Achromobacter insolitus sp. nov. and *Achromobacter spanius* sp. nov., from human clinical samples

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A polyphasic taxonomic study (employing whole-cell protein and fatty acid analyses, 16S rDNA sequencing, DNA–DNA hybridization, determination of DNA G + C content, antibiotic susceptibility testing and extensive phenotypic characterization) was performed on 10 isolates that appeared to be related to *Alcaligenes faecalis*. The isolates were recovered from diverse environments that included human clinical samples. 16S rDNA sequence analysis indicated that these isolates belonged to the genus *Achromobacter*. Whole-cell protein analysis distinguished two groups, which were confirmed by DNA–DNA hybridization. Based on the results of this study, the organisms were classified as two novel *Achromobacter* species, *Achromobacter insolitus* sp. nov. (type strain, LMG 6003^T) and *Achromobacter spanius* sp. nov. (type strain, LMG 5911^T). *Achromobacter insolitus* can be distinguished from *Achromobacter spanius* by its ability to grow on acetamide and to assimilate mesaconate and aconitate, and by its inability to assimilate diaminobutane. Various tests allow the differentiation of both novel species from other *Achromobacter* species, including growth on acetamide, denitrification and assimilation of D-glucose, D-xylose, mesaconate, aconitate and diaminobutane.

The genus *Achromobacter* was described by Yabuuchi & Yano (1981) and originally contained a single species, *Achromobacter xylosoxidans*. Following a polyphasic taxonomic study, Yabuuchi *et al.* (1998) transferred the species *Alcaligenes ruhlandii* and *Alcaligenes piechaudii* to the genus *Achromobacter*. The same authors also transferred *Alcaligenes denitrificans* to the genus *Achromobacter* as *Achromobacter xylosoxidans* subsp. *denitrificans*. However, the reclassification of *Alcaligenes denitrificans* as a subspecies of *Achromobacter xylosoxidans* contradicts previous work that showed that there is sufficient evidence to consider both taxa as distinct species (Vandamme *et al.*, 1996). We recently proposed to formally reclassify *Alcaligenes denitrificans* as *Achromobacter denitrificans* (Coenye *et al.*, 2003). *Achromobacter xylosoxidans* is widespread in aquatic habitats, but has also been involved in opportunistic infection of humans (Kerstens & De Ley, 1984). For example,

Achromobacter xylosoxidans is capable of causing persistent respiratory tract infection in cystic fibrosis patients (Liu *et al.*, 2002). *Achromobacter piechaudii* can be isolated from soil and human clinical samples, including blood (Kiredjian *et al.*, 1986), whereas *Achromobacter ruhlandii* is a soil commensal that is not known to be pathogenic to humans (Kerstens & De Ley, 1984).

We performed a polyphasic taxonomic study to elucidate the taxonomic positions of 10 isolates that were recovered from diverse environments, including human clinical samples. These isolates resembled *Alcaligenes faecalis* phenotypically, but whole-cell protein and fatty acid analyses suggested that they belonged to the genus *Achromobacter*. We show that these isolates belong to two novel *Achromobacter* species, for which we propose the names *Achromobacter insolitus* sp. nov. and *Achromobacter spanius* sp. nov.

Strains used in this study are listed in Table 1. Reference strains of other taxa have been described previously (Vandamme *et al.*, 1995, 1996; Foss *et al.*, 1998; Yabuuchi *et al.*, 1998; Coenye *et al.*, 2003). All strains were grown aerobically on trypticase soy agar (TSA; BBL) at 37 °C unless indicated otherwise. For SDS-PAGE of whole-cell proteins, strains were grown on TSA for 48 h at 37 °C.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 6003^T and LMG 5911^T are AY170847 and AY170848, respectively.

A picture of the protein profiles of *Achromobacter spanius* and *Achromobacter insolitus* strains and tables showing their fatty acid compositions and MIC values are available as supplementary material in IJSEM Online.

Table 1. List of strains studied

API, Appareils et Procédés d'Identification, Montalieu-Vercieu, France; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Gent, Belgium.

