *Arthrobacter echigonensis* sp. nov. (type strain LC10^T=IAM 15385^T=CCTCC AB 206017^T) and *Arthrobacter albidus* sp. nov. (type strain LC13^T=IAM 15386^T=CCTCC AB 206018^T) are proposed.

The genus *Arthrobacter* was established by Conn & Dimmick (1947) and includes most of the bacteria that exhibit a rod (in young cultures)–coccus (in older cultures) morphological cycle, although some members of the genus are spheres, occurring in pairs and tetrads, such as *Arthrobacter agilis*. The members of this genus have been isolated from various environments, such as air, soil, fresh water, oil, brine, airborne infections, tobacco leaves, human skin, mural paintings, sewage and activated sludge (Sgueros, 1955; Li *et al.*, 2004; Conn & Dimmick, 1947; Takeuchi & Yokota, 1991; Stackebrandt *et al.*, 1983; Westerberg *et al.*, 2000; Koch *et al.*, 1995; Heyrman *et al.*, 2005; Skerman *et al.*, 1980; Margesin *et al.*, 2004; Rzechowska, 1976). It has been reported that some poisonous contaminants and difficult-to-degrade chemical substances (such as PCB, dioxin and oil) can be degraded by species of the genus *Arthrobacter* (Rzechowska, 1976; Marks *et al.*, 1984; Singer *et al.*, 2000; Duarte *et al.*, 2001; Fukatsu *et al.*, 2005; Jussila *et al.*, 2006). Denitrification

activity has been described for some species of the genus *Arthrobacter* (Carter *et al.*, 1995). At the present time, there are more than 50 recognized species of the genus *Arthrobacter*. This study describes four high G+C, Gram-positive bacterial strains, LC4^T, LC6^T, LC10^T and LC13^T which were isolated from a filtration substrate and, from the results of a polyphasic taxonomic approach, are suggested to represent four novel species of the genus *Arthrobacter*.

The bacterial strains were isolated from a filtration substrate made from volcanic soil using NY medium (1.6 g nutrient broth l⁻¹ and 0.5 g yeast extract l⁻¹) containing 0.05 g cycloheximide l⁻¹, 0.1 g kabicidin l⁻¹, 1.5 % agar and adjusted to pH to 7.0. All isolates were maintained on NY medium. The reference strains used in this study, *Arthrobacter methylotrophus* IAM 15313^T, *Arthrobacter atrocyaneus* IAM 12339^T and *Arthrobacter chlorophenolicus* JCM 12360^T were obtained from the IAM Culture Collection (University of Tokyo, Japan) and JCM (Japan Collection of Microorganisms). All strains were cultured at 30 °C. Genomic DNA extraction (Sambrook *et al.*, 1989) and PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described previously (Ding & Yokota, 2002, 2004). Sequence alignment was performed using CLUSTAL_X

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of *Arthrobacter niigatensis* LC4^T, *Arthrobacter alkaliphilus* LC6^T, *Arthrobacter echigonensis* LC10^T and *Arthrobacter albidus* LC13^T reported in this paper are AB248526, AB248527, AB248531 and AB248533, respectively.

v1.83 (Thompson *et al.*, 1994). The evolutionary distance matrix was calculated using Kimura's two-parameter method (Kimura, 1980). A phylogenetic tree was constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood methods (Felsenstein, 1981). The topology of the phylogenetic tree was evaluated by bootstrap analysis with 1000 replications according to the method of Felsenstein (1985). The G+C content of the DNA was determined by HPLC as described by Mesbah *et al.* (1989). DNA–DNA relatedness experiments were conducted using a modification of the microplate method of Ezaki *et al.* (1989) and using photobiotin-labelled DNA and microdilution wells as described by Willems *et al.* (2001). The DNA–DNA hybridization temperature was 55 °C.

Analysis of cell-wall amino acids was performed by suspending 300 mg of dried cells in 10–20 ml of water and then around 1 g of glass beads (0.11–0.12 mm in diameter) was added and the suspension was treated at 180 W for 30 min using an ultrasonicator (201M ultrasonic oscillator; Kubota). The crude cell wall preparation was washed with water, centrifuged in 4 % SDS solution to eliminate proteins and the purified cell walls were freeze-dried. The amino acid composition was determined by TLC followed by HPLC. For two dimensional TLC, 2-propanol/acetic acid/water (75:10:15, v/v) was used in the first dimension and methanol-pyridine/10 M hydrochloric acid/water (64:8:2:14, v/v) was used for the second dimension. The amino acids of the cell-wall peptidoglycan were determined according to the methods of Schleifer & Kandler (1972) and Harper & Davis (1979). The cellular fatty acids were extracted according to the protocol of the MIDI system (Microbial ID Inc.). The bacteria were incubated in TSBA medium for 48 h at 30 °C. The fatty acid methyl esters (FAMES) were obtained from the cells by saponification, methylation and extraction. Analysis by GC was controlled by MIS software (Microbial ID Inc.) and the peaks were automatically integrated and identified by the Microbial Identification software package (Sasser, 1990). Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) and purified by TLC using *n*-hexane-diethyl ether (85:15, v/v) as the solvent and the menaquinone fraction was analysed by HPLC.

