Arcanobacterium abortisuis sp. nov., isolated from a placenta of a sow following an abortion

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A Gram-positive, short diphtheroid-shaped organism was isolated from a sow's placenta of an abortion. This novel isolate, strain Murakami^T, was examined physiologically, chemotaxonomically and phylogenetically. Cells had an irregular V-shaped or palisade arrangement. Colonies appeared translucent on TMVL agar. Cells were strictly anaerobic, negative for catalase and gelatin decomposition and positive for nitrate reduction and soluble starch hydrolysis. Fourteen sugars including glucose were utilized as carbon sources for growth, but 15 sugars including arabinose were not. α -Galactosidase, β -galactosidase, α -glucosidase and leucine arylamidase were produced, but β -glucosidase was not. Fermentation products were lactic, succinic and acetic acids. Sugars of whole cells consisted of rhamnose and ribose. The amino-acid composition of the peptidoglycan was glutamic acid, alanine and lysine in the molar ratio of 1:2:1. The main fatty acid components of whole cells were C_{14:0}, C_{16:0}, C_{16:1 ω 7} and C_{18:1 ω 9}. The bacterial menaquinone was MK-10(H₄). The polar lipids were phosphatidylethanolamine and two unknown phosphatidylinositol mannosides. The G + C content of the genomic DNA of strain Murakami^T was 63.8 mol%. Phylogenetic analysis of 16S rRNA gene sequences from strain Murakami^T and other members of the genus *Arcanobacterium* supported the phenotypic findings that strain Murakami^T represents a novel species, for which the name *Arcanobacterium abortisuis* sp. nov. is proposed. The type strain is Murakami^T (=ATCC BAA-1522^T =DSM 19515^T =JCM 14813^T).

During June and November 1999, intermittent abortions were observed at a pig farm in Chiba Prefecture, Japan, on which are raised approximately 600 sows. One case was subjected to pathological and bacteriological examinations, which revealed suppurative bronchopneumonia and suppurative placentitis. The lesions in three fetuses and the placenta from an abortion were found to contain a number of Gram-positive filamentous and/or rod-shaped bacteria. The bacteria were immunolabelled with a primary antiserum against this micro-organism. Samples were cultured on TMVL agar slants at 37 °C under flushing

with 5 % CO₂ (Azuma & Suto, 1970; Azuma *et al.*, 1979; Azuma & Ito, 1987) and a novel *Actinomyces*-like micro-organism, strain Murakami^T, was isolated from a sample from the placenta.

Physiological examinations (Azuma & Suto, 1970; Cowan, 1974) were conducted on cultures of strain Murakami^T prepared on TMVL medium, in which Tween 80 is added to MVL medium (Cote, 1992; Azuma *et al.*, 1979). Cells of strain Murakami^T were short and diphtheroid-shaped and mainly had an irregular V-shape or palisade arrangement. Colonies were translucent and smooth with a diameter of about 2 mm on TMVL agar. The isolate was strictly anaerobic and negative for catalase activity and indole

The GenBank/EMBL/DDBJ accession number for the complete 16S rRNA gene sequence of strain Murakami^T is AB305159.

production, but positive for nitrate reduction. Cells were negative for gelatin decomposition (Kohn, 1953) and positive for soluble starch hydrolysis. The strain utilized fructose, galactose, glucose, cellobiose, lactose, maltose, sucrose, trehalose, melezitose, raffinose, inulin, soluble starch, adonitol and glycerol as sole carbon sources for growth. It did not utilize arabinose, ribose, xylose, rhamnose, mannose, sorbose, melibiose, amygdalin, arbutin, salicin, dulcitol, erythritol, mannitol, sorbitol or inositol. With the Rapid ID 32A system (bioMérieux), the strain was positive for α -galactosidase, β -galactosidase, α -glucosidase and leucine arylamidase production, but negative for urease, β -glucosidase and pyroglutamic acid arylamidase.

Fermentation products were examined (Tabaru *et al.*, 1988). After deproteinization of the sample, it was eluted with 3 mM HClO₄ using a HPLC equipped with a stainless-steel column packed with cation-exchange resin (RSKC-8119; Showa denko). Separated organic acids were detected with bromothymol blue solution in 15 mM Na₂HPO₄. Succinic acid (24.7 mM), lactic acid (38.4 mM) and acetic acid (19.8 mM) were shown to be present in strain Murakami^T.