Species and strain designation	Depositor	Source
<i>Achromobacter insolitus</i> :		
LMG 5997 (= CCUG 47056 = Gilardi 3599 = API 195-3-84)	D. Monget	Laboratory sink
LMG 6003 ^T (= CCUG 47057 ^T = Gilardi 3038 ^T = API 201-3-84 ^T)	D. Monget	Leg wound
LMG 6000 (= CCUG 47058 = Gilardi 3401 = API 198-3-84)	D. Monget	Urine
LMG 5999 (= CCUG 47059 = Gilardi 3505 = API 197-3-84)	D. Monget	Wound
LMG 6001 (= CCUG 47060 = Gilardi 3370 = API 199-3-84)	D. Monget	
LMG 6010 (= CCUG 47061 = Gilardi 4207 = API 72-4-82)	D. Monget	
<i>Achromobacter spanius</i> :		
LMG 14525 (= CCUG 24722 = Gilardi 5347)	G. L. Gilardi	Blood
LMG 5911 ^T (= CCUG 47062 ^T = API 198-2-84 ^T)	D. Monget	
LMG 5896 (= CCUG 47063 = API 151-2-84)	D. Monget	
LMG 5909 (= CCUG 47064 = API 187-2-84)	D. Monget	

Preparation of whole-cell proteins and SDS-PAGE were performed as described previously (Pot *et al.*, 1994). Densitometric analysis, normalization and interpolation of protein profiles and numerical analysis with the Pearson product-moment correlation coefficient were performed by using GelCompar 4.2 software (Applied Maths). Sequences of the 16S rRNA genes of strains LMG 6003^T and LMG 5911^T were determined as described previously (Coenye *et al.*, 1999). Phylogenetic and bootstrap analyses (1000 replicates) were performed by using Kodon software (Applied Maths); a phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987). Preparation of high-molecular-mass DNA for DNA-DNA hybridization experiments and determination of the degree of DNA-DNA binding by the initial renaturation rate method were performed as described previously (De Ley *et al.*, 1970; Vandamme *et al.*, 1992). Each value is the mean of at least two hybridization experiments. Total DNA concentration was 65 µg ml⁻¹ and the optimal renaturation temperature in 2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) was 79 °C. Alternatively, high-molecular-mass DNA was prepared as described by Pitcher *et al.* (1989) and DNA-DNA hybridization was performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS 7000 BioAssay Reader (PerkinElmer) for fluorescence measurements. Hybridization temperature was 50 °C. Reciprocal experiments were performed for every pair of strains. For determination of DNA base composition, DNA (prepared as described above) was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture was then separated by HPLC, using a Waters SymmetryShield C8 column thermostatted at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated λ-phage DNA (Sigma) was used as the calibration reference. After 24 h incubation at 35 °C, a loopful of well-grown cells was harvested; fatty acid methyl esters were prepared as described previously

(Vandamme *et al.*, 1992) and separated and identified by using the Sherlock Microbial Identification system (version 3.0; MIDI). API galleries (API 50CH, API 50AO and API 50AA; bioMérieux) were used to determine assimilation of 147 organic compounds as sole carbon sources, as described previously (Kerstens *et al.*, 1984). Classical phenotypic tests were performed as described previously (Vandamme *et al.*, 1993). API 20NE and API ZYM tests were performed according to the recommendations of the manufacturer (bioMérieux). MIC values towards levofloxacin, ciprofloxacin, ofloxacin, sparfloxacin, erythromycin, roxithromycin, clarithromycin, azithromycin, cefotaxime, ceftiofime and rifampicin were determined for all strains listed in Table 1 by using the agar dilution method, conforming to NCCLS (1995) guidelines. Strains were grown on Mueller-Hinton agar (BRL) for 16–20 h at 35 °C.

Reproducibility of SDS-PAGE of whole-cell proteins was checked by preparing protein extracts in duplicate. The correlation level between patterns was >93 % (data not shown). After numerical analysis and visual comparison, two clusters could be delineated, whilst reference strains of other *Achromobacter* and *Alcaligenes* species occupied separate positions in the dendrogram (Fig. 1). One cluster contained *Achromobacter spanius* LMG 5896, LMG 14525, LMG 5911^T and LMG 5909. *Achromobacter insolitus* strains LMG 5997, LMG 6000, LMG 5999, LMG 6003^T, LMG 6010 and LMG 6001 formed the second cluster. The protein patterns of strains in both clusters were also clearly different from those of *Bordetella* and *Kerstersia* strains (data not shown). The 16S rDNA sequences of isolates LMG 5911^T and LMG 6003^T (representatives of the two protein-electrophoretic groups) were determined and compared with available 16S rDNA sequences of other β-Proteobacteria (Fig. 2). The 16S rDNA sequences of LMG 5911^T and LMG 6003^T were very similar to each other (98.3 % similarity) and were related closely to those of *Achromobacter piechaudii* (98.6 and 98.0 % similarity, respectively), *Achromobacter*