The biochemical profile was determined with API ZYM and API CORYNE strips (bioMérieux). The optimum temperature and the pH for growth were also determined. The range of test temperatures was 5, 10, 20, 30, 37, 40, 45 and 50 °C and the pH range tested was 4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11 and 12. The results of NaCl tolerance tests were obtained with 3, 5 and 7 % (w/v) NaCl in nutrient broth and the incubation temperature was 27 °C.

Colonies of the novel isolates on nutrient agar were round, convex, glossy, with entire margins and had a light grey or light yellow colour. Cells were Gram-positive, aerobic, exhibited a rod–coccus growth cycle, produced non-fluorescent pigment and were non-spore-forming and non-motile. The growth temperature range of strains

LC4^T and LC6^T was 5–40 °C, however, strains LC10^T and LC13^T grew between 10 and 40 °C. The optimal temperature for growth was 30 °C for all of the novel strains. The pH range for growth of strains LC4^T and LC6^T was pH 6–11 and was 6–10 for strains LC10^T and LC13^T. Strains LC4^T, LC6^T and LC13^T could grow on nutrient broth medium with 7 % NaCl. Strain LC10^T could grow on medium with 5 % NaCl, but was unable to grow in the presence of 7 % NaCl.

The 16S rRNA gene sequences of all members of the genus *Arthrobacter* and related genera obtained from DDBJ/EMBL/GenBank were compared and a phylogenetic tree was constructed. The phylogenetic analysis included all recognized species of the genus *Arthrobacter*. The analysis based on 16S rRNA gene sequences indicated that all of the novel isolates belonged to the genus *Arthrobacter*. As can be seen in Fig. 1, the four novel isolates were divided into three clusters. At the 16S rRNA gene sequence level, strains LC4^T, LC6^T, LC10^T and LC13^T had sequence similarities of 97.0–97.4 % compared with *A. chlorophenolicus* JCM 12360^T (GenBank accession no. AF102267), *A. methylo-trophus* IAM 15313^T (AF235090) and *A. atrocyaneus* IAM 12339^T (X80746).

In order to determine the genotypic relatedness between the novel strains and recognized species of the genus *Arthrobacter*, DNA–DNA hybridization analyses were performed. The DNA–DNA relatedness value between strain LC4^T and *A. chlorophenolicus* was 32.1 %, between strain LC6^T and *A. methylo-trophus*, 14.1 %, between strain LC10^T and *A. atrocyaneus*, 11.2 %, between strain LC10^T and LC13^T, 25.3 %, and between strain LC13^T and *A. atrocyaneus*, 9.6 %. DNA–DNA relatedness below 70 % is considered to be the threshold level for the separation of species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002) and so these results show that strains LC4^T, LC6^T, LC10^T and LC13^T all represent novel genospecies.

The morphological and biochemical characteristics of the novel strains are shown in Table 1. In the genus *Arthrobacter*, little or no acid is reported to be produced from glucose (Keddie *et al.*, 1984), however, in this study, glucose could be oxidized by isolates LC4^T, LC6^T and LC10^T and strain LC13^T showed weak activity. Isoprenoid menaquinone composition, fatty acid content and DNA G+C content are shown in Table 2. All four novel strains had lysine as the diagnostic diamino acid of the cell-wall peptidoglycan. The major amino acids of the cell wall were lysine, alanine, serine and threonine. The major isoprenoid menaquinone for the novel isolates was MK-9(H₂). The major fatty acid was anteiso-C_{15:0} (44.0–74.4 %), but anteiso-C_{17:0} (5.0–29.8 %), iso-C_{15:0} (3.0–35.5 %) and iso-C_{16:0} (2.5–12.8 %) were also present. The DNA G+C contents of strains LC4^T, LC6^T, LC10^T and LC13^T were 70.8, 69.0, 71.8 and 70.8 mol%, respectively.