The chemotaxonomic properties of strain Murakami^T were determined using methods accepted for use with actinomycetes (Slack & Gerencser, 1975). The whole-cell sugar components were determined according to Stanek & Roberts (1974). After growth in TMVLG (TMVL plus 1 % glucose instead of 0.2 %), pelleted cells were obtained from 50 ml culture. They were resuspended with 3 ml 2 M H₂SO₄ and adjusted to pH 5.0–5.5 with saturated Ba(OH)₂. After centrifugation, the culture supernatant was evaporated to about 0.3 ml. The sample was applied to an Avicel TLC plate (Funakoshi Co.) and mixtures of standards were applied to the same plate. The chromatogram was developed using ethylene acetate/pyridine/acetic acid/water (5:5:1:1, by vol.). After drying, the spots were visualized by spraying with acid aniline phthalate and heating at 105 °C for 5 min. Whole-cell sugars of Murakami^T consisted of rhamnose and ribose.

The amino-acid composition of the cell-wall peptidoglycan was determined for strain Murakami^T grown in TMVLG. Pelleted cells from 50 ml culture were treated with 10 % trichloroacetic acid at 80 °C for 30 min. After centrifugation, the sediment was treated with actinase E (KA-002; Kaken-pharma) in 10 ml 0.1 M phosphate buffer (pH 7.2) at 37 °C for 6 h. The sample was purified with three washings, using distilled water followed by centrifugation, and then hydrolysed with 6 M HCl at 110 °C for 24 h. After hydrolysis, the HCl was removed under reduced pressure and the residue was dissolved in sodium citrate buffer (pH 3.25). Analysis was performed using an amino-acid analyser (JLC-300; JASCO). The amino-acid composition was determined as glutamic acid, alanine and lysine in the molar ratio of 1:2:1.

The fatty-acid composition of whole cells was determined according to Ikemoto *et al.* (1978). Pelleted cells from

50 ml TMVLG culture were vacuum-dried and underwent methanolysis with 2 ml of 3 % HCl/methanol solution at 100 °C for 3 h. The sample was extracted three times with 1 ml petroleum ether. The ether layer was harvested, washed with 1 ml distilled water, dehydrated with Na₂SO₄, concentrated under a stream of N₂ gas and dissolved in 1 ml acetone. The fatty acids were analysed with a gas chromatograph (Agilent 6890; Hewlett-Packard) equipped with a flame-ionization detector and a fused silica, open tubular column coated with polyethylene glycol-HT (GL Sciences), which has been calibrated with mixtures of standards containing C_{11:0}, C_{12:0}, C_{13:0}, C_{14:0}, C_{15:0}, C_{16:0}, C_{16:1} ω 7, C_{17:0}, C_{18:0}, C_{18:1} ω 9, C_{18:2} ω 6, C_{18:3} ω 3 and C_{19:0} (1021-58108, 1201-58202, 1201-58203 and 1201-58205; GL Science Inc.). The major fatty acids of strain Murakami^T were C_{18:1} ω 9 (47.4 %), C_{16:0} (17.3 %), C_{14:0} (12.0 %), C_{16:1} ω 7 (5.2 %) and C_{18:0} (3.8 %).

Menaquinone analysis was performed according to Collins *et al.* (1977) and Collins & Jones (1981). About 50 mg vacuum-dried cells, obtained from 250 ml TMVLG culture, was mixed with a chloroform/methanol solution (1:1) overnight. After filtration, the clear liquid was dried using a rotary evaporator at 35 °C and dissolved with 0.3 ml acetone. The sample was applied on to a silica-gel TLC plate (Kieselgel 60 F₂₅₄; Merck). A solution of petroleum ether/diethyl ether (85:15, v/v) was used as the developing solvent. After development, the plate was dried. Menaquinones were detected with UV (254 nm) and removed from the plate by scraping. Purified menaquinones were obtained by dissolving the scraped silica powders in 5 ml acetone, filtering through a cartridge filter, concentrating in a rotary evaporator at 35 °C and dissolving in 0.3 ml acetone. For liquid chromatography mass spectrometry, an API 3200 mass spectrometer (Applied Biosystems) was used in the positive-ion mode with atmospheric pressure chemical ionization. Separation of the menaquinone fraction was carried out with an L-column (Chemicals Evaluation and Research Institute, Japan). The major menaquinone component of strain Murakami^T was MK-10(H₄).