Fig. 1. Dendrogram derived from UPGMA linkage of correlation coefficients between protein patterns of strains studied. Correlation coefficient is expressed as percentage similarity for convenience.

ruhlandii (98.0 and 98.5 %, respectively), *Achromobacter xylosoxidans* (98.1 and 98.4 %, respectively) and *Achromobacter denitrificans* (97.6 and 98.0 %, respectively). Bootstrap analysis indicated that strains LMG 5911^T and LMG 6003^T formed a stable phylogenetic group with other *Achromobacter* species (bootstrap value, 99.9 %).

As 16S rDNA sequence similarity of representative isolates of both protein-electrophoretic groups was well above 97 %, DNA–DNA hybridization was performed to further clarify their relatedness (Stackebrandt & Goebel, 1994). DNA was prepared from isolates LMG 6003^T, LMG 6001, LMG 6010, LMG 6000 and LMG 5911^T and from reference strains of *Achromobacter piechaudii*, *Achromobacter xylosoxidans*, *Achromobacter denitrificans*, *Achromobacter ruhlandii*, *Alcaligenes faecalis*, *Bordetella hinzii* and *Kerstersia gyiorum*. DNA binding values are shown in Table 2; they confirm that strains from both electrophoretic groups formed two separate novel *Achromobacter* species. The DNA G+C content of isolates LMG 6003^T, LMG 6001, LMG 6010, LMG 6000 and LMG 5911^T was 64.9, 65.3, 64.9, 65.5 and 64.9 mol%, respectively.

Cellular fatty acid compositions of all isolates of *Achromobacter spanius* and *Achromobacter insolitus* were determined. Fatty acid compositions of other *Achromobacter* species and of *Alcaligenes faecalis* were available from previous studies (Coenye *et al.*, 2003). Predominant fatty acids that were present in both novel *Achromobacter* species were C_{16:0}, C_{17:0} cyclo, summed feature 2 (comprising C_{14:0} 3-OH, iso-C_{16:1} I, an unidentified fatty acid with equivalent chain-length of 10.928 and/or C_{12:0} ALDE) and summed feature 3 (comprising C_{16:1} ω7c and/or iso-C_{15:0} 2-OH). Detailed results of the fatty acid composition analysis are available as supplementary material in IJSEM Online.

All strains examined showed oxidase, catalase, C₄-esterase and leucine arylamidase activities but no lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, gelatinase, amylase, β-galactosidase or DNase activity. All strains were capable of growth between 28 and 37 °C and could grow at NaCl concentrations from 0 to 4.5 %. All strains reduced nitrate but none reduced nitrite or were capable of denitrification. No haemolysis, hydrolysis of aesculin or production of indole, H₂S or acid on TSI agar was observed.

Fig. 2. Phylogenetic tree (based on 16S rDNA sequences) showing positions of *A. insolitus* and *A. spanius*. Bar, 10% sequence dissimilarity.

Table 2. DNA–DNA binding values (%) of all strains examined

Strain	DNA binding values with strain:				
	1	2	3	4	5
1. <i>Achromobacter insolitus</i> LMG 6003 ^T	100				
2. <i>Achromobacter insolitus</i> LMG 6001		100			
3. <i>Achromobacter insolitus</i> LMG 6010	76*		100		
4. <i>Achromobacter insolitus</i> LMG 6000	89*	78*		100	
5. <i>Achromobacter spanius</i> LMG 5911 ^T	44	6*	18*	21*	100
6. <i>Achromobacter piechaudii</i> LMG 1873 ^T	33				47
7. <i>Achromobacter xylosoxidans</i> LMG 1863 ^T	34				36
8. <i>Achromobacter denitrificans</i> LMG 1231 ^T	42				34
9. <i>Achromobacter ruhlandii</i> LMG 1866 ^T	35				42
10. <i>Alcaligenes faecalis</i> LMG 1229 ^T	4				7
11. <i>Bordetella hinzii</i> LMG 13501 ^T	18				25
12. <i>Kerstersia gyiorum</i> LMG 5906 ^T	9				12

*Values obtained with the initial renaturation rate method; other values were obtained with the microplate method.

