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Kroppenstedtia pulmonis sp. nov. and *Kroppenstedtia sanguinis* sp. nov., isolated from human patients

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Abstract

Three human clinical strains (W9323^T, X0209^T and X0394) isolated from lung biopsy, blood and cerebral spinal fluid, respectively, were characterized using a polyphasic taxonomic approach. Comparative analysis of the 16S rRNA gene sequences showed the three strains belonged to two novel branches within the genus *Kroppenstedtia*: 16S rRNA gene sequence analysis of W9323^T showed closest sequence similarity to *Kroppenstedtia eburnea* JFMB-ATE^T (95.3 %), *Kroppenstedtia guangzhouensis* GD02^T (94.7 %) and strain X0209^T (94.6 %); sequence analysis of strain X0209^T showed closest sequence similarity to *K. eburnea* JFMB-ATE^T (96.4 %) and *K. guangzhouensis* GD02^T (96.0 %). Strains X0209^T and X0394 were 99.9 % similar to each other by 16S rRNA gene sequence analysis. The DNA-DNA relatedness was 94.6 %, confirming that X0209^T and X0394 belong to the same species. Chemotaxonomic data for strains W9323^T and X0209^T were consistent with those described for the genus *Kroppenstedtia*: whole-cell peptidoglycan contained LL-diaminopimelic acid; the major cellular fatty acids were *iso*-C_{15} and *anteiso*-C_{15}; and the major menaquinone was MK-7. Different endospore morphology, carbon utilization profiles, and whole cell wall sugar patterns of strains W9323^T and X0209^T supported by phylogenetic analysis enabled us to conclude that the strains represent two new species within the genus *Kroppenstedtia*, for which the names *Kroppenstedtia pulmonis* sp. nov. (type strain W9323^T = DSM 45752^T = CCUG 68107^T) and *Kroppenstedtia sanguinis* sp. nov. (type strain X0209^T = DSM 45749^T = CCUG 38657^T) are proposed.

Introduction

The family *Thermoactinomycetaceae* was established to accommodate new taxa (*Mechercharimyces* spp.) and the previously described genera *Thermoactinomyces*, *Laceyella*, *Thermoflavimicrobium*, *Seinonella* and *Planifilum* (Matsuo et al. 2006). The family now encompasses 19 genera, with representatives isolated from clinical specimens (e.g. *Desmospora*, *Marinithermofilum*, *Hazenella*) or environmental sources (e.g. *Mechercharimyces*, *Lihuaxuella*, *Geothermomicrobium* and *Risungbinella*) (Matsuo et al. 2006; Yassin et al. 2009; Yu et al. 2012; Buss et al. 2013; Zhou et al. 2014; Kim et al. 2015; Zhang et al. 2015). Salient properties of the family *Thermoactinomycetaceae* include thermotolerant growth up to 60 °C, Gram positivity, non-acid fastness and formation of single spores that may be sessile or on simple sporophores with the structure and properties of endospores. The description of the family was emended by establishing the 16S rRNA gene sequence signature nucleotides as required for suprageneric assignment (Yassin et al. 2009). Von Jan et al. (2011) further emended the description of the family *Thermoactinomycetaceae* (to contain either LL-diaminopimelic acid or *meso*-diaminopimelic acid) when they described a new genus and species, *Kroppenstedtia eburnea*, that contained the isomer LL-diaminopimelic acid in its whole-cell peptidoglycan.

Kroppenstedtia species have been isolated from environmental and clinical sources. Although the type strain of *K. eburnea* was isolated from an environmental source (plastic surface in a contract manufacturing company in Germany), Barker et al. (2012) identified 14 strains of this species in clinical (blood, skin, peritoneal fluid, cerebral spinal fluid) samples in the United States. Another species, *Kroppenstedtia guangzhouensis*, was isolated from soil in south China (Yang et al. 2013). Three human clinical strains of *Kroppenstedtia* species were identified in a retrospective evaluation of 16S rRNA gene sequences at the Special Bacteriology Reference Laboratory (SBRL). Genotypic and phenotypic data suggest that strain W9323^{T} represents a new species, for which we propose the name *Kroppenstedtia pulmonis* sp. nov., and that strains X0209^{T} and X0394 both represent another new species, for which we propose the name *Kroppenstedtia sanguinis* sp. nov.

