Pseudoxanthomonas mexicana sp. nov. and *Pseudoxanthomonas japonensis* sp. nov., isolated from diverse environments, and emended descriptions of the genus *Pseudoxanthomonas* Finkmann *et al.* 2000 and of its type species

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Three mesophilic bacteria (strains AMX 26B^T, UR374_02 and 12-3^T) isolated respectively from an anaerobic digester, human urine and urban riverside soil were characterized. Cells were Gram-negative, motile, non-sporulating, straight to curved rods with one polar flagellum and had a strictly respiratory metabolism with O₂ as the preferential terminal electron acceptor. Phylogenetic analysis based on 16S rRNA gene sequences revealed that all strains clustered within the *Xanthomonadaceae* branch of the *Proteobacteria*. Isolates AMX 26B^T and UR374_02 exhibited 100 % 16S rRNA gene sequence similarity and both were related to strain 12-3^T (99.6 % similarity). The closest relative of all the isolates was *Pseudoxanthomonas broegbernensis* DSM 12573^T (similarity 97.1–97.5 %), and they were equidistantly related to *Xanthomonas* species (95.4–96.6 %), *Stenotrophomonas* species (95.3–96.1 %) and *Pseudoxanthomonas taiwanensis* ATCC BAA-4040^T (95.3–95.4 %). Chemotaxonomic and biochemical data (branched-chain cellular fatty acid pattern without C_{13:0} iso 3-OH, ubiquinone with eight isoprenoid units, limited range of substrates used, ability to reduce nitrite but not nitrate with the production of N₂O) supported their affiliation to the genus *Pseudoxanthomonas*. The results of

Published online ahead of print on 15 June 2004 as DOI 10.1099/ijs.0.02810-0.

Abbreviation: CFA, cellular fatty acids.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains AMX 26B^T, UR374_02 and 12-3^T are respectively AF273082, AY124375 and AB008507.

Transmission electron micrographs, graphs showing anoxic growth in the presence of nitrite and a dendrogram comparing CFA profiles are available as supplementary material in IJSEM Online.

DNA–DNA hybridization and/or phenotypic analysis allowed them to be differentiated from the two *Pseudoxanthomonas* species with validly published names and showed that strain 12-3^T was genomically and phenotypically distinct from the other two isolates. On the basis of these results, two novel species of the genus *Pseudoxanthomonas* are proposed: *Pseudoxanthomonas mexicana* sp. nov., consisting of strains AMX 26B^T (=ATCC 700993^T=CIP 106674^T=JCM 11524^T) (type strain) and UR374_02 (=DSM 15133), and *Pseudoxanthomonas japonensis* sp. nov., consisting of strain 12-3^T (=CCUG 48231^T=CIP 107388^T=JCM 11525^T). The report of these two novel species leads to the emendation of the description of the genus *Pseudoxanthomonas* and the re-evaluation of the phenotype of *P. broegbernensis* DSM 12573^T necessitates the emendation of its description.

The creation of the genus *Pseudoxanthomonas* was initially based on the description of a single species, *Pseudoxanthomonas broegbernensis*, which shared phenotypic traits of *Xanthomonas* and *Stenotrophomonas* species (colour of the colonies, cell shape, Gram staining, presence of a branched-chain fatty acid pattern and of an ubiquinone with eight isoprenoid units) but which could be easily differentiated from them by its ability to reduce nitrite but not nitrate to N₂O and by the lack of C_{13:0} iso 3-OH fatty acid characteristic of these genera (Yang *et al.*, 1993; Finkmann *et al.*, 2000; Assih *et al.*, 2002). More recently, the isolation of two novel N₂O-producing strains from hot springs led to the description of a second species of the genus, *Pseudoxanthomonas taiwanensis*, which differs from *P. broegbernensis* mainly by the fact that it is not motile and that its optimum growth temperature is 50 °C instead of 30 °C (Chen *et al.*, 2002). The two species also exhibit differences in the spectrum of substrates used and in their cellular fatty acid (CFA) profiles. Within the framework of a study on the role of aerobic bacteria in anaerobic digesters, a strain identified as AMX 26B^T was isolated in Mexico. This strain could not be assigned to any known species on the base of classical biochemical tests (conventional and API 20NE, Biotype 100) and CFA profiles. Analysis of its 16S rRNA gene sequence revealed that it was closely related to a strain (12-3^T) previously isolated in Japan (Iizuka *et al.*, 1998) and also to another strain (UR374_02) isolated in Germany and that it was moderately related to *P. broegbernensis*. In order to clarify their taxonomic position, all strains were subjected to more detailed study. In this paper, we report the characterization of two novel species of *Pseudoxanthomonas*, *Pseudoxanthomonas mexicana* sp. nov., including strains AMX 26B^T and UR374_02, and *Pseudoxanthomonas japonensis* sp. nov., including strain 12-3^T.