Polar lipid extraction and analysis were performed according to Kudo (2001) with cells prepared in TMVLG medium. Harvested cells (0.5 g) were washed by shaking for 15 min with 2 ml methanol/0.3 % NaCl solution (100:10) and 2 ml petroleum ether in a screw-capped test tube. After centrifugation at 3000 r.p.m. for 10 min, the lower layer was collected, washed a second time with 1 ml petroleum ether and collected again after centrifugation. The lower layer was then boiled at 100 °C for 5 min and the polar lipids were extracted by shaking for 1 h with 2.3 ml chloroform/methanol/distilled water (90:100:30, by vol.). After centrifugation, the upper layer was retained and extraction was performed twice more on the lower layer by shaking for 30 min with 0.75 ml chloroform/methanol/distilled water (50:100:40, by vol.). The upper layers from each extraction were combined and washed by shaking with 2.6 ml chloroform/distilled water (1:1, v/v). After centrifugation, the lower layer was dried by flushing

with N₂ and dissolved in 60 µl chloroform/methanol (2:1). TLC was performed with 5 µl sample on a silica-gel TLC plate (10 × 10 cm Kieselgel 60 F₂₅₄). The solvent system was chloroform/methanol/water (65:25:4, by vol.) for the first dimension and chloroform/acetic acid/methanol/water (80:15:12:4, by vol.) for the second dimension. Another plate was treated with standards diphosphatidylglycerol, phosphatidylethanolamine, (phosphatidylglycerol, phosphatidylinositol and phosphatidylcholine) (C5646, P7693, P0514, P5766 and P3644, respectively; Sigma). TLC was performed in triplicate. Plates were sprayed with the reagents anisaldehyde (Kudo, 2001), α -naphthol and ninhydrin (N0094; Tokyo Kasei Co). Extracts from strain Murakami^T exhibited the presence of phosphatidylethanolamine and two unknown phosphatidylinositol mannosides.

The DNA G + C content was determined according to the methods of Ezaki *et al.* (1990). Purified DNA (10 µg) was dissolved in 40 µl distilled water and denatured by boiling for 10 min. Digestion was performed with 40 µl P1 nuclease (5 U ml⁻¹; Takara) at 50 °C for 1 h followed by the addition of 40 µl alkaline phosphatase (2.4 U ml⁻¹ P-4252; Sigma-Aldrich) and further incubation at 37 °C for 1 h. A 10 µl aliquot was injected into an HPLC (LC-10 system; Shimadzu) connected with a Union Uk-18 column (Imtakt). The G + C content of strain Murakami^T was determined to be 63.8 mol%.

Genomic DNA was extracted using a yeast and Gram-positive bacteria DNA purification kit (Gentra Systems) following the instructions of the manufacturer. The extracted DNA was used for *in vitro* amplification of the 16S rRNA gene by PCR (Saiki *et al.*, 1988) with 16S rRNA gene-specific primers 5'-AGTTGATCCTGGCTC-3' (sense positions 10–25 in the *Escherichia coli* numbering system; Brosius *et al.*, 1978) and 5'-AAGGAGGTGATCCAGCC-3' (antisense positions 1541–1525) and a Takara PCR amplification kit (Takara Syuzo). Amplified products were sequenced using an ABI Prism 3100 automated sequencer (Applied Biosystems) following the manufacturer's instructions, by double-strand sequencing with the primer walking technique. The 16S rRNA sequence of strain Murakami^T was aligned with those of other members of the family Actinomycetaceae and 16S rRNA gene sequence similarities were determined by using the CLUSTAL W program (Higgins & Sharp, 1988) supported by DDBJ (<http://www.ddbj.nig.ac.jp>). A phylogenetic tree was constructed with the neighbour-joining method (Saitou & Nei, 1987). Gaps and regions of ambiguous sequence alignment were excluded from subsequent analysis. A bootstrap confidence analysis was performed with 1000 replicates to determine the reliability of the tree topology (Felsenstein, 1985). The phylogenetic tree of 16S rRNA gene sequences is shown in Fig. 1.

The phylogenetic analysis and the pattern of enzyme activities show that strain Murakami^T is related to the genus *Arcanobacterium*. It is interesting to note that the *Arcanobacterium* species that have been described to date

Fig. 1. Phylogenetic neighbour-joining tree based on 16S rRNA gene sequences. The sequence of *Actinomyces suimastitidis* CCUG 39276^T was used as an outgroup. Numbers at branch points are percentages of 1000 bootstrap replicates. Bar, 0.01 substitutions per site.

are facultative anaerobes, whereas strain Murakami^T is strictly anaerobic. There are quantitative differences in the cellular fatty acid patterns from strain Murakami^T and other *Arcanobacterium* species: strain Murakami^T contains a rather higher percentage of C_{18:1} (47.3 %) and a smaller percentage of C_{18:0} (3.8 %), compared with 20.9–37.0 and 12.3–24.7 %, respectively, in other species (Lehnen *et al.*, 2006). However, strain Murakami^T contains the same menaquinone component, MK-10(H₄), as that reported for *Arcanobacterium* species (Lehnen *et al.*, 2006). The polar lipid pattern appears to belong to phospholipid type PII (Lechevalier & Lechevalier, 1980). Table 1 shows the chief characteristics that differentiate between strain Murakami^T and the type strains of other *Arcanobacterium* species.

