Staphylococcus petrasii subsp. pragensis subsp. nov., occurring in human clinical material

Pavel Svec,1 Annelies De Bel,2 Ivo Sedlacik,1 Petr Petras,3 Tereza Gelbicova,1 Jitka Cernohlavkova,1 Ivana Maslanova,4 Margo Cnockaert,5 Ivana Varbanovova,3 Fedoua Echahidi,2 Peter Vandamme5 and Roman Pantucek4

Correspondence Pavel Svec
mpavel@sci.muni.cz

1Czech Collection of Microorganisms, Department of Experimental Biology, Faculty of Science, Masaryk University, Kamenice 5, Bl. A25, 625 00 Brno, Czech Republic
2Department of Microbiology and Infection Control, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel (VUB), Laarbeeklaan 101, 1090 Brussels, Belgium
3Reference Laboratory for Staphylococci, National Institute of Public Health, Sobrava 48, 100 42 Prague 10, Czech Republic
4Division of Genetics and Molecular Biology, Department of Experimental Biology, Faculty of Science, Masaryk University, Kolafska 2, 611 37 Brno, Czech Republic
5Department of Biochemistry and Microbiology, Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium

Seven coagulase-negative, oxidase-negative and novobiocin-susceptible staphylococci assigned tentatively as Staphylococcus petrasii were investigated in this study in order to elucidate their taxonomic position. All strains were initially shown to form a genetically homogeneous group separated from remaining species of the genus Staphylococcus by using a repetitive sequence-based PCR fingerprinting with the (GTG)5 primer. Phylogenetic analysis based on 16S rRNA gene, hsp60, rpoB, dnaJ, gap and tuf sequences showed that the group is closely related to Staphylococcus petrasii but separated from the three hitherto known subspecies, S. petrasii subsp. petrasii, S. petrasii subsp. croceilyticus and S. petrasii subsp. jettensis. Further investigation using automated ribotyping, MALDI-TOF mass spectrometry, fatty acid methyl ester analysis, DNA–DNA hybridization and extensive biotyping confirmed that the analysed group represents a novel subspecies within S. petrasii, for which the name Staphylococcus petrasii subsp. pragensis subsp. nov. is proposed. The type strain is NRL/St 12/356T (=CCM 8529T =LMG 28327T).

Staphylococcus petrasii is a recently described coagulase-negative, oxidase-negative and novobiocin-susceptible species. At the time of description of S. petrasii, two subspecies, namely Staphylococcus petrasii subsp. petrasii and Staphylococcus petrasii subsp. croceilyticus were recognized (Pantucek et al., 2013). Recently, another coagulase-negative species Staphylococcus jettensis (De Bel et al., 2013) was revealed to be a member of the species S. petrasii and was reclassified as Staphylococcus petrasii subsp. jettensis (De Bel et al., 2014).

The present taxonomic study deals with seven strains (Table 1) assigned tentatively to the species S. petrasii, but revealing characteristics differentiating them from the three hitherto known subspecies of S. petrasii. Six isolates (NRL/St 12/356T, NRL/St 93/321, NRL/St 94/190, NRL/St 13/623, NRL/St 14/114 and NRL/St 14/221) were obtained from human clinical material in different clinical laboratories in the Czech Republic and were referred to the National Reference Laboratory for Staphylococci (National Institute of Public Health, Prague) for identification. The remaining strain LMG 28328 was isolated in 2013 from a

Abbreviations: MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; NRL/St, National Reference Laboratory for Staphylococci (Prague, Czech Republic).

The GenBank/EMBL/DDBJ accession numbers for Staphylococcus petrasii subsp. pragensis subsp. nov. strains NRL/St 12/356T, NRL/St 93/321, NRL/St 94/190, NRL/St 13/623, NRL/St 14/114, NRL/St 14/221 and LMG 28328 are KMB73669–KMB73675 (16S rRNA gene); KMB73676–KMB73682 (hsp60); KMB73683–KMB73688 (rpoB); KMB73690–KMB73696 (dnaJ); KMB73697–KMB73703 (gap); and KMB73704–KMB73710 (tuf). Two supplementary tables and two supplementary figures are available with the online version of this paper.
The 16S rRNA gene (Pantuček phylogenetically closest species. Recognized species of the genus Microorganisms covering representatives of all recognized taxa of the genus Staphylococcus were obtained from the Czech Collection of Microorganisms (Masaryk University, Brno) and/or from the BCCM/LMG Bacteria Collection (Ghent University, Belgium).