Isolation and cultivation

Unless otherwise indicated, isolation and culture of microorganisms to perform phenotypic tests were done under aerobic conditions at 35 °C, pH 7. All analyses were performed at least in duplicate. AMX 26B^T was isolated from a laboratory-scale upflow anaerobic sludge blanket (UASB) reactor treating the wastewater of a cheese factory. Isolation was performed by serial dilution of grounded

reactor sludge in a buffered salt solution (g l⁻¹: MgCl₂·6H₂O, 0·405; KH₂PO₄, 0·042; pH 7·2) and surface inoculation of Petri dishes containing medium R2A with 0·1 ml of the highest dilutions (10⁻⁵–10⁻¹⁰). Purification was later obtained by streaking single colonies on Petri dishes filled with the same medium. Strain UR374_02 was isolated from the urine of a 10-year-old boy presenting bladder extrophy, a congenital birth defect that requires long-term catheterization and may occasionally cause urinary tract infections. As a routine follow up of the patient, 1 µl urine was plated on trypticase soy agar (TSA) supplemented with 5 % (v/v) sheep blood and incubated at 37 °C. Strain 12-3^T was obtained from a sample of polluted urban soil, collected at the riverside of the Tamagawa river in the Tokyo metropolitan area, Japan, after a procedure (direct plating following filtration through a 0·45 µm membrane without enrichment) specially designed to select for extremely small, free-living bacteria. A detailed description of the isolation and purification procedures for this strain is given by Iizuka *et al.* (1998).

16S rRNA gene sequence analyses, DNA–DNA hybridization and G+C content

DNA extraction, 16S rRNA gene amplification, purification and sequencing were done as described previously by Assih *et al.* (2002) for strain AMX 26B^T, Iizuka *et al.* (1998) for strain 12-3^T and Relman *et al.* (1992) for strain UR374_02. A non-redundant BLASTN search of full sequences through GenBank (Altschul *et al.*, 1990; Benson *et al.*, 1999) identified the closest relatives. Sequences used in the phylogenetic analysis were obtained from the RDP II (Maidak *et al.*, 2001) and GenBank (Benson *et al.*, 1999). The alignment was produced by importing sequences into BioEdit version 5.0.9 (Hall, 1999) and using the RDP II Sequence Aligner program (Maidak *et al.*, 2001). Positions of sequence and alignment ambiguity were omitted and a masked dataset of 1410 unambiguous nucleotides was produced. Pairwise evolutionary distances were calculated using the method of Jukes & Cantor (1969). Dendrograms were constructed using the neighbour-joining method (Saitou & Nei, 1987) as implemented in the TreeCon for Windows package (Van de Peer & De Wachter, 1994). Confidence in the tree topology was determined by bootstrapping 100 replicates (Felsenstein, 1985) and expressed as a percentage near the

branching point. Determination of G + C content and DNA renaturation studies were performed by the DSMZ using HPLC and spectrophotometric methods, respectively, as described by Assih *et al.* (2002). Hybridization percentages obtained by the spectrophotometric technique presented a standard deviation of less than 2.5 %. In any case, hybridizations between 60 and 75 % were repeated in order to confirm the results.

16S rRNA gene sequences of 1464, 1466 and 1540 nucleotides, respectively, were determined for strains 12-3^T, UR374_02 and AMX 26B^T. Phylogenetic analysis revealed that the three isolates clustered within the *Xanthomonas*–*Stenotrophomonas*–*Pseudoxanthomonas* branch of the *Proteobacteria* (Fig. 1). The sequences of isolates AMX 26B^T and UR374_02 were 100 % identical and they both shared 99.6 % similarity with that of strain 12-3^T. The three strains were related almost equidistantly to *Xanthomonas* (95.4–96.6 %) and *Stenotrophomonas* (95.3–96.1 %) species. *P. broegbernensis* DSM 12573^T, which shared 97.5 % sequence similarity with strains AMX 26B^T and UR374_02 and 97.1 % with strain 12-3^T, appeared to be their closest described relative. Within the genus *Pseudoxanthomonas*, the strains were only moderately related to *P. taiwanensis* ATCC BAA-4040^T (95.3–95.4 % similarity), which was itself only moderately related to *P. broegbernensis* DSM 12573^T (95.8 % similarity). In order to clarify definitively the phylogenetic position of the three isolates, DNA–DNA

hybridization experiments were performed between them and with *P. broegbernensis* DSM 12573^T. Strain AMX 26B^T hybridized at 80.4 % with UR374_02 and 58.7–61.3 % with 12-3^T. It showed a much lower level of hybridization with *P. broegbernensis* DSM 12573^T (33.7 %), similar to that observed between this species and strain 12-3^T (37 %). The DNA G + C content of the isolates is given in the species description and was similar to that of the two *Pseudoxanthomonas* species described to date.

Morphological, biochemical and physiological characteristics

The strain morphology and results of biochemical and physiological analyses are given in the genus and species descriptions as well as in Supplementary Fig. A (available in IJSEM Online) and Table 1. Cell morphology and motility were deduced from direct observations of fresh cultures using a Nikon phase-contrast microscope. The presence, number and position of flagella were determined by flagellar staining (Kodaka *et al.*, 1982; Mayfield & Inniss, 1977) and/or transmission electron microscopy after negative staining. To detect the presence of spores, slant cultures of the strains were prepared on yeast-peptone medium and one drop of the condensation water at the bottom of the slants was observed by optical microscopy. Gram staining was performed according to Murray *et al.* (1994). The capacity of the strains to hydrolyse Tween 80 and ONPG as well as the presence of arginine dihydrolase and lysine and ornithine decarboxylases were determined using the traditional techniques outlined by Marchal *et al.* (1987). All other classical biochemical phenotypic tests were performed with conventional procedures in Petri plates or tubes as described by Smibert & Krieg (1994). The ranges of temperature and NaCl concentration that allowed growth of the strains were examined on solid media. TSA, which contains 5 g NaCl l⁻¹, was used for the temperature measurement, while nutrient agar was used for the salt concentration assay. For the range of pH, a liquid medium (nutrient broth with no NaCl added) was utilized instead of a solid one. The profile of substrates used by the strains was obtained by inoculating API 20NE (12 carbon sources) and Biotype 100 (98 carbon sources) strips according to the manufacturer's instructions (bioMérieux). The strips were incubated at 30 °C and the readings were done after 1–4 days. The API 20NE strips also allowed cross-checking of some of the classical phenotypic tests and presented 11 common substrates with Biotype 100. A good correlation was found between these different tests, since a discrepancy was only observed for gelatin hydrolysis in the case of strain 12-3^T.