Materials and methods

Bacterial strains

Strain X0209^T was obtained from the Culture Collection University of Göteborg (CCUG), Göteborg, Sweden as '*Thermoactinomyces sanguinis*' (CCUG 38657^T). Two strains, W9323^T and X0394, were sent to the SBRL, Centers for Disease Control and Prevention for identification. *K. eburnea* DSM 45196^T was used as a phenotypic, chemotaxonomic and genetic control and *K. guangzhouensis* GD02^T and *Melghirimyces algeriensis* NariEX^T were used as chemotaxonomic and genetic controls throughout this study. The GenBank accession numbers for the 16S rRNA gene sequences and the patients' data associated with these strains are given in Table 1.

Phenotypic analyses

Morphological, cultural, physiological and biochemical analyses

To examine morphologic features, strains were grown aerobically using brain heart infusion (BHI) broth, heart infusion agar (HIA; Becton, Dickinson and Co, Sparks, MD) supplemented with 5 % rabbit blood, HIA slants, trypticase soy agar (TSA) supplemented with 5 % sheep blood (Becton, Dickinson and Co) at 35 and 45 °C for 3 to 7 days and then examined for microscopic and macroscopic features. Gram and modified Kinyoun acid-fast staining were conducted as described previously (Berd 1973). Cultures were examined for optimal growth at 35, 45, 50 and 60 °C for 7 days on HIA slants with 5 % rabbit blood. Optimal growth was determined by comparative observation of the amount of cell mass production at each temperature. All phenotypic studies were performed under optimal growth conditions (at 45 °C in air).

Decomposition tests for adenine, casein, esculin, hypoxanthine, tyrosine, urea and xanthine, utilization of 22 carbohydrates as sole source of carbon, utilization of acetamide and citrate, arylsulfatase production and nitrate reduction and growth in the presence of lysozyme were performed as described previously (Conville and Witebsky 2007; Conville et al. 2008; Weyant et al. 1996; Yassin et al. 1995). The decomposition of casein was compared with casein plus 0.5 % NaCl as described by von Jan et al. (2011).

Antimicrobial susceptibility testing

Since no guidelines were available for the genus *Kroppenstedtia*, the MICs to 10 antimicrobial agents were determined following interpretative breakpoints as recommended for aerobic actinomycetes (Clinical and Laboratory Standards Institute 2011) for amikacin, amoxicillin-clavulanate, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, moxifloxacin and trimethoprim-sulfamethoxazole; the interpretive breakpoint for ampicillin used was that recommended for *Enterobacteriaceae* (Clinical and Laboratory Standards Institute 2015). The preparation of the inoculum suspension followed the guidelines as described previously (Clinical and Laboratory Standards Institute 2003).

Chemotaxonomic analyses

Assays of diaminopimelic acid stereoisomers and whole-cell sugars were performed by thin-layer chromatography using methods described previously (Lechevalier and Lechevalier 1970; Rhuland et al. 1955; Staneck and Roberts 1974). Isoprenoid quinones and polar lipids were extracted and purified and analyzed by the method of Minnikin et al. (1984). Analysis of isoprenoid quinones by HPLC was performed as described by Kroppenstedt (1982, 1985). Cellular fatty acids were prepared by the method of Klatte et al. (1994) and the fatty acid methyl esters were then separated as described by Sasser (1990) using the Microbial Identification System (MIDI, Inc., Sherlock version 6.1). Standardization of the physiological age of the study and reference strains was obtained by choosing the sector from a quadrant streak of culture plates.