Initial genotypic screening of the isolates using the repetitive sequence-based PCR (rep-PCR) fingerprinting with the (GTG)$_5$ primer was performed as described previously (Svec et al., 2010). The seven isolates revealed visually similar fingerprints (75 % similarity), grouped in a single cluster and separated from the remaining entries included in the in-house fingerprint database of the Czech Collection of Microorganisms covering representatives of all recognized species of the genus Staphylococcus. The dendrogram (Fig. S1, available in the online Supplementary Material) shows differentiation of the analysed strains from all three hitherto described subspecies of S. petrasii and the phylogenetically closest species.

The 16S rRNA gene (Pantuček et al., 2005), hsp60 (Kwok & Chow, 2003), rpoB (Mellmann et al., 2006), dnaJ (Shah et al., 2007), gap (Yugueros et al., 2000), and tuf (Bergeron et al., 2011) amplification from crude boiled cell extracts was performed as described previously. Sequencing was performed in the Eurofins MWG Operon sequencing facility (Ebersberg, Germany) and the obtained sequences were compared with those of other taxa of the genus Staphylococcus retrieved from the GenBank/EMBL/DDJB database. Determination of the closest relatives of the seven isolates was performed by using the NCBI BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were reconstructed according to the neighbour-joining and maximum-likelihood methods to confirm the tree topology, implementing the Kimura-2 substitution model and using bootstrap values based on 1000 replications, with the MEGA software version 6 (Tamura et al., 2013).

The topology of the neighbour-joining tree reconstructed with 16S rRNA gene sequences (Fig. 1) was similar to that of the maximum-likelihood tree (not shown) and showed that the isolates belonged to the species S. petrasii. However, the isolates clustered separately from the three subspecies S. petrasii subsp. petrasii (99.5 % 16S rRNA gene sequence similarity), S. petrasii subsp. croceilyticus (99.8 %), and S. petrasii subsp. jettenensis (99.4 %). Because the 16S rRNA gene analysis has limited discriminatory power for the identification of some staphylococcal species, the phylogenetic position of the novel isolates was also assessed using concatenated multi-locus sequence data of five routinely used housekeeping genes, hsp60, rpoB, dnaJ, gap and tuf, as well as the corresponding amino acid sequences. The portions of analysed gene sequences corresponded to the following gene coordinates of Staphylococcus aureus subsp. aureus NCTC 8325 (GenBank accession no. NC_007795): 260–822 for hsp60, 1443–2263 for rpoB, 29–886 for dnaJ, 46–926 for gap, and 378–1019 for tuf. Neighbour-joining and maximum-likelihood phylogenetic trees for the five housekeeping genes including the closest relatives had a very similar topology, which corresponded with that of the 16S rRNA gene sequence tree. Both nucleotide and amino acid sequence-based phylogenetic analyses showed that the seven isolates represented a well-delineated group within the S. petrasii cluster, clearly separate from the three established subspecies (Fig. 2).

The capacity of matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to distinguish among subspecies of S. petrasii was examined. For this purpose, a main reference spectrum of the strains S. petrasii subsp. petrasii CCM 8418$^T$, S. petrasii subsp. croceilyticus CCM 8421$^T$, S. petrasii subsp. jettenensis LMG 26879$^T$ and NRL/St 12/356$^T$ was added to the commercial database (version 3.3.1.0) of a Microflex instrument (Bruker) after cultivation of these strains on tryptic soy agar (Lab M) with 5 % horse blood (E&O Laboratories). Subsequently, ten clinical isolates were examined: three isolates of S. petrasii subsp. petrasii (Pantuček et al., 2013), two isolates each of S. petrasii subsp. croceilyticus (Pantuček et al., 2013) and S. petrasii subsp. jettenensis (De Bel et al., 2013), and three human clinical isolates of the present study. Nine of these strains yielded good species level identification results