The isolates were able to use 17–24 of the 99 substrates assayed (Table 1). Strain UR374_02 appeared to be the least versatile and the most fastidious. It could use only 9–11 substrates when the Biotype test was performed with Biotype medium 1 and could use 21–22 substrates in the presence of medium Biotype 2, which contains more growth factors (37 instead of 16, including vitamins and amino

Fig. 1. Phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of *P. mexicana* sp. nov. AMX 26B^T and UR374_02 and *P. japonensis* sp. nov. 12-3^T within the genus *Pseudoxanthomonas*. The tree was constructed using the neighbour-joining method based on a masked dataset of 1410 nucleotides. Each bootstrap is represented as a percentage of 100 replicates. Values above 70 % are given at branching points. Accession numbers are shown in parentheses. Bar, 10 % sequence divergence.

Table 1. Morphological, biochemical and physiological characteristics of strains AMX 26B^T, UR374_02 and 12-3^T compared with those of the type strains of *Pseudoxanthomonas* species

Strains: 1, *P. mexicana* sp. nov. AMX 26B^T; 2, *P. mexicana* sp. nov. UR374_02; 3, *P. japonensis* sp. nov. 12-3^T; 4, *P. broegbernensis* DSM 12573^T; 5, *P. taiwanensis* ATCC BAA-404^T. PNPG, *p*-Nitrophenyl β -D-galactoside; +, positive test or growth occurred; –, negative test or no growth; v, variable growth between duplicates; W, weak response; ND, not done. *P. broegbernensis* DSM 12573^T and *P. taiwanensis* ATCC BAA-404^T shared the following characteristics with the isolates: presence of an aesculin and DNA hydrolase and ability to use as carbon sources D-cellobiose and D-glucose but not D-raffinose, L-rhamnose, glycerol, *myo*-inositol, D-sorbitol or D-malate. In addition, *P. broegbernensis* DSM 12573^T shared with the isolates the following characteristics not tested for *P. taiwanensis* ATCC BAA-404^T: ability to use as growth substrates *N*-acetyl-D-glucosamine, gentiobiose and maltotriose and inability to use several alcohols (adonitol, D-arabitol, L-arabitol, dulcitol, i-erythritol, D-mannitol, xylitol), amines (ethanolamine, tryptamine), amino acids (D-alanine, L-alanine, tryptophan), benzenic and heterocyclic aromatic compounds (benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, *m*-coumarate, gentisate, phenylacetate, 3-phenylpropionate, protocatechuate, trigonelline), organic acids (*cis*-aconitate, *trans*-aconitate, adipate, 5-aminovalerate, caprate, caprylate, D-galacturonate, D-gluconate, 2-ketogluconate, 5-ketogluconate, D-glucuronate, glutarate, DL-glycerate, itaconate, malonate, mucate, quinate, D-saccharate, L-tartrate, D-tartrate, *meso*-tartrate, tricarballoylate) and sugars plus related compounds (L-fucose, hydroxyquinoline β -glucuronide, D-lyxose, D-melezitose, 1-O-methyl α -galactoside, 3-O-methyl D-glucose, D-ribose, L-sorbose, D-tagatose) tested. Data for *P. taiwanensis* ATCC BAA-404^T were taken from Chen *et al.* (2002). All the other data were determined in this study.

Characteristic	1	2	3	4	5
Flagellation (single and polar) and motility	+	+	+	+	–
Catalase	+ W	+	–	+	+
Growth at 10 °C	+	+	+	+	–
Growth at 41 °C	–	–	–	–	+
Optimum temperature for growth	30–37	30–37	30–37	30	50
Hydrolysis of:					
ONPG/PNPG	–	–	+	+	+
Gelatin or casein	+	+	+	–	–
Tween 80	+	–	+	+	+
Assimilation of carbon compounds					
Sugars:					
D-Fructose, D-mannose	+	–	–	+	–
Lactose, D-melibiose, sucrose	–	–	–	+	–
L-Arabinose	–	–	–	+	–
D-Galactose	–	–	+	+	–
Maltose	+	+	+	+	–
D-Xylose	–	–	+	+	+
D-Trehalose	+	–	+	+	+
D-Glucosamine, lactulose	–	–	+	+	ND
Maltitol, palatinose, D-turanose	–	–	–	+	ND
Organic acids:					
L-Malate	–	–	+	–	–
Succinate	–	+*	+	–	–
4-Aminobutyrate	–	+*	v	–	ND
Citrate	–	–	–	+	ND
3-Hydroxybutyrate	+	+*	+	–	ND
Fumarate	–	–	+	–	ND
2-Oxoglutarate	–	+*	–	–	ND
DL-Lactate	–	+*	+	–	ND
Propionate	+	+	+	–	ND
Amino acids:					
L-Glutamate	+	+*	+	–	+
L-Proline	+	+*	–	+	+
L-Serine	+	+	v	–	–
L-Aspartate, L-histidine	+	+*	+	–	ND
L-Tyrosine	–	+*	–	–	ND
Others:					
Histamine, putrescine	–	+*	–	–	ND

*Growth occurred only with medium Biotype 2.

acids). Despite the poor carbon source utilization by the strains, several sugars, amino acids and organic acids supported growth (Table 1). The test performed on MEVAG medium indicated that glucose was metabolized by oxidation and that none of the strains, including *P. broegbernensis* DSM 12573^T, could ferment it.

