Taxonomic Note

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Pannonibacter phragmitetus, described from a Hungarian soda lake in 2003, had been recognized several decades earlier from human blood cultures as *Achromobacter* groups B and E

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We performed a polyphasic taxonomic study on isolates previously tentatively classified as *Achromobacter* groups B and E in comparison with the type strain of *Pannonibacter phragmitetus*, LMG 22736^T=NCTC 13350^T. Comparative 16S rRNA gene sequence analysis suggested that strains of *Achromobacter* groups B and E belong to *P. phragmitetus* (similarity levels were higher than 99%). DNA–DNA hybridization experiments and other genotypic and phenotypic analyses confirmed that the three taxa represent a single species. Whilst *P. phragmitetus* was described in 2003 from a Hungarian soda lake, it had been observed in human blood cultures in the UK since 1975. We present here the characteristics of the organism to facilitate its recognition in human clinical specimens and hence to determine its clinical significance.

In a numerical taxonomic analysis of the phenotypic characters of 81 strains of *Achromobacter* or *Achromobacter*-like strains, Holmes & Dawson (1983) discerned six phena that clustered separately from the reference strains of named species. They designated these strains as *Achromobacter* groups A–F. These six taxa were included in the probabilistic identification matrix of Holmes *et al.* (1986). Subsequent studies (Holmes *et al.*, 1988) established groups A, C and D as biovars of a single novel species, for which the name *Ochrobactrum anthropi* was proposed. Some genotypically (but not phenotypically) distinct strains in *O. anthropi* were subsequently proposed as a separate species *Ochrobactrum intermedium* by Velasco *et al.* (1998).

Thirty-two strains of *Achromobacter* groups B, E and F were studied by numerical analysis of their electrophoretic protein patterns (Holmes *et al.*, 1990a). Two major clusters were observed, one comprising 30 strains of groups B and E and the other containing the two strains of group F. A

similar observation was made by a study of representative strains of groups B, E and F by comparison of their DNA restriction endonuclease digest and rRNA gene probe patterns (Holmes *et al.*, 1990b). Thus, *Achromobacter* groups B and E appeared to be biovars of a single unnamed taxon, which was confirmed by subsequent characterization of representative strains of the two biovars by cellular fatty acid analysis (Holmes *et al.*, 1993).

In the present study, we describe how a comparison of 16S rRNA gene sequences indicated that the *Achromobacter* group B and E taxon corresponded to the organism described by Borsodi *et al.* (2003, 2005) as *Pannonibacter phragmitetus*, an alkalitolerant bacterium isolated from decomposing reed rhizomes in a Hungarian soda lake. We present the characteristics of this organism so that its role in human clinical infections may be properly determined.

The strains examined in the present study include *P. phragmitetus* LMG 22736^T (=NCTC 13350^T), *Achromobacter* group B strains LMG 5410 (=CL264/81), LMG 5411 (=CL299/79), LMG 5412 (=CL43/77) and LMG 5421 (=CL622/77) and *Achromobacter* group E strains LMG 5430 (=CL731/80) and LMG 5431 (=CL616/80). All *Achromobacter* group B and E strains were isolated from blood cultures in the UK. Further details of the origin of these isolates were provided by Holmes *et al.*

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Achromobacter* group B strains LMG 5410, LMG 5411, LMG 5412 and LMG 5421 and *Achromobacter* group E strains LMG 5430 and LMG 5431 are respectively AF227158, AF227157, AM269446, AM269447, AF227160 and AF227159.

A comparison of the fatty acid profiles of seven strains assigned to *P. phragmitetus* is available as supplementary material in IJSEM Online.

(1990a). All strains were grown aerobically on ordinary nutrient agar at 37 °C.

DNA for 16S rRNA gene sequencing was prepared by heating one or two colonies at 95 °C for 15 min in 20 µl lysis buffer containing 0.25 % (w/v) SDS and 0.05 M NaOH. Following lysis, 180 µl distilled water was added to the lysate. The 16S rRNA gene was amplified using oligonucleotide primers complementary to highly conserved regions of bacterial 16S rRNA genes. The forward primer was 5'-AGAGTTTGATCCTGGCTCAG-3' (hybridizing at positions 8–27, according to the *Escherichia coli* numbering system) and the reverse primer was 5'-AAGGAGGTG-ATCCAGCCGCA-3' (hybridizing at positions 1541–1522). PCR products were purified by using a NucleoFast 96 PCR Clean-up kit (Macherey-Nagel). Sequencing reactions were performed by using a BigDye Terminator cycle sequencing kit (Applied Biosystems) and purified by using a Montage SEQ₉₆ sequencing reaction cleanup kit (Millipore). Sequencing was performed by using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The eight sequencing primers used are listed in Coenye *et al.* (1999). Sequence assembly was performed using the program AutoAssembler (Applied Biosystems). Sequences were compared and aligned with sequences retrieved from GenBank using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic analyses were subsequently performed using the BioNumerics 4.5 software package (Applied Maths).