### Table 1. Origin of strains of Staphylococcus petrasii subsp. pragensis subsp. nov.

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Specimen</th>
<th>Diagnosis</th>
<th>Sex (age)</th>
<th>Locality</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRL/St 12/356$^T$</td>
<td>Ejaculate</td>
<td>Prostatitis</td>
<td>M (58y)</td>
<td>Prague, CZ</td>
<td>May 2012</td>
</tr>
<tr>
<td>(=CCM 8529$^T$=LMG 28327$^T$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRL/St 93/321 (=CCM 8530)</td>
<td>Wound pus</td>
<td>Hand wound infection</td>
<td>F (22y)</td>
<td>Prague, CZ</td>
<td>April 1993</td>
</tr>
<tr>
<td>NRL/St 94/190</td>
<td>Appendix pus</td>
<td>Appendicitis</td>
<td>M (52y)</td>
<td>Prague, CZ</td>
<td>February 1994</td>
</tr>
<tr>
<td>NRL/St 13/623</td>
<td>Blood</td>
<td>Pancreatitis</td>
<td>M (55y)</td>
<td>Ostra, CZ</td>
<td>October 2013</td>
</tr>
<tr>
<td>NRL/St 14/114</td>
<td>Blood</td>
<td>Sepsis</td>
<td>M (45y)</td>
<td>Pilsen, CZ</td>
<td>February 2014</td>
</tr>
<tr>
<td>NRL/St 14/221</td>
<td>Wound pus</td>
<td>Phlegmona</td>
<td>M (56y)</td>
<td>Strakonice, CZ</td>
<td>March 2014</td>
</tr>
<tr>
<td>LMG 28328 (=CCM 8531)</td>
<td>Lambic beer</td>
<td>N/A</td>
<td>N/A</td>
<td>Brussels, Belgium</td>
<td>May 2013</td>
</tr>
</tbody>
</table>

NA, Not applicable; F, female; M, male; CZ, Czech Republic.

Lambic beer brewery air sample in Brussels (Belgium). The type and reference strains included in individual experiments and representing all three hitherto described subspecies of S. petrasii as well as representatives of the other related species of the genus Staphylococcus were obtained from the Czech Collection of Microorganisms (Masaryk University, Brno) and/or from the BCCM/LMG Bacteria Collection (Ghent University, Belgium).

The topology of the neighbour-joining tree reconstructed with 16S rRNA gene sequences (Fig. 1) was similar to that of the maximum-likelihood tree (not shown) and showed that the isolates belonged to the species S. petrasii. However, the isolates clustered separately from the three subspecies S. petrasii subsp. petrasii (99.5 % 16S rRNA gene sequence similarity), S. petrasii subsp. croceilyticus (99.8 %), and S. petrasii subsp. jettenensis (99.4 %). Because the 16S rRNA gene analysis has limited discriminatory power for the identification of some staphylococcal species, the phylogenetic position of the novel isolates was also assessed using concatenated multi-locus sequence data of five routinely used housekeeping genes, hsp60, rpoB, dnaJ, gap and tuf, as well as the corresponding amino acid sequences. The portions of analysed gene sequences corresponded to the following gene coordinates of Staphylococcus aureus subsp. aureus NCTC 8325 (GenBank accession no. NC_007795): 260–822 for hsp60, 1443–2263 for rpoB, 29–886 for dnaJ, 46–926 for gap, and 378–1019 for tuf. Neighbour-joining and maximum-likelihood phylogenetic trees for the five housekeeping genes including the closest relatives had a very similar topology, which corresponded with that of the 16S rRNA gene sequence tree. Both nucleotide and amino acid sequence-based phylogenetic analyses showed that the seven isolates represented a well-delineated group within the S. petrasii cluster, clearly separate from the three established subspecies (Fig. 2).

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The similarity values between individual ribotypes from the obtained ribotypes clustered all seven isolates into a single cluster separated from the remaining phylogenetically closely related species of the genus *Staphylococcus* (Fig. S2). The similarity values between individual ribotypes from the studied strains ranged from 64.2 % to 95.9 %. The closest ribotype pattern was revealed by *Staphylococcus devriesii* CCM 7896T (55.8 % similarity).

A Hewlett Packard 6890N gas chromatograph equipped with a flame-ionization detector and the Microbial Identification System (MIDI) was used to analyse the cellular fatty acid components. For this, bacterial cells were harvested from tryptic soy agar plates (BD) incubated for 48 h at 28 °C, and were subsequently analysed according to the manufacturer’s instructions. The predominant fatty acid was anteiso-C₁₅ : ₀, whilst iso-C₁₅ : ₀, iso-C₁₇ : ₀, anteiso-C₁₇ : ₀ and C₁₈ : ₀ were present in variable and moderate amounts. These results are in line with previous reported