The differences in biochemical and chemotaxonomic characteristics indicated that the four new isolates LC4^T,

Fig. 1. Neighbour-joining tree based on almost complete 16S rRNA gene sequences, showing the phylogenetic position of strains LC4^T, LC6^T, LC10^T and LC13^T within the radiation of recognized *Arthrobacter* species. Asterisks indicate branches of the tree that were also found using the maximum-likelihood (Felsenstein, 1981) tree-making algorithms. Numbers at nodes indicate percentage levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values over 60% are given. Bar, 0.01 substitutions per nucleotide position.

LC6^T, LC10^T and LC13^T were distinct from the recognized species of the genus *Arthrobacter* and supported the proposal that the strains should be classified as four novel species of the genus *Arthrobacter*, for which the names *Arthrobacter niigatensis* sp. nov., *Arthrobacter alkaliphilus* sp. nov., *Arthrobacter echigonensis* sp. nov., and *Arthrobacter albidus* sp. nov., are proposed, respectively.

Description of *Arthrobacter niigatensis* sp. nov.

Arthrobacter niigatensis (ni.i.ga.ten'sis. N.L. masc. adj. *niigatensis* pertaining to the Niigata region, Japan).

Cells are non-motile and non-spore-forming. Gram-positive, catalase-positive, oxidase-negative, shows a rod-coccus growth cycle and produces non-fluorescent pigment. Growth occurs on nutrient broth agar at 5–40 °C, optimal temperature for growth is 30 °C. Grows in

the presence of 3–7% (w/v) NaCl. The pH range for growth is 6–11 and the optimum pH is 7.5. Colonies are round, convex, glossy, with entire margins and are light grey or light yellow. Using the API CORYNE system, a positive reaction is observed for reduction of nitrate, activities of pyrazinamidase, pyrrolidonyl arylamidase and urease, hydrolysis of gelatin and for the utilization of glucose, ribose, lactose and sucrose. A negative reaction is obtained for utilization of maltose and the utilization of mannitol, xylose and glycogen is weak. Using the API ZYM system, activity is detected for alkaline phosphatase, acid phosphatase, leucine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, esterase C4, esterase lipase C8, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase and α -mannosidase. No activity is detected for lipase C14, α -galactosidase, *N*-acetyl- β -glucosaminidase, α -chymotrypsin or α -fucosidase. Activities of valine

Table 1. Differential phenotypic characteristics of the novel strains

Taxa: 1, *A. niigatensis* sp. nov. LC4^T; 2, *A. alkaliphilus* sp. nov. LC6^T; 3, *A. echigonensis* sp. nov. LC10^T; 4, *A. albidus* sp. nov. LC13^T; 5, *A. chlorophenolicus* JCM 12360^T (data from Westerberg *et al.*, 2000); 6, *A. methylotrophus* IAM 15313^T (Borodina *et al.*, 2002); 7, *A. atrocyaneus* IAM 12339^T (Kuhn & Starr, 1960). G, Grey; W, white; LY, light yellow; LG, light grey; PG, pearl grey; CY, creamy yellow; ND, no data; UK, contaminant on an agar plate; +, positive; (+), weakly positive; –, negative.

Characteristic	1	2	3	4	5	6	7
Isolation source	Filtration substrate	Filtration substrate	Filtration substrate	Filtration substrate	Soil	Soil	UK
Colony colour	LG/LY	LY	LG/LY	W/LY	PG	CY	G/W
Optimum pH	7.5	8.5	7	7	ND	7.2–7.5	6
Optimum temperature	25–37	25–30	25–37	25–37	20–30	25	18–37
Reduction of nitrate	+	–	–	–	–	(+)	+
Pyrrolidonyl arylamidase	+	+	+	+	–	+	–
Urease	+	+	–	+	+	+	–
Hydrolysis of gelatin	+	–	–	–	+	(+)	(+)
Utilization of:							
Maltose	–	+	(+)	–	–	–	+
Mannitol	(+)	+	–	+	+	–	–
Ribose	+	(+)	+	+	+	+	–
Sucrose	+	–	+	(+)	+	+	–
Xylose	(+)	–	–	(+)	–	(+)	+
Esterase (C4)	+	–	+	(+)	(+)	+	–
Cystine arylamidase	(+)	–	+	+	+	–	+
α-Chymotrypsin	–	(+)	–	–	+	+	–
α-Galactosidase	–	+	–	–	+	–	–
β-Glucosidase	+	–	–	+	+	–	(+)

arylamidase and cystine arylamidase are weak. The predominant fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The diamino acid of the cell-wall peptidoglycan is lysine and major components are lysine, serine, threonine and alanine. Menaquinones are MK-9(H₂), MK-8(H₂) and MK-8(H₄) (approx. 64, 17 and 15 %, respectively).