Genetic analyses

16S rRNA gene sequence analysis

Purification of whole-cell DNA, amplification of near full-length 16S rRNA gene fragments, primers and nucleotides for PCR, purification of amplicons and DNA cycle sequencing were described previously (Lasker et al. 2011). Consensus 16S rRNA gene sequences were assembled and edited using Sequencher version 4.10.1 software. To identify related gene sequences in the GenBank database, consensus sequences were submitted to GenBank using BLASTn software

(https://www.ncbi.nlm.nih.gov/blast/). A multiple sequence alignment was created using Clustal W (within Geneious 8.1.4), from which gaps and 5' and 3' ends were trimmed. The distance matrix was calculated using DNADIST (Kimura 2-correction parameter) (Felsenstein 1989). Phylogenetic trees were constructed using the neighbor-joining (NEIGHBOR), maximum likelihood (DNAML with global rearrangements) and maximum parsimony (DNAPARS with global rearrangements) methods available in the PHYLIP package (Felsenstein, 1989). Stability of groupings within the neighbor-joining tree was estimated by bootstrap analysis (1000 replications) using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein 1989). The phenograms were visualized by using the program DRAWGRAM (Felsenstein 1989). The sequence of the type strain of *Bacillus subtilis* was used as the outgroup.

DNA-DNA hybridization and DNA G + C content

Cells for hybridization and G+C mol% determination were disrupted using a Constant Systems TS 0.75 KW (IUL Instruments, Germany). DNA in the crude lysates was purified on hydroxyapatite by chromatography as described by Cashion et al. (1977). DNA-DNA hybridization was carried out in duplicate as described by De Ley et al. (1970) as modified by Huss et al. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostat 6 x 6 multicell changer and a temperature controller with *in-situ* temperature control. DNA-DNA hybridization studies were between the clinical strains X0209^T and X0394 to confirm they were the same genomic species. DNA-DNA hybridization studies between X0209^T and W9323^T and their respective closest phylogenetically related neighbors were not conducted because of the low probability inferred from 16S rRNA gene similarities observed (Meier-Kolthoff et al. 2013).

DNA G+C content

The method of Mesbah et al. (1989) was performed to determine the G+C content of the novel type strains.

Results and discussion

Strains W9323^T, X0209^T and X0394 were aerobic, mesophilic to thermophilic, Gram-positive bacteria but were not acid fast. Substrate hyphae were filamentous and branched, and could be seen as fringes around the colony margins; no aerial hyphae were observed. Rare elongated (paddle shaped) endospores on long, unbranched sporophores were seen on Gram-stained smears of W9323^T; single globose endospores on unbranched sporophores were seen on Gram-stained smears of X0209^T and

X0394. Colonies of all three strains showed beta hemolysis on TSA with 5 % sheep blood at 45 °C. Growth occurred at 35 and 45 °C but not at 50 °C with optimum growth at 45 °C; the optimal growth at 45 °C was consistent with the type strain of *K. eburnea* but differed from the optimal growth at 50 °C of the *K. guangzhouensis* type strain. Pale yellow colonies on 7-day TSA with 5 % sheep blood had irregular margins with random surface ridges at 35 and 45 °C. Except for the production of paddle-shaped endospores on long, unbranched sporophores of strain W9323^T, the microscopic morphology was consistent with the type strains of *K. eburnea* and *K. guangzhouensis*. The macroscopic morphology of W9323^T, X0209^T and X0394 was consistent with that described for the type strains of *K. eburnea* and *K. guangzhouensis*, however, none of the three strains produced aerial hyphae as reported by von Jan et al. (2011) and Yang et al (2013).

Table 2 gives the differential phenotypic, chemotaxonomic and genetic characteristics of the study strains and their closest phylogenetic relatives. Strains X0209^T and X0394 both were able to utilize D-mannitol; W9323^T was the only strain able to utilize D-glucose and sucrose. Strain X0209^T utilized cellobiose and salicin but the related strain X0394 did not.