Fig. 1. Unrooted neighbour-joining tree based on 16S rRNA gene sequence comparison, showing the phylogenetic relationships of isolates of *Staphylococcus petrasii* subsp. *pragensis* subsp. nov. and reference type strains of related staphylococcal taxa. *Staphylococcus aureus* subsp. *aureus* ATCC 12600T sequence was used as an outgroup. Bootstrap probability values (percentages of 1000 tree replications) are indicated at branch points. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. There were 1422 positions in the final dataset.
Fig. 2. a) Maximum-likelihood tree based on Kimura’s two-parameter model reconstructed using concatenated partial gene sequences of five housekeeping genes (hsp60, rpoB, dnaJ, gap and tuf), showing the relationships of isolates of *Staphylococcus petrasii* subsp. *pragensis* subsp. nov. and staphylococcal reference type strains. The tree with the highest log-likelihood is shown and the percentage of trees from 1000 replicates in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were 3656 positions in the final dataset.
b) Neighbour-joining tree reconstructed using concatenated partial sequences of five proteins Hsp60, RpoB, DnaJ, Gap and EF Tu, showing the relationships of isolates of *Staphylococcus petrasii* subsp. *pragensis* subsp. nov. and staphylococcal reference type strains. The bootstrap consensus tree inferred from 1000 replicates is shown and bootstrap probability values are indicated at branch points. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. There were 1243 amino acid positions in the final dataset.
51 ± 6 %, which is clearly below the threshold value of 70 % generally accepted for bacterial species delineation.

Extensive phenotypic characterization using API 50CH, API Staph, ID 32 Staph, API ZYM (bioMérieux) and STAPHYtest 24 (Erba Lachema) commercial kits, phenotypic fingerprinting using the Biolog system with the Gram-positive identification test panel GP2 MicroPlate (Biolog), and conventional biochemical, physiological and growth tests relevant for the genus Staphylococcus were done as described by Pantůček et al. (2013). The obtained results are listed in the subspecies description. Extensive testing of clumping factor production using slide coagulase test and different commercial kits showed test-dependent results for S. petrasii subsp. petrasii. All strains revealed positive agglutination reactions when tested with Pastorex Staph plus test (Bio-Rad) and Staph Plus (DiaMondiaL), but negative results were obtained using the slide coagulase test and Staphylo La Seiken (Denka Seiken), Staphytect plus (Oxoid) and Staphylase test (Oxoid) kits. Representatives of S. petrasii subsp. croceilyticus and S. petrasii subsp. jettensis, as well as all seven studied isolates, revealed negative agglutination reactions using all aforementioned methods. Table 2 shows phenotypic characteristics differentiating the novel taxon represented by strain NRL/St 12/356T from the phylogenetically closely related and phenotypically similar species. Table 3 shows the tests enabling differentiation of individual subspecies of S. petrasii. Susceptibility to antibiotics was tested by the disc diffusion method (Oxoid discs) on Mueller–Hinton agar (Oxoid, AST media) and interpreted according to the EUCAST v.4.0 (EUCAST 2014). Antimicrobial susceptibility profiles of the strains under study are given in the subspecies description.

In summary, data from the present study demonstrate that although the seven isolates represented by strain NRL/St 12/356T belong to the species S. petrasii, they represent a taxon that can be distinguished both genotypically and phenotypically from established subspecies of S. petrasii. We therefore propose to classify these isolates as the novel subspecies Staphylococcus petrasii subsp. pragensis, with strain NRL/St 12/356T (=CCM 8529T=LMG 28327T) as the type strain.

### Table 2. Key tests for phenotypic differentiation of Staphylococcus petrasii subsp. pragensis subsp. nov. from phylogenetically related and phenotypically similar species of the genus Staphylococcus

Species: 1, S. petrasii subsp. pragensis subsp. nov. (data from this study); 2, S. petrasii (including subspecies petrasii, croceilyticus and jettensis) (Pantůček et al., 2013; De Bel et al., 2013, 2014); 3, S. haemolyticus (Schleifer & Bell, 2009); 4, S. warneri (Schleifer & Bell, 2009); 5, S. hominis subsp. hominis (Schleifer & Bell, 2009); 6, S. pasteuri (Schleifer & Bell, 2009); 7, S. devriesei (Supré et al., 2010); 8, S. hominis subsp. novobiosepticus (Schleifer & Bell, 2009); 9, S. lugdunensis (Schleifer & Bell, 2009). +, 90 % or more strains positive; −, 90 % or more strains negative; d, 11–89 % strains positive; (+), delayed reaction; w, weak reaction; td, test dependent reaction; in the case of S. petrasii subsp. pragensis subsp. nov. the test results are: +, 100 % strains positive; −, 100 % strains negative.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clumping factor</td>
<td>−</td>
<td>td*</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Novobiocin resistance (5 μg)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Thioglycolate (growth)</td>
<td>−</td>
<td>−</td>
<td>(+)</td>
<td>+</td>
<td>−w</td>
<td>+</td>
<td>−w</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Pyrrolidonyl arylamidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Urease</td>
<td>−</td>
<td>d</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>+</td>
<td>d</td>
</tr>
<tr>
<td>Haemolysis</td>
<td>w</td>
<td>d</td>
<td>(+)</td>
<td>d</td>
<td>−w</td>
<td>+</td>
<td>−</td>
<td>w</td>
<td>−</td>
</tr>
</tbody>
</table>

*Data obtained in this study; strains of S. petrasii subsp. petrasii reveal positive agglutination reaction when tested with Pastorex Staph plus test (Bio-Rad) and Staph Plus (DiaMondiaL), while negative when tested using slide coagulase test and Staphylo La Seiken (Denka Seiken), Staphytect plus (Oxoid) and Staphylase test (Oxoid) kits.