Growth was never observed on 10 % lactose, Tween 80 or oxidation–fermentation (OF) medium supplemented with glucose, maltose, adonitol, fructose or xylose. C₈-ester-lipase, C₁₄-lipase, valine arylamidase, cysteine arylamidase, trypsin, chymotrypsin, phosphoamidase, α -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activities were never observed. All strains assimilated the following substrates: gluconate, acetate, propionate, butyrate, isobutyrate, *n*-valerate, succinate, fumarate, adipate, pimelate, suberate, azelate, sebacate, DL-lactate, DL-3-hydroxybutyrate, D-malate, L-malate, meso-tartrate, pyruvate, citraconate, itaconate, citrate, phenylacetate, *m*-hydroxybenzoate, D- α -alanine, L- α -alanine, L-leucine, L-isoleucine, L-serine, L-threonine, L-phenylalanine, L-histidine, L-aspartate, L-glutamate, L-proline, DL-4-aminobutyrate and 2-aminobenzoate. None of the strains assimilated the following substrates: glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β -D-xyloside, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, D-melezitose, raffinose, starch, glycogen, xylitol, β -gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate, 5-ketogluconate, caprylate, oxalate, malonate, glycolate, levulinate, *o*-hydroxybenzoate, D-mandelate, L-mandelate, phthalate, isophthalate, terephthalate, trigonelline, L-arginine, betaine, creatine, DL-5-aminovalerate, 2-aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, urea, acetamide, sarcosine, ethylamine, butylamine, amylamine, ethanolamine, benzylamine, spermine, histamine or glucosamine. Urease activity, growth on acetamide and assimilation of isovalerate, *n*-caproate,

heptanoate, pelargonate, caprate, malate, glutarate, DL-glycerate, D-tartrate, L-tartrate, 2-oxoglutarate, mesaconate, aconitate, benzoate, *p*-hydroxybenzoate, glycine, L-norleucine, L-valine, DL-norvaline, DL-2-aminobutyrate, L-cysteine, L-methionine, L-tyrosine, D-tryptophan, L-tryptophan, L-ornithine, L-lysine, L-citrulline, DL-kynurenine, β -alanine, DL-3-aminobutyrate, diaminobutane and tryptamine were strain-dependent characteristics.

The range of MIC values and MIC₅₀ and MIC₉₀ of the strains are available as supplementary data in IJSEM Online.

Previous studies have indicated that SDS-PAGE of whole-cell proteins is a valuable tool for identification of members of the family *Alcaligenaceae* (Vancanneyt *et al.*, 1995; Vandamme *et al.*, 1995, 1996; Coenye *et al.*, 2003). By using whole-cell protein analysis, both *Achromobacter insolitus* and *Achromobacter spanius* could be differentiated easily from each other, from known *Achromobacter* species and from members of the genera *Alcaligenes*, *Bordetella* and *Kerstersia* (Fig. 1 and data not shown). Both quantitative and qualitative differences occur in the fatty acid compositions of different *Achromobacter* species; however, these differences are small and it seems questionable whether they will suffice to identify strains at the species level. Biochemically, both novel *Achromobacter* species are difficult to separate from each other, from other *Achromobacter* species and from *Alcaligenes faecalis*. Biochemical characteristics that are useful for the differentiation of *Achromobacter insolitus* and *Achromobacter spanius* from each other and from other *Achromobacter* species are given in Table 3. In addition, differentiation of *Achromobacter insolitus* and *Achromobacter spanius* from *Alcaligenes faecalis* is possible due to the absence of assimilation of azelate, D-malate and adipate in the latter.