Results of antimicrobial susceptibility testing showed strains W9323^T, X0209^T and X0394 were resistant (MIC, ≥ 8 µg/ml) to clarithromycin but were susceptible to amikacin (MIC, ≤ 8), amoxicillin-clavulanate (MIC, ≤ 8/4 µg/ml), ampicillin (MIC, ≤ 4 µg/ml), ceftriaxone (MIC, ≤ 8 µg/ml), ciprofloxacin (MIC, ≤ 1 µg/ml), imipenem (MIC, ≤ 4 µg/ml), linezolid (MIC, ≤ 8 µg/ml), minocycline (MIC, ≤ 1 µg/ml) , moxifloxacin (MIC, ≤ 1 µg/ml) and trimethoprim-sulfamethoxazole (MIC, ≤ 2/38 µg/ml). Except for resistance to ampicillin of *K. eburnea* JFMB-ATE^T, susceptibility results of the three study strains were consistent with *K. eburnea* JFMB-ATE^T. The antimicrobial susceptibility test results in our study were consistent with the MIC results of 14 clinical strains of *K. eburnea* reported by Barker et al. (2012); for example, all their strains were susceptible to all antimicrobial agents tested except for clarithromycin.

Whole-cell wall hydrolysates contained LL-diaminopimelic acid, ribose and traces of galactose (X0209^T and X0394); *K. eburnea* JFMB-ATE^T contained ribose and glucose. The predominant menaquinones of W9323^T were MK-7 (95 %) and MK-8 (5 %); the predominant menaquinones of X0209^T and X0394 were identified as MK-7 (97 %) and MK8 (3 %). Polar lipids were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylmethylethanolamine (PME), two unknown phospholipids (PL) (4PL, W9323^T and X0394; 5PL, X0209^T) and one unknown glycolipid (GL) (1GL, W9323^T, X0209^T and X0394). The fatty acid profile of the three strains consisted predominantly of *iso*-$C_{15:0}$ (74-75 %) and *anteiso*-$C_{15:0}$ (11-13 %).

DNA-DNA hybridization studies between the clinical strains X0209^T and X0394 confirmed they were the same genomic species with relatedness value of 93.6% ± 6.5%. The genomic DNA G+C content of strains W9323^T and X0209^T was 45.9 and 50.6 mol%, respectively. These values fall within the range of genomic DNA G+C content reported for the genus *Kroppenstedtia* as it exists presently (46 to 56 mol%) (von Jan et al. 2011, Yang et al 2013).

Based on 16S rRNA gene sequence analysis, the clinical strains were assigned within the subclade for the genus *Kroppenstedtia*, within the family *Thermoactinomycetaceae* (Fig. 1). The highest sequence similarity with strain W9323^T was to *K. eburnea* JFMB-ATE^T (95.3 % sequence similarity), *K. guangzhouensis* GD02^T (94.7 % sequence similarity), *Melghirimyces thermohalophilus* Nari11A^T (94.5 % sequence similarity), *M. algeriensis* DSM 45474^T (94.3 % similarity), and *Desmospora activa* DSM 45169^T (94.2 % similarity). The 16S rRNA gene sequences for strains X0209^T and X0394 were 99.9 % identical to each other. Strain X0209^T showed the highest sequence similarity to *K. eburnea* JFMB-ATE^T (96.4 % similarity), *K. guangzhouensis* GD02^T (96.0 % similarity), *M. algeriensis* DSM 45474^T (94.3 % similarity), *M. thermohalophilus* Nari11A^T and *D. activa* DSM 45169^T (93.7 % similarity). The 16S rRNA gene sequences of strain X0209^T and *K. eburnea* JFMB-ATE^T differed by 59 bp; the sequences of strain W9323^T and *K. eburnea* JFMB-ATE^T differed by 66 bp.

From the results of our phenotypic, chemotaxonomic and genetic studies, it is proposed that strains W9323^T and X0209^T be classified in the genus *Kroppenstedtia* as *Kroppenstedtia pulmonis* sp. nov. and *Kroppenstedtia sanguinis* sp. nov., respectively.

Description of *Kroppenstedtia pulmonis* sp. nov.