Nearly complete 16S rRNA gene sequences were determined for *Achromobacter* group B strains LMG 5410, LMG 5411, LMG 5412 and LMG 5421 and *Achromobacter* group E strains LMG 5430 and LMG 5431. The 16S rRNA gene sequences of all *Achromobacter* group B and E strains were >99.8 % similar and about 99.5 % similar to that of *P. phragmitetus* LMG 22736^T (GenBank accession no. AJ400704), suggesting that they represent the same species.

The G+C contents of representatives of *Achromobacter* groups B and E and of *P. phragmitetus* were determined as described by Mesbah *et al.* (1989). DNA was degraded enzymically into nucleosides. The nucleoside mixture obtained was then separated by HPLC using a Waters Symmetry Shield C8 column thermostatted at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage (Sigma) was used as the calibration reference. The DNA G+C contents of strains LMG 5412 (63.6 mol%), LMG 5421 (63.2 mol%), LMG 5431 (63.0 mol%) and LMG 22736^T (63.2 mol%) proved very similar and agree well with the value of 64.6 mol% determined for the type strain of *P. phragmitetus* by Borsodi *et al.* (2003).

In order to confirm the tentative identification of *Achromobacter* groups B and E as *P. phragmitetus*, we performed DNA–DNA hybridization experiments among strains LMG 5412 and LMG 5421 (*Achromobacter* group B), LMG 5430 and LMG 5431 (*Achromobacter* group E) and *P. phragmitetus* LMG 22736^T. High-molecular-mass DNA was prepared as described by Pitcher *et al.* (1989) and DNA–DNA

hybridizations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989) using an HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. The hybridization temperature was 45 °C. Reciprocal experiments were performed for every pair of strains. All hybridization values obtained were in the range of 73 to 96 % (data not shown).

Cellular fatty acid analysis was carried out with a loopful of well-grown cells after an incubation period of 48 h. Fatty acid methyl esters were prepared, separated and identified using the Microbial Identification System (Microbial ID, Inc.) as described by Vandamme *et al.* (1992). All seven strains tested proved highly similar in their cellular fatty acid profiles, with dominant features 14:0 3-OH, 16:0, 17:0, 18:0, 18:1 ω 7c, 18:0 3-OH (see Supplementary Table S1 in IJSEM Online). These results are comparable with those of Holmes *et al.* (1993). The observed quantitative differences may be explained by different cultivation and incubation conditions.

All strains were characterized biochemically in most of a range of 68 conventional biochemical tests by methods described previously by Holmes *et al.* (1975). The features presented below are based on data obtained for a total of 33 strains of *Achromobacter* groups B and E studied by Holmes *et al.* (1990a, b, 1993), including the isolates of the present study and the *P. phragmitetus* type strain. Colonies are circular, low-convex, entire, opaque or translucent, shiny and smooth. All strains tested were positive for acid production (in ammonium salt sugar medium) from glucose, adonitol, arabinose, cellobiose, fructose, inositol, maltose, rhamnose and xylose, catalase production, cytochrome oxidase production, β -galactosidase production (ONPG test), growth at 37 °C, growth at room temperature (18–22 °C), growth on β -hydroxybutyrate, growth on MacConkey agar, motility at room temperature (hanging drop preparation at 18–22 °C), urease production and utilization of citrate (Christensen's medium). Most strains tested (exceptions given in parentheses) were positive for acid from ethanol (LMG 5430, LMG 5431, CL515/79, CL158/81), glycerol (CL109/78), mannitol (LMG 22736^T, LMG 5430, LMG 5431, CL515/79, CL221/85, CL139/87), salicin (LMG 22736^T, LMG 5431), sorbitol (LMG 22736^T, LMG 5430, LMG 5431, CL515/79), sucrose (LMG 22736^T) and trehalose (CL221/85), aesculin hydrolysis (CL90/88), growth at 42 °C (LMG 5411, CL775/77, CL547/78, CL515/79), hydrolysis of Tween 20 (LMG 22736^T, LMG 5410, LMG 5412, LMG 5430, CL 299/79, CL515/79, CL734/79, CL752/80), hydrolysis of tyrosine (LMG 5410, LMG 5411, CL373/78, CL515/79, CL734/79, CL752/80, CL26/84, CL27/84, CL28/84, CL43/84, CL137/87, CL139/87, CL221/85, CL90/88, CL329/88), lipid inclusions after growth on β -hydroxybutyrate (LMG 5411, LMG 5430, LMG 5431, CL556/75, CL166/76, CL109/78, CL373/78, CL752/80, CL329/88), motility at 37 °C (hanging drop preparation) (CL166/76, CL176/87), nitrate reduction (CL90/88), nitrite reduction (LMG 5430, LMG 5431, CL90/88), oxidative metabolism in the Hugh and Leifson O-F test (LMG 5431, CL775/77, CL43/84, CL98/88) and utilization of citrate (Simmons' medium;