Discrepancies were found between some of the characteristics reported in the original description of *P. broegbernensis* (Lipski *et al.*, 1992; Finkmann *et al.*, 2000) and observations in the present study: hydrolysis of aesculin (positive instead of negative), growth at 37 °C and the use of L-arabinose, D-fructose and L-proline as carbon sources. Except for aesculin hydrolysis, however, our results are in line with those of Chen *et al.* (2002), who also re-evaluated the phenotype of this strain. Considering that *P. broegbernensis* DSM 12573^T has repeatedly given a positive result in the aesculin hydrolysis assay performed in our different laboratories when using API 20NE and Biotype 100 strips but also in conventional tests in tubes or Petri plates, it seems that the negative result reported by Finkmann *et al.* (2000) is in fact restricted to the microtitration plate technique (Lipski *et al.*, 1992) that they used to determine this parameter and the criteria that they selected to consider that the response to the test was positive or negative. Beyond the specific scope of this paper, this suggests that, in order to avoid evaluation-dependent phenotypes and so to improve stability in the description of strains, the use of widely available commercial kits or conventional techniques easily reproducible everywhere should be recommended for analysis of physiological properties.

Nitrate and nitrite reduction

The ability of the strains to reduce nitrate and nitrite was first investigated by the standard technique with the Griess reagent (Smibert & Krieg, 1994) and *Escherichia coli* W3110 (=ATCC 27325), a bacterium able to reduce nitrate to nitrite but not nitrite, as a control. Since no clear and reproducible result could be obtained from the previous experiments between our different laboratories, despite the fact that the control worked well (the same situation was observed with the API 20NE NO₃⁻ test), a new assay was prepared. This time, the strains were cultivated in trypticase soy broth (TSB), a medium of choice to study denitrification (Tiedje, 1988). The medium was pre-reduced by boiling and distributed under a nitrogen atmosphere in hermetically closed Hungate tubes. NaNO₃ or NaNO₂ was added to the tubes (three per strain and electron acceptor) immediately prior to inoculation (10 % v/v). Five concentrations were tested for NO₂⁻ (1.47, 5, 10, 15, 20 mM) and one for NO₃⁻ (10 mM). One tube per strain was inoculated but not amended with either nitrate or nitrite. These tubes served as negative controls. Another series of tubes prepared with unreduced TSB under an air atmosphere was also inoculated to check the viability of the strains. Growth was followed daily or every 2–3 days by measuring OD₅₈₀. The capacity of the strains to grow anoxically in presence of NO₃⁻ or NO₂⁻ but not in their

absence was considered to indicate their ability to use them as electron acceptors. If no growth occurred after 46 days, the test was considered negative. Tubes showing growth were subcultured at least once in the same conditions in order to confirm the results. The reduction of NO₃⁻ and NO₂⁻ was later validated by measuring their final concentrations in the tubes using Quantofix nitrate/nitrite paper test sticks (Macherey-Nagel). The presence of N₂O in the tube gas phase was also investigated qualitatively by GC with a thermal conductivity detector.

The three isolates showed the capacity to grow under anoxic conditions in the presence of nitrite at all the concentrations tested but not in its absence (Supplementary Fig. B; results for only some concentrations are shown) or in the presence of 10 mM nitrate (data not shown). Less than 0.43 mM NO₂⁻ was detected in the tubes initially amended with 5 and 10 mM NO₂⁻ after 46 days of incubation for the primary cultures and 25–30 days of incubation for the subcultures. At higher initial concentrations (15 and 20 mM), the amount of nitrite remaining in the tubes after the same length of time was above the upper concentration detectable (1.74 mM) by the Quantofix test sticks. The presence of N₂O in the gas phase of all the tubes containing nitrite definitively established the ability of the strains to reduce it. However, the conditions of the test, performed with a nitrogen atmosphere and a complex medium containing organic nitrogen that may liberate NH₄⁺ upon degradation, did not allow us to determine whether nitrous oxide was the sole and final product of nitrite reduction. The growth efficiency of the three isolates under anoxic conditions with nitrite as the electron acceptor was much lower than under aerobic conditions. Indeed, the increase in OD was limited to 1.2–1.7 times that of the inoculated control without added nitrite, while the maximal OD achieved represented only one-fifth to one-third of that reached in the aerobic viability controls. All the strains also presented a lag phase ranging from 4 to 12 days before starting to grow when transferred from an aerobic to an anoxic environment. This lag phase decreased or even disappeared upon subculturing anoxically with nitrite. *P. broegbernensis* DSM 12573^T, used as a positive control for NO₂⁻ reduction and absence of NO₃⁻ reduction, behaved similarly to the novel isolates. Nevertheless, its growth in the presence of NO₂⁻ appeared to be much more efficient, since it was characterized by shorter lag phases, at least in the case of the primary cultures, and it attained higher OD for the same nitrite concentrations. In the case of *P. broegbernensis* DSM 12573^T, the maximal OD reached by the cultures increased with the concentration of nitrite (Supplementary Fig. B). This indicates that, in contrast to what was originally hypothesized (Finkmann *et al.*, 2000), the reduction of nitrite to N₂O by this strain is not only a detoxification process but is also coupled to energy production, since it influences the biomass yield. The positive correlation between OD and nitrite concentration was less evident for strains AMX 26B^T, UR374_02 and 12-3^T (data not shown). Nevertheless, the fact that they

could grow anoxically only in presence of this electron acceptor shows that they can also get energy from its reduction.