In summary, data from the present study demonstrate that although the seven isolates represented by strain NRL/St 12/356T belong to the species S. petreasii, they represent a taxon that can be distinguished both genotypically and phenotypically from established subspecies of S. petrasii. We therefore propose to classify these isolates as the novel subspecies Staphylococcus petrasii subsp. pragensis, with strain NRL/St 12/356T (=CCM 8529T=LMG 28327T) as the type strain.

### Description of Staphylococcus petrasii subsp. pragensis subsp. nov.

Staphylococcus petrasii subsp. pragensis [prag.en’sis. N. L. masc. adj. pragensis of Praga, the Latin name for Prague (capital of the Czech Republic), pertaining to Prague where the type strain and two other strains were isolated].

Cells are Gram-stain-positive, non-spor-forming, non-motile, spherical cocci, occurring predominantly in pairs and clusters. Colonies on nutrient agar are circular with whole margins, flat, smooth, shiny, whitish, 1–3 mm in diameter and aerobic. Weak haemolytic activity on sheep-blood agar, production of δ-haemolysin revealed in a synergistic test with a β-haemolysin-producing strain. Grows at 45 °C; weak growth is observed at 15 °C. Growth in the presence of 12 % NaCl is strain-dependent. No growth occurs in thioglycolate medium. Positive results in tests for catalase, pyrrolidinyl arylamidase, arginine arylamidase, Voges–Proskauer reaction (acetoin) and hydrolysis of DNA. Negative results in tests for coagulase, clumping factor, hyaluronidase, thermonuclease, oxidase, urease and ornithine decarboxylase. Susceptible to novobiocin, polymyxin B and furazolidone, but resistant to bacitracine. Susceptible to lysostaphin and resistant to lysozyme. Hydrolysis of aesculin, Tween 80 and...
gelatin is negative. Esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present, but not lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, α-mannosidase or α-fucosidase activities. Acid is produced from glycerol, D-glucose, maltose and sucrose, but not from erythritol, D-arabinose, L-arabinose, D-xyllose, L-xyllose, adonitol, methyl β-D-xylside, mannose, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, methyl D-glucoside, N-acetylglucosamine, amygdalin, arbutin, cellobiose, melibiose, inulin, raffinose, starch, glycogen, xylitol, β-gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, L-arabitol, gluconate, and 2-ketogluconate. Test results were strain-dependent for acid production from ribose (3 strains positive among 7 strains), galactose (2), fructose (3), mannitol (1), salicin (1), lactose (1), trehalose (6), melezitose (4), turanose (5), and 5-ketogluconate; 2-activity of alkaline phosphatase (5), leucine arylamidase (5), valine arylamidase (2), cystine arylamidase (1), β-glucuronidase (1), α-glucosidase (5), β-glucosidase (2), N-acetyl-β-glucosaminidase (1), arginine dihydrolase (3) and nitrate reduction (1). Carbon source utilization results via respiration determined in the Biolog GP2 MicroPlate test panel is shown in Table S2. Tested strains were susceptible to cefoxitin, ciprofloxacin, clindamycin, erythromycin, trimethoprim/sulfamethoxazole, fusidic acid, gentamicin, rifampicin, tigecycline, minocycline, tetracycline, chloramphenicol, linezolid, benzylpenicillin, vancomycin and teicoplanin with the exception of strain NRL/St 12/356 T (resistant to erythromycin and tetracycline) and strain NRL/St 13/623 (resistant to benzylpenicillin).

The type strain NRL/St 12/356 T (=CCM 8529 T=LMG 28327 T) was retrieved from an ejaculate specimen from a 58-year-old male suffering with prostatitis in Prague (Czech Republic) in May 2012. The DNA G+C content of the type strain is 33.0 mol% (HPLC). Most characteristics of the type strain are in agreement with the general subspecies description. In addition, strain NRL/St 12/356 T displays the following characteristics: acid production from trehalose, melezitose and turanose, but not from ribose, galactose, fructose, mannitol, salicin, lactose or 5-ketogluconate; exhibits α-glucosidase activity, but not alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase or arginine dihydrolase activity; negative for nitrate reduction and growth in 12 % NaCl.

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