The type strain, strain LC4^T (=IAM 15382^T=CCTCC AB 206012^T), was isolated from a filtration substrate made from volcanic rock from Niigata, Japan. The DNA G+C content of the type strain is 70.8 mol%.

Description of *Arthrobacter alkaliphilus* sp. nov.

Arthrobacter alkaliphilus [al.ka.li'phi.lus. N.L. n. *alkali* (from the Arabic word *al-qaliy*) the ashes of saltwort; Gr. adj. *philos* loving; N.L. masc. adj. *alkaliphilus* loving alkaline environments].

Cells are non-motile and non-spore-forming. Gram-positive, catalase-positive, oxidase-negative, shows a rod-coccus growth cycle and produces non-fluorescent pigment. Growth occurs on nutrient broth agar at 5–40 °C, optimal temperature for growth is 30 °C. Grows in the presence of 3–7 % (w/v) NaCl. The pH range for growth is 6–11 and the optimum pH is 8.5. Colonies are round, convex, glossy, with entire margins and are light yellow. Using the API CORYNE system, positive reactions are observed for activities of pyrazinamidase, pyrrolidonyl

arylamidase and urease and for the utilization of glucose, ribose, maltose and mannitol. Negative reactions are obtained for nitrate reduction, for hydrolysis of gelatin and for utilization of glycogen, sucrose and xylose. Lactose gives a weak reaction. Using the API ZYM system, activity is detected for alkaline phosphatase, acid phosphatase, esterase lipase C8, leucine arylamidase, valine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase and α-mannosidase. No activity is detected for esterase C4, lipase C14, cystine arylamidase, β-glucosidase, N-acetyl-β-glucosaminidase or α-fucosidase. The activity of α-chymotrypsin is weak. Predominant fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The diamino acid of the cell-wall peptidoglycan is lysine and the major components are lysine, serine, threonine and alanine. The menaquinone content is MK-9(H₂), MK-10(H₂) and MK-8(H₄) (approx. 77, 17 and 3 %, respectively).

The type strain, strain LC6^T (=IAM 15383^T=CCTCC AB 206013^T), was isolated from a filtration substrate made from volcanic rock from Niigata, Japan. The DNA G+C content of the type strain is 69.0 mol%.

Description of *Arthrobacter echigonensis* sp. nov.

Arthrobacter echigonensis (echi.go.nen'sis. N.L. masc. adj. *echigonensis* pertaining to the Echigo region, in Japan).

Table 2. Chemotaxonomic characteristics of the novel species of the genus *Arthrobacter*

Taxa: 1, *A. niigatensis* sp. nov. LC4^T; 2, *A. alkaliphilus* sp. nov. LC6^T; 3, *A. echigonensis* sp. nov. LC10^T; 4, *A. albidus* sp. nov. LC13^T; 5, *A. chlorophenolicus* JCM 12360^T (data from Westerberg *et al.*, 2000); 6, *A. methylotrophus* IAM 15313^T (Borodina *et al.*, 2002); 7, *A. atrocyaneus* IAM 12339^T (Koch *et al.*, 1995). Menaquinone and fatty acid content data were obtained in this study. Lys, lysine; Ser, serine; Thr, threonine; Ala, alanine. Amino acids separated by hyphens indicate results from partial sequencing of peptidoglycan structure. Amino acids separated by commas indicate results of composition studies with no sequence study.

Characteristic	1	2	3	4	5	6	7
Peptidoglycan amino acid composition	Lys, Ser, Thr, Ala	Lys, Ser, Thr, Ala	Lys, Ser, Ala	Lys, Ser, Ala	Lys-Ser-Thr-Ala	Lys-Ala	Lys-Ser-Ala
Menaquinones (%):							
MK-7(H ₂)						2	
MK-8					7.2		
MK-8(H ₂)	16.7	2.1	4.0	2.4		6.0	
MK-8(H ₄)	14.8	2.9					
MK-9					23.7	4.0	
MK-9(H ₂)	64.2	77.0	82.6	82.9	60.9	79.0	Major
MK-10(H ₂)		17.0	5.7	14.0		7.0	
MK-11					8.1		
MK-11(H ₂)						1.0	
Fatty acids (%):							
iso-C _{14:0}	1.6	0.7	0.9	0.4	4.0	1.9	1.1
C _{14:0}	1.2	0.2	0.2	0.2	2.0	0.9	0.2
iso-C _{15:0}	8.4	4.5	14.7	14.5	7.0	7.1	29.0
anteiso-C _{15:0}	51.2	58.0	55.0	43.5	66.0	74.4	38.8
iso-C _{16:0}	12.8	7.1	5.8	5.3	10.0	5.7	9.5
C _{16:0}	5.6	1.1	0.8	1.1	3.0	1.6	0.9
Summed feature 3*	0.7				1.0		
iso-C _{17:0}	1.9	1.4	2.5	4.8		0.3	4.7
anteiso-C _{17:0}	14.8	26.1	20.1	29.8	5.0	6.8	15.5
anteiso-C _{17:1} ω9c	0.6				1.0		
DNA G + C content (mol%)	70.8	69.0	71.8	70.8	65.1	61.0	69.5–70.3