Chemotaxonomic analyses

The quinone systems of strains AMX 26B^T and 12-3^T were extracted with an organic solvent mixture, evaporated, purified by TLC and separated by reverse-phase TLC (Hiraishi *et al.*, 1984). Both strains were characterized by the absence of menaquinones and the presence of a ubiquinone with eight isoprenoid units as sole ubiquinone. The accuracy of this result was checked by extracting and analysing in parallel the quinones of *P. broegbernensis* DSM 12573^T, known to contain exclusively Q-8 (Lipski *et al.*, 1992; Finkmann *et al.*, 2000), as well as those of *E. coli* W3110 (= ATCC 27325), which contain MK-8 and Q-8.

The CFA composition was determined by Microbial ID using the fully automated GC Sherlock Microbial Identification System (MIDI) and MIDI standard procedures (Sasser, 1990) for strain cultivation (24 h at 28 °C in TSB supplemented with 15 g agar l⁻¹) as well as CFA extraction and analysis. Some 24–29 different CFA were detected in the novel isolates (Table 2). Nevertheless, eight to ten of them appeared only in very small amounts (0.07–0.32 %) and represented altogether less than 1.6 % of the total CFA content. Saturated, hydroxy and unsaturated linear fatty acids were almost absent, while methyl-branched fatty acids corresponded to 66–71 % of the total CFA. In order of decreasing abundance of this class were C_{15:0} iso, C_{16:0} iso, C_{11:0} iso, C_{16:1} iso H and C_{17:0} iso. The branched, unsaturated fatty acid C_{17:1} iso *cis*7 and the branched hydroxy fatty acid C_{11:0} iso 3-OH were also found in large amounts, particularly the first, which represented 18–20 % of the total fatty acid content. The branched saturated and branched hydroxy fatty acid patterns obtained for the three strains were similar to those of *Xanthomonas* and *Stenotrophomonas* species, but they lacked C_{13:0} iso 3-OH. Qualitatively and quantitatively, the CFA profiles of the three strains were almost indistinguishable to the eye. Their comparison by unweighted arithmetic average clustering together with the CFA profiles of closely related species from the genera *Pseudoxanthomonas*, *Stenotrophomonas* and *Xanthomonas* showed that strains AMX 26B^T and UR374_02 had the most similar profiles, since they linked at a Euclidean distance of 3.9, and that they linked together at a distance of 6.8 with strain 12-3^T (Supplementary Fig. C). This last value nevertheless remained well below the usual cut-off limit of 10 found by MIDI between species. In the same analysis, *P. broegbernensis* DSM 12573^T linked with them at a Euclidean distance of over 25. Qualitatively, *P. broegbernensis* DSM 12573^T can be distinguished from the novel isolates by the fact that it possesses unsaturated (C_{16:1} *cis*7, C_{17:1} *cis*9) and hydroxy (C_{10:0} 3-OH, C_{12:0} 3-OH) fatty acids and that, within the methyl-branched fatty acids, it contains C_{11:0} anteiso but not C_{16:1} iso H or C_{18:1} iso H. Quantitative differences can also be found. For instance, C_{16:0} iso and C_{17:1}

iso *cis*7 are respectively 3–5 and 1.7–1.9 times more abundant in strains AMX 26B^T, UR374_02 and 12-3^T than in *P. broegbernensis* DSM 12573^T, while the latter strain contains 15–22 times more C_{16:0} and 2.4–3.75 times more C_{16:1} *cis*9/C_{15:0} iso 2-OH mixture than the others (Table 2). In addition, the ratio of C_{15:0} iso/C_{15:0} anteiso is completely different between *P. broegbernensis* DSM 12573^T and the three strains (1.89 against 8.5–19).

Antibiotic susceptibility

Susceptibility to antibiotics was tested using the Kirby–Bauer disc diffusion method (Bauer *et al.*, 1966) on Mueller–Hinton solid medium (bioMérieux) with *E. coli* CIP 76.24 and *Pseudomonas aeruginosa* CIP 76.110 as controls. Inhibition diameters were recorded after 18–24 h incubation at 37 °C. MICs were extrapolated from the inhibition diameters using adapted software. The isolates showed a similar response and were found to be highly susceptible (low MIC values) to most of the antibiotics tested except those characterized by a mechanism of action based on the inhibition of protein synthesis and pipemidic acid, a quinolone, acting on the synthesis of nucleic acids (Table 3). Within the antibiotics that affect protein synthesis, the strains seemed to be particularly resistant (very high MIC) to the aminoglycosides but remained sensitive to doxycycline (MIC = 0.01 mg l⁻¹). Despite its medical origin, strain UR374_02 appeared to be the most susceptible of the three isolates. For instance, it presented low MICs for gentamicin, fusidic acid and erythromycin, in contrast to the others. Strain AMX 26B^T had the lowest MIC for penicillin G. *P. broegbernensis* DSM 12573^T produced a susceptibility pattern completely different from those of the new isolates (Table 3). It was the only one to show low MICs to the aminoglycosides. Nevertheless, it must be noted that, among this antibiotic class, it was reported as resistant to gentamicin and kanamycin in the original published description (Finkmann *et al.*, 2000). In the same study, it was also reported as resistant to penicillin G, while we found it susceptible. Since Finkmann *et al.* (2000) did not report MIC values or inhibition zone diameters for the antibiotics that they tested, and these parameters cannot be estimated from the given resistant and susceptible categories, their results are not in fact comparable to those obtained here. The antibiotic susceptibility profile presented in Table 3 for *P. broegbernensis* DSM 12573^T therefore corresponds in reality to the first antibiogram defined on an adequate basis for this strain and which can serve as a reference for future comparisons.