LMG 22736^T, LMG 5431, CL556/75, CL98/76, CL514/77, CL775/77, CL815/77, CL373/78, CL547/78, CL515/79, CL100/80, CL752/80). All strains tested were negative for acid production from dulcitol and raffinose, acid from 10 % (w/v) lactose, both acid and gas production from glucose in peptone water medium, casein digestion, fluorescence on King's B medium, gelatinase production (plate and stab methods), gluconate oxidation, growth at 5 °C, H₂S production (by both lead acetate paper and triple-sugar iron agar methods), hydrolysis of starch and of Tween 80, lysine decarboxylase production, malonate utilization, ornithine decarboxylase production, phenylalanine deamination, pigment production, production of extracellular DNase, production of 3-ketolactose and reduction of 0.4 % (w/v) selenite. Most strains tested (exceptions in parentheses) were negative for acid from lactose (CL98/76, CL166/76, CL514/77) and from 10 % (w/v) glucose (LMG 5410, LMG 5431, CL317/77, CL514/77, CL815/77, CL734/79, CL158/81, CL221/85, CL176/87, CL98/88), arginine dihydrolase production (by Moeller's method) (LMG 5430, LMG 5431, CL556/75, CL98/76, CL515/79, CL100/80, CL137/87, CL139/87, CL221/85, CL90/88), arginine dihydrolase production (by Thornley's method) (LMG 5430, LMG 5431, CL98/76), growth on cetrimide agar (LMG 22736^T, CL556/75, CL514/77, CL815/77, CL109/78, CL515/79, CL734/79, CL27/84,

CL221/85, CL176/87, CL90/88, CL98/88), KCN tolerance (CL28/84, CL90/88), lecithinase production (CL373/78) and production of a brown melanin-like pigment on tyrosine agar (LMG 5411, CL556/75, CL109/78, CL547/78). These results are comparable with those obtained by Borsodi *et al.* (2003) except that we found that most strains hydrolysed aesculin. The type strain is somewhat atypical in that it was the only one of the 33 to fail to produce acid from sucrose and was one of only two that failed to produce acid from salicin.

In summary, the 16S rRNA gene sequence data, DNA–DNA hybridization results, G + C content determinations, cellular fatty acid analysis and biochemical characterizations presented above all confirm that *Achromobacter* group B, *Achromobacter* group E and *P. phragmitetus* are the same taxon. *P. phragmitetus* is thus an organism that has the potential to cause serious bloodstream infections in humans. A case of replacement valve endocarditis (McKinley *et al.*, 1990) and two cases of septicaemia (Holmes *et al.*, 1992) have been reported. *P. phragmitetus* belongs to the *Alphaproteobacteria* and is closely related to taxa such as *Agrobacterium* and *Ochrobactrum* that occur in human clinical specimens. Differential characteristics between *P. phragmitetus* and phenotypically similar organisms from human clinical material (*Agrobacterium tumefaciens* and *O. anthropi*) are shown in Table 1.

Table 1. Phenotypic characteristics useful for the differentiation of *P. phragmitetus* from phenotypically similar taxa of clinical interest

Results from Holmes *et al.* (1986). +, 80 % or more of strains positive; –, 20 % or fewer of strains positive; d, 21–79 % of strains positive.

Characteristic	<i>P. phragmitetus</i>		<i>A. radiobacter</i> / <i>A. tumefaciens</i>	<i>O. anthropi</i>		
	Biovar B	Biovar E		Biovar A	Biovar C	Biovar D
Acid in ASS medium from:						
Adonitol	–	–	+	+	+	–
Cellobiose	+	+	+	+	+	–
Dulcitol	–	–	+	+	+	+
Ethanol	+	–	+	+	+	+
Lactose	–	–	+	–	+	–
Maltose	+	+	+	+	+	–
Mannitol	+	–	+	+	d	d
Raffinose	–	–	+	–	–	–
Salicin	+	d	+	–	d	–
Sorbitol	+	–	+	d	+	–
Sucrose	+	+	+	+	+	–
Trehalose	+	+	+	+	+	–
Acid from 10 % glucose	d	–	–	d	d	+
Aesculin hydrolysis	+	+	+	–	–	–
β-Galactosidase production	+	+	d	–	+	–
Growth at 42 °C	+	+	–	–	+	–
H ₂ S production (lead acetate paper)	–	–	+	d	+	–
Lipid inclusions after growth on β-hydroxybutyrate	+	–	–	–	–	–
Nitrite reduction	+	–	d	+	–	+
Tyrosine hydrolysis	d	+	–	–	+	–
3-Ketolactose production	–	–	+	–	–	–

As well as the type strain of *P. phragmitetus*, LMG 22736^T = NCTC 13350^T, several additional strains originally deposited as *Achromobacter* groups B or E are available from both the NCTC (<http://www.hpa.org.uk/nctc/>) and BCCM/LMG (<http://bccm.belspo.be/>) culture collections.

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