*Summed feature 3, C_{16:1}ω7c/iso-C_{15:0} 2-OH.

Cells are non-motile and non-spore-forming. Gram-positive, catalase-positive, oxidase-negative, shows a rod-coccus growth cycle and produces non-fluorescent pigment. Growth occurs on nutrient broth agar at 10–40 °C, optimal temperature for growth is 30 °C. Grows in presence of 3–5 % NaCl (w/v), but no growth occurs in the presence of 7 % NaCl. The pH range for growth is 6–10 and the optimum pH is 7. Colonies are round, convex, glossy, with entire margins and are light grey or light yellow. Using the API CORYNE system, positive reactions are observed for activities of pyrazinamidase, pyrrolidonyl arylamidase and for the utilization of glucose, ribose and sucrose. Negative reactions are obtained for nitrate reduction, hydrolysis of gelatin, urease activity and for the utilization of xylose, mannitol, lactose and glycogen. Utilization of maltose is weak. By using the API ZYM system, activity is detected for alkaline phosphatase, acid phosphatase, esterase C4, esterase lipase C8, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase β-galactosidase, β-glucuronidase, α-glucosidase and α-mannosidase. No activity detected for lipase C14, α-chymotrypsin, α-

galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase or α-fucosidase. The predominant fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{15:0}. The diamino acid of the cell-wall peptidoglycan is lysine and the major components are lysine, serine and alanine. The menaquinone content is MK-9(H₂), MK-10(H₂) and MK-8(H₂) (approx. 83, 6 and 4 %, respectively).

The type strain, strain LC10^T (=IAM 15385^T=CCTCC AB 206017^T), was isolated from a filtration substrate made from volcanic rock from Niigata, Japan. The DNA G + C content of the type strain is 71.8 mol%.

Description of *Arthrobacter albidus* sp. nov.

Arthrobacter albidus (al'bi.dus. L. masc. adj. *albidus* whitish, referring to the colour of the colonies).

Cells are non-motile and non-spore-forming. Gram-positive, catalase-positive, oxidase-negative, shows a rod-coccus growth cycle and produces non-fluorescent pigment. Growth occurs on nutrient broth agar at 10–40 °C, optimal temperature for growth is 30 °C. Grows in

the presence of 3–7 % (w/v) NaCl, but no growth occurs in the presence of 7 % NaCl. The pH range for growth is 6–10, and the optimum pH is 7. Colonies are round, convex, glossy, with entire margins and are white or light yellow. Using the API CORYNE system, positive reactions are observed for activities of pyrazinamidase, pyrrolidonyl arylamidase and urease and for the utilization of ribose and mannitol. Negative reactions are obtained for nitrate reduction, hydrolysis of gelatin and for the utilization of maltose, lactose and glycogen. Utilization of glucose, xylose and sucrose is weak. By using the API ZYM system, activity is detected for alkaline phosphatase, esterase lipase C8, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase and α -mannosidase. No activity is detected for lipase C14, α -chymotrypsin, α -galactosidase, *N*-acetyl- β -glucosaminidase or α -fucosidase. Esterase C4 and acid phosphatase activities are weak. The predominant fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{15:0}. The diagnostic diamino acid of the cell-wall peptidoglycan is lysine and the major components are lysine, serine and alanine. The menaquinone content is MK-9(H₂), MK-10(H₂) and MK-8(H₄) (approx. 83, 14 and 2 %, respectively).

The type strain, strain LC13^T (=IAM 15386^T=CCTCC 206018^T), was isolated from a filtration substrate made from volcanic rock from Niigata, Japan. The DNA G+C content of the type strain is 70.8 mol%,

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