Taxonomic considerations

The characterization of the three isolates clearly showed that they are phylogenetically affiliated to the genus *Pseudoxanthomonas* and that they possess the main phenotypic traits of its members. Nevertheless, within this group, the strains cannot be assigned to either of the two existing species. In particular, they cannot belong to *P. taiwanensis*, which is not motile, lacks flagellation, does not use maltose,

Table 2. Fatty acid profiles of strains AMX 26B^T, UR374_02 and 12-3^T and that of their closest phylogenetic relative, *P. broegbernensis* DSM 12573^T

Values are percentages of total fatty acids. tr, Trace, corresponding arbitrarily to less than 0.34 % of total CFA.

Fatty acid	AMX 26B ^T	UR374_02	12-3 ^T	<i>P. broegbernensis</i> DSM 12573 ^T
Saturated				
10:0	tr	tr	tr	0.72
14:0	0.40	0.34	0.38	1.79
15:0			tr	1.15
16:0	0.48	0.53	0.67	10.32
Unsaturated				
15:1 <i>cis</i> 7				tr
15:1 <i>cis</i> 9				tr
16:1 <i>cis</i> 7				0.96
17:1 <i>cis</i> 11			tr	
17:1 <i>cis</i> 9				0.67
18:1 <i>cis</i> 9				tr
18:1 <i>cis</i> 11	tr	tr	0.48	tr
Methyl-branched				
10:0 iso	tr	tr	tr	tr
11:0 iso	5.41	4.89	4.69	5.39
11:0 anteiso				0.72
12:0 iso	tr	tr	tr	tr
13:0 iso	tr	tr	tr	tr
13:0 anteiso				tr
14:0 iso	2.11	2.86	2.37	0.52
15:1 iso F	1.30	1.63	1.49	1.49
15:0 iso	39.69	36.82	32.95	26.70
15:0 anteiso	2.57	1.94	3.85	14.12
16:1 iso H	3.89	4.91	4.05	
16:0 iso	9.70	11.79	12.67	2.57
17:0 iso	4.87	3.53	3.46	3.55
17:0 anteiso	0.39	tr	0.58	0.93
17:0 10-methyl	tr	tr		
18:1 iso H	0.72	0.77	0.72	
Branched-chain unsaturated				
17:1 iso <i>cis</i> 7	18.46	18.74	19.91	10.68
17:1 anteiso <i>cis</i> 7	tr		0.46	tr
Hydroxy				
10:0 3-OH				0.38
11:0 2-OH	tr			
12:0 3-OH				0.37
13:0 2-OH				tr
Branched-chain hydroxy				
11:0 iso 3-OH	6.53	6.02	5.22	5.88
12:0 iso 3-OH	tr	tr	tr	tr
13:0 iso 3-OH				tr
Unresolved				
13:0 3-OH/15:1 iso I/H	tr	tr	tr	
16:1 <i>cis</i> 9/15:0 iso 2-OH	3.15	2.9	4.47	10.86
17:1 iso I/anteiso B	0.34	0.7	0.48	

cannot hydrolyse proteins and is thermophilic, while the novel isolates possess these characteristics and are typically mesophilic (Table 1). Moreover, their 16S rRNA gene sequences show a difference of 4.6–4.7 % from that of

P. taiwanensis ATCC BAA-4040^T, which is greater than the 3 % difference recognized as sufficient to separate bacteria in distinct species (Stackebrandt & Goebel, 1994). The same criteria would indicate that all isolates could be members

Table 3. MICs (mg l⁻¹) of selected antibiotics against isolates AMX 26B^T, UR374_02 and 12-3^T and *P. broegbernensis* DSM 12573^T

Antibiotics are listed under mode of action and class. Values in parentheses after the name of the antibiotics correspond to the disc charge. S, R and I in parentheses after the MICs mean that the strains are respectively susceptible, resistant or intermediately susceptible to the antibiotics based on the guidelines of CASFM (2002). Such classification is given simply as an indication and should be used with caution for novel species that were not considered in the definition of the clinical categories.

Antibiotic	AMX 26B ^T	UR374_02	12-3 ^T	<i>P. broegbernensis</i> DSM 12573 ^T
Inhibition of protein synthesis				
Aminoglycosides:				
Amikacin (30 µg)	45 (R)	362 (R)	256 (R)	0·01 (S)
Gentamicin (10 IU, 15 µg)	32 (R)	3 (S)	11 (R)	0·01 (S)
Kanamycin (30 IU)	91 (R)	362 (R)	256 (R)	0·01 (S)
Netilmicin (30 µg)	181 (R)	362 (R)	256 (R)	0·01 (S)
Tobramycin (10 µg)	128 (R)	128 (R)	128 (R)	0·01 (S)
Fusidic acid (10 µg)	16 (I)	2 (S)	128 (R)	3 (I)
Macrolides:				
Erythromycin (15 IU)	1·74 (I)	0·11 (S)	1·74 (I)	7 (R)
Tetracyclines:				
Doxycycline (30 IU)	0·01 (S)	0·01 (S)	0·01 (S)	0·01 (S)
Inhibition of nucleic acid synthesis				
Fluoroquinolones:				
Ciprofloxacin (5 µg)	0·04 (S)	0·05 (S)	0·04 (S)	0·04 (S)
Ofloxacin (5 µg)	0·03 (S)	0·2 (S)	0·04 (S)	0·04 (S)
Quinolones:				
Pipemidic acid (20 µg)	28 (R)	12 (I)	32 (R)	11 (I)
Disruption of cell membranes				
Polypeptides:				
Colistin (50 µg)	<2 (S)	<2 (S)	<2 (S)	<2 (S)
Inhibition of folate synthesis				
Sulfonamides + diaminopyrimidines:				
Trimethoprim/sulfamethoxazole (1·25/23·75 µg)	0·02 (S)	0·2 (S)	0·02 (S)	0·02 (S)
Inhibition of peptidoglycan synthesis				
Carbapenems:				
Imipenem (10 µg)	0·57 (S)	1·74 (S)	5 (I)	0·57 (S)
Cephems:				
Cephalophin (30 µg)	0·13 (S)	1·59 (S)	4 (S)	0·13 (S)
Cefotaxime (30 µg)	0·02 (S)	0·12 (S)	0·02 (S)	0·02 (S)
Ceftazidime (30 µg)	0·02 (S)	0·18 (S)	0·02 (S)	0·02 (S)
Penams:				
Amoxicillin (25 µg)	0·21 (S)	0·67 (S)	0·21 (S)	0·21 (S)
Amoxicillin/clavulanic acid (20/10 µg)	0·21 (S)	0·67 (S)	0·21 (S)	0·21 (S)
Piperacillin (75 µg)	0·13 (S)	0·21 (S)	0·1 (S)	0·13 (S)
Piperacillin/tazobactam (75/10 µg)	0·09 (S)	0·17 (S)	0·09 (S)	0·09 (S)
Penicillin G (10 IU, 6 µg)	0·21 (S)	1 (I)	1 (I)	0·06 (S)
Ticarcillin (75 µg)	0·09 (S)	4 (S)	0·12 (S)	0·12 (S)

of *P. broegbernensis*, since their 16S rRNA gene sequences have only 2·5–2·9% difference from that of *P. broegbernensis* DSM 12573^T. This conclusion is strengthened by the fact that the type strain of this species shares numerous phenotypic traits with the novel strains (see legend to Table 1), especially that of being mesophilic and motile by means of a single polar flagellum. Nevertheless, beside these similarities, strains AMX 26B^T, UR374_02 and 12-3^T present more phenotypic differences from *P. broegbernensis*

DSM 12573^T at the level of substrate utilization (at least 13 differences, when each strain uses only 17–24 carbon sources of the 99 tested), CFA composition and antibiotic susceptibility. These differences, combined with the fact that the DNA of strains AMX 26B^T and 12-3^T hybridized with that of *P. broegbernensis* DSM 12573^T at a level significantly below the value of 70% proposed for species delineation (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002), support definitively the conclusion that these novel isolates

are not members of this species. DNA–DNA hybridization also revealed that the three strains could be divided in two genomic groups. The first was composed of AMX 26B^T and UR374_02 and the second of 12-3^T. The two genomic groups could not be easily distinguished by their morphology, antibiotic susceptibility or CFA profiles. Notwithstanding this observation, and despite the heterogeneous biochemical profile presented by the members of the first group (Table 1), strain 12-3^T could be clearly distinguished from it by other phenotypic properties such as a catalase-negative reaction, the inability to grow in the presence of 40 g NaCl l⁻¹ and the capacity to hydrolyse ONPG/PNPG and to use D-galactose, D-glucosamine, lactulose, D-xylose, fumarate and L-malate as carbon sources but not L-proline (Table 1).

Taking into account the phenotypic and phylogenetic characteristics described above, we propose that strains AMX 26B^T and UR374_02 represent a novel species within the genus *Pseudoxanthomonas*, *Pseudoxanthomonas mexicana* sp. nov., with AMX 26B^T as the type strain, and that strain 12-3^T represents another novel species of the genus, *Pseudoxanthomonas japonensis* sp. nov. The genus *Pseudoxanthomonas* now contains four species. Three of them, *P. broegbernensis*, *P. japonensis* and *P. mexicana*, form a phenotypically and phylogenetically homogeneous group, since they are all mesophilic and motile by means of a single polar flagellum and share 97.1–99.6 % 16S rRNA gene sequence similarity. In this scheme, the thermophilic and non-motile *P. taiwanensis* appears isolated. The phylogenetic distance (>4.5 % difference in 16S rRNA gene sequence) that separates it from the others indicates that a new genus could be set up to accommodate it. Nevertheless, this splitting appears premature, since only one thermophilic *Pseudoxanthomonas* species has been described so far. As a consequence, it seems judicious to wait for the description of more thermophilic and mesophilic *Pseudoxanthomonas* species, in order to determine whether they clearly form two independent homogeneous groups. In any case, the additional information brought by the description of *P. japonensis* and *P. mexicana* and also the re-evaluation of the phenotype of *P. broegbernensis* DSM 12573^T in this work indicate the necessity of emending the descriptions of the genus *Pseudoxanthomonas* and *P. broegbernensis*. The new data brought by the recent description of *P. taiwanensis* will be also incorporated in the emended description of the genus.