Chemotaxonomic and phylogenetic analyses have demonstrated that strain Murakami^T is a member of a novel species, for which the name *Arcanobacterium abortusis* sp. nov. is proposed.

Description of *Arcanobacterium abortusis* sp. nov.

Arcanobacterium abortusis (a.bor.ti.su'is. L. n. *abortus* -us an abortion; L. n. *sus suis* a pig; N.L. gen. n. *abortusis* from an abortion of a pig).

Cells are short and diphtheroid-shaped and are arranged in either V-shapes or palisades. Colonies have a translucent smooth surface and are strictly anaerobic. Catalase- and indole-negative. Reduces nitrate. Soluble starch is hydrolysed but gelatin is not. Utilizes fructose, galactose, glucose, cellobiose, lactose, maltose, sucrose, trehalose, melezitose, raffinose, inulin, soluble starch, adonitol and glycerol. Does not utilize arabinose, ribose, xylose, rhamnose, mannose, sorbose, melibiose, amygdalin, arbutin, salicin, dulcitol, erythritol, mannitol, sorbitol or inositol. Produces α -galactosidase, β -galactosidase, α -glucosidase and leucine arylamidase but not urease, β -glucosidase or pyroglutamic acid arylami-

Table 1. Characteristics of strain Murakami^T and some species of the genus *Arcanobacterium*

Strains/species: 1, Murakami^T; 2, *A. bernardiae* DSM 9152^T; 3, *A. pluranimalium* DSM 13483^T; 4, *A. phocae* DSM 10002^T; 5, *A. hippocoleae* DSM 15539^T; 6, *A. haemolyticum* DSM 20595^T; 7, *A. pyogenes* DSM 20630^T; 8, *A. bialowienzense* (n=4); 9, *A. bonasi* (n=9). Data for reference strains were taken from Lehnen *et al.* (2006). +, Positive; –, negative; w, weakly positive; (+), mostly positive; (–), mostly negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Catalase	–	–	+	–	–	–	–	–	–
α-Galactosidase	+	–	–	+	–	–	–	–	–
β-Galactosidase	+	–	–	+	+	+	+	–	–
α-Glucosidase	+	+	–	–	+	+	+	–	–
β-Glucosidase	–	–	w	w	–	–	–	–	–
β-Glucuronidase	+	–	–	–	+	w	+	+	+
Leucine arylamidase	+	–	–	+	+	–	+	+	w
Utilization of:									
Ribose	–	+	+	+	–	+	+	+	–
Xylose	–	–	–	w	–	–	+	–	–
Fructose	+	–	–	–	–	–	–	+	+
Lactose	+	–	–	w	+	+	+	–	w
Maltose	+	+	w	w	–	+	+	(–)	w
Sucrose	+	–	–	–	–	w	w	–	–
Adonitol	+	–	–	–	–	–	–	(–)	w
Erythritol	–	–	–	–	–	–	–	–	w
Glycerol	+	–	–	–	–	–	–	(+)	w
Mannitol	–	–	–	w	–	–	–	–	–
Gelatin	–	–	+	–	–	–	+	–	–
Fatty acid content (%)									
C _{14:0}	12.0	ND	ND	ND	ND	ND	8.5	7.8–7.5	1.6–1.8
C _{16:1ω7}	5.2	ND	ND	ND	ND	ND	3.7	1.7–1.8	0.0–0.4
C _{16:0}	17.3	ND	ND	ND	ND	22.5	33.2	30.6–33.4	24.4–25.2
C _{18:1ω9}	47.3	ND	ND	ND	ND	37	33.2	20.9–24.7	30.4–32.4
C _{18:0}	3.8	ND	ND	ND	ND	24.7	12.3	20.6–16.4	22.0–19.8
Major menaquinone	MK-10(H ₄)	ND	ND	ND	ND	MK-9(H ₄)	MK-10(H ₄)	MK-10(H ₄)	MK-10(H ₄)

dase. Fermentation products are lactic, succinic and acetic acids. Whole-cell sugars include rhamnose and ribose. Cell-wall peptidoglycan contains glutamic acid, alanine and lysine in the molar ratio of 1:2:1. Major fatty acids are C_{14:0}, C_{16:1ω7}, C_{16:0} and C_{18:1ω9}. The major menaquinone is MK-10(H₄). Polar lipids are phosphatidylethanolamine and two unknown phosphatidylinositol mannosides. The G+C content of the type strain is 63.8 mol %.

The type strain is Murakami^T (=ATCC BAA-1522^T =DSM 19515^T =JCM 14813^T), isolated from a sow's placenta after an abortion.

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