Table 3. Phenotypic characteristics that are useful for the differentiation of *A. insolitus* and *A. spanius* from other *Achromobacter* species

Taxa: 1, *A. insolitus*; 2, *A. spanius*; 3, *A. xylosoxidans*; 4, *A. denitrificans*; 5, *A. piechaudii*; 6, *A. ruhlandii*. +, Positive; –, negative; v, strain-dependent; ND, not determined. Data for taxa 3–6 were taken from Kersters & De Ley (1984), Yabuuchi *et al.* (1998) and our own unpublished data.

Characteristic	1	2	3	4	5	6
Growth on acetamide	+	–	–	v	v	ND
Denitrification	–	–	+	+	–	–
Assimilation of:						
D-Glucose	–	–	+	–	–	+
D-Xylose	–	–	+	–	–	+
Mesaconate	+	–	+	v	+	ND
Aconitate	+	–	+	+	+	+
Diaminobutane	–	+	ND	ND	v	ND

Description of *Achromobacter insolitus* sp. nov.

Achromobacter insolitus (in.so'li.tus. L. masc. adj. *insolitus* unusual, uncommon, referring to the fact that these organisms are found only rarely in human clinical samples).

Gram-negative, small (1–2 µm long) and coccoid cells that occur as a single unit, in pairs or in short chains. Motility is strain-dependent. On nutrient agar, colonies are flat or slightly convex with smooth margins and range from white to light brown in colour. Oxidase- and catalase-positive, but no urease, β-galactosidase or DNase activity is present. Nitrate reduction is present. Other biochemical characteristics are listed in the Results section above. In addition, *Achromobacter insolitus* grows on acetamide and assimilates isovalerate, malate, glutarate, DL-glycerate, mesaconate, aconitate, L-norleucine, L-valine, L-cysteine, L-tryptophan and tryptamine, but does not assimilate D-tartrate or diaminobutane. The following fatty acid components are present in significant amounts: C_{12:0}, C_{12:0} 2-OH, C_{14:0}, C_{16:0}, C_{17:0} cyclo, C_{18:0}, C_{18:1}ω7c and summed features 2 and 3. G+C content of the DNA is 64.9–65.5 mol%. Isolated from various human clinical samples.

The type strain, LMG 6003^T (=API 201-3-84^T=CCUG 47057^T), was isolated from a leg wound. Characteristics of the type strain are the same as those for the species. In addition, the type strain assimilates *n*-caproate, pelargonate, caprate, L-tartrate, 2-ketoglutarate, benzoate, DL-norvaline, DL-2-aminobutyrate, L-tyrosine, L-lysine, β-alanine and DL-3-aminobutyrate. DNA G+C content of the type strain is 64.9 mol%.

Description of *Achromobacter spanius* sp. nov.

Achromobacter spanius (spa'ni.us. N.L. masc. adj. *spanius* from Gr. adj. *spanios* rare, scarce, referring to the fact that, so far, only a few strains have been found).

Gram-negative, small (1–2 µm long), coccoid cells that occur as a single unit, in pairs or in short chains. Motility is strain-dependent. On nutrient agar, colonies are flat or slightly convex with smooth margins and range from white to light brown in colour. Oxidase- and catalase-positive, but no urease, β-galactosidase or DNase activity is present. Nitrate reduction is present. Other biochemical characteristics are listed in the Results section above. In addition, *Achromobacter spanius* does not grow on acetamide, assimilates 2-ketoglutarate, L-tyrosine, β-alanine, DL-3-aminobutyrate, diaminobutane and tryptamine and does not assimilate mesaconate, aconitate, *n*-caproate, heptanoate, pelargonate, caprate, benzoate, *p*-hydroxybenzoate, glycine, L-methionine, L-ornithine, L-lysine, L-citrulline or DL-kynurenine. The following fatty acid components are present in significant amounts: C_{12:0} 2-OH, C_{14:0}, C_{16:0}, C_{16:0} 2-OH, C_{17:0} cyclo, C_{18:0}, C_{18:1}ω7c and summed features 2 and 3. G+C content of the DNA is 64.9 mol%.

The type strain, LMG 5911^T (=API 198-2-84^T=CCUG 47062^T), was isolated from blood. Characteristics of the type strain are the same as those for the species. In addition, the type strain utilizes glutarate, DL-glycerate and L-tryptophan. DNA G+C content of the type strain is 64.9 mol%.

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