Ecological aspects

The isolation of strains AMX 26B^T, UR374_02 and 12-3^T broadens the number of biotopes known to be occupied by *Pseudoxanthomonas* species and indicates that they are widely distributed over the world, since they have been found in three continents (Europe, Asia and America). The case of *P. mexicana* also shows that each species may colonize different environments. The presence of this species in an anaerobic digester seems on first inspection to be casual. Indeed, its growth under anoxic conditions is

only possible with nitrite as the electron acceptor, a compound unlikely to be found in sufficient concentrations in the cheese factory effluent used to feed the reactor. This suggests that *P. mexicana* was probably introduced in this biotope as a transient micro-organism through the wastewater. Nevertheless, it is surprising to see that an as-yet uncultured bacterium (clone SH-39) phylogenetically very close to *P. mexicana* (99.7 % 16S rRNA gene sequence similarity; Fig. 1) was also detected in an anaerobic reactor (Schlötterburg, 2001) and that other respiratory denitrifiers have been found to survive for very long periods of time (even years) in such nitrate-/nitrite-free anaerobic environments (Jørgensen & Tiedje, 1993). The question then remains open as to whether this species can or can not be an autochthonous member of the microflora of this biotope, as was previously observed in the case of another denitrifying member of the family *Xanthomonadaceae*, *Stenotrophomonas acidaminiphila* (Assih *et al.*, 2002). The presence of *P. mexicana* UR374_02 in the urine of a child at a titre compatible with urinary tract infections (10⁵ c.f.u. ml⁻¹) also raises the question of its potential role (and that of other *Pseudoxanthomonas* species) as an opportunistic pathogen. The fact that it was obtained in mixed culture with environmental or saprophytic bacterial species (*Enterococcus* sp., *Acinetobacter* sp., coagulase-negative staphylococci and *Corynebacterium* sp.) and the absence of infective signs at the moment of isolation suggest that it came from contamination (i.e. saprophytic skin flora) and propagated in the bag used to collect the catheter urine. *P. mexicana* therefore appears not to have clinical significance. In any case, it seems to be highly susceptible to treatment by antibiotics, as are the other mesophilic *Pseudoxanthomonas* species. It is also worth noting that, similarly to *P. mexicana*, uncultured bacteria (clones KL-15-2-16 and KL-59-7-1) phylogenetically close to *P. japonensis* (99.2–99.3 % 16S rRNA gene sequence similarity; Fig. 1) have been found in a completely different biotope (spacecraft assembly facilities). This suggests that novel strains belonging to the known species should soon be isolated or that novel species of the genus should be described.

Emended description of the genus *Pseudoxanthomonas* Finkmann *et al.* 2000

Members of the genus are non-spore-forming rods, usually 0.4–0.8 × 0.9–1.5 µm, which stain Gram-negative. They have a strictly respiratory type of metabolism with O₂ as preferential terminal electron acceptor and can reduce nitrite but not nitrate. N₂O is always a main product of nitrite reduction if not the only one. Most species are mesophilic (optimum temperature 30–37 °C), slightly alkalophilic (optimum pH over 7 and preferentially around 8) and motile by means of a single polar flagellum; however, one species is thermophilic (optimum temperature 50 °C) and non-motile. Colonies on solid media are generally yellow to pale yellow or beige. Oxidase-positive and heterotrophic. They use a limited range of carbon sources, including some sugars, organic and amino acids.

The cellular fatty acids are of the iso/anteiso type with 15:0 iso normally predominating and 13:0 iso 3-OH always absent. Quinones are generally of the Q8 type. DNA G+C content is 65–70 mol%. Members of the genus are widely distributed in nature. The type species is *Pseudoxanthomonas broegbernensis*.

Emended description of *Pseudoxanthomonas broegbernensis* Finkmann *et al.* 2000

Exhibits all of the characteristics of the mesophilic members of the genus. Tests for catalase and hydrolysis of ONPG/PNPG, aesculin, DNA and Tween 80 are positive, while those for gelatin, starch and urea hydrolysis, indole production, lysine and ornithine decarboxylase but also arginine dihydrolase are negative. Substrates utilized and susceptibility to antibiotics are detailed in Tables 1 and 3. Can be distinguished from the other mesophilic species by its ability to use L-arabinose, lactose, maltitol, D-melibiose, palatinose, sucrose, D-turanose and citrate. Predominant fatty acids are, in decreasing abundance, 15:0 iso, 15:0 anteiso, 16:1 *cis*9, 17:1 iso *cis*7 and 16:0. The fatty acid 17:0 cyclo is absent. Reduces nitrite to nitrous oxide as sole end product. Growth is possible with 0–40 g NaCl l⁻¹, at 10–37 °C and pH 6.5–9.75 and is optimal at 30 °C, pH 8 and without NaCl. The DNA G+C content is 66.5 ± 0.8 mol%.

The type strain, B1616/1^T (=ATCC BAA-10^T=CCUG 46890^T=CIP 107227^T=DSM 12573^T), was isolated from an experimental biofilter supplied with the waste gas of an animal-rendering plant.

Description of *Pseudoxanthomonas mexicana* sp. nov.

Pseudoxanthomonas mexicana (me.xi.ca'na. N.L. fem. adj. *mexicana* pertaining to Mexico, where the type strain was isolated).

Exhibits all of the characteristics of the mesophilic members of the genus. Tests for catalase and hydrolysis of aesculin, gelatin and DNA are positive, while those for ONPG/PNPG and urea hydrolysis, indole production