K. pulmonis (N. L. fem. adj. *pul.mo'nis*. L. n. pulmo-onis, lung; L. gen. n. pulmonis of a lung, isolated from a lung).

Cells are Gram-positive, non-acid-fast and filamentous. Elongated spores (paddle shaped) are formed singly on sessile sporophores on substrate hyphae. Pale yellowish-gray colonies are wrinkled with random ridges in 3 to 7 days at 35 and 45 °C on trypticase soy agar with 5 % sheep blood. Colonies are beta hemolytic but do not form aerial hyphae. Growth occurs at 35 and 45 °C but not at 50 °C. D-Glucose and sucrose are utilized but i-adonitol, L-arabinose, cellobiose, citrate, dulcitol, i-erythritol, D-fructose, D-galactose, glycerol, i-*myo*-inositol, lactose, maltose, D-mannitol, mannose, melibiose, raffinose, L-rhamnose, salicin, D-sorbitol, trehalose and xylose are not utilized. Grows in the presence of lysozyme but has no

arylsulfatase activity and does not reduce nitrate. Hydrolyses casein with 0.5 % NaCl but does not hydrolyse acetamide, adenine, casein, hypoxanthine, tyrosine, urea or xanthine. Esculin hydrolysis is positive by browning but negative by UV light absorption. Major fatty acids (>10 %) are *iso*-$C_{15:0}$ (75 %) and *anteiso*-$C_{15:0}$ (11 %). Whole-cell hydrolysates contain LL-diaminopimelic acid and the sugar ribose. The predominant menaquinones of strain W9323^T are MK-7 (95 %) and MK-8 (5 %). Polar lipids are phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylmethylethanolamine (PME), one unknown phospholipid (4PL) and one unknown glycolipid (1GL). The type strain (W9323^T = DSM 45752^T = CCUG 68107^T) was isolated from lung biopsy of a 78-year-old male patient from New York, USA. The G+C content of its genomic DNA is 45.9 mol%.

Description of *Kroppenstedtia sanguinis* sp. nov.

K. sanguinis (san'gui.nis L. n. sanguis-inis, blood; L. gen. n. sanguinis, of blood).

Cells are Gram-positive, non-acid-fast and filamentous. Globose spores are formed singly on sessile sporophores on substrate hyphae. Pale yellowish gray colonies are wrinkled with random ridges in 3 to 5 days at 35 and 45 °C on trypticase soy agar with 5 % sheep blood. Colonies are beta hemolytic but do not form aerial hyphae. Growth occurs at 35 and 45 °C but not at 50 °C. Cellobiose, D-mannitol, mannose and salicin are utilized but i-adonitol, L-arabinose, citrate, dulcitol, i-erythritol, D-fructose, D-galactose, D-glucose, glycerol, i-*myo*-inositol, lactose, maltose, melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose and D-xylose are not utilized. Grows in the presence of lysozyme but has no arylsulfatase activity and does not reduce nitrate. Hydrolyses casein with 0.5 % NaCl but does not hydrolyse acetamide, adenine, casein, hypoxanthine, tyrosine, urea or xanthine. Esculin hydrolysis is positive by browning but negative by UV light absorption. The predominant menaquinone is MK-7 (95 %). Whole-cell hydrolysates contain LL-diaminopimelic acid and traces of the sugar galactose. Major fatty acids (>10 %) are *iso*-$C_{15:0}$ (74 %) and *anteiso*-$C_{15:0}$ (12 %). Whole-cell hydrolysates contain LL-diaminopimelic acid and traces of galactose and ribose. Polar lipids are phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylmethylethanolamine (PME), one unknown phospholipid (5PL), and one unknown glycolipid (1GL). The type strain (X0209^T = DSM 45749^T = CCUG 38657^T) was isolated from the blood of a 59-year-old male from Gävle, Sweden. The G+C content of its genomic DNA is 50.6 mol%.

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Figure Legend

Figure 1. Neighbor joining tree showing the positions of *Kroppenstedtia sanguinis* sp. nov. and *Kroppenstedtia pulmonis* sp. nov. within the family *Thermoactinomycetaceae*. The tree was constructed based on an analysis of ~1480 nt. Bootstrap values shown at the nodes are expressed as a percentage of 1000 replications (neighbor joining); only values >70 % are shown. ML, nodes common to the neighbor joining and maximum likelihood analyses; MP, nodes common to the neighbor joining and maximum parsimony analyses (Felsenstein 1989). The sequence of the type strain *Bacillus subtilis* was used as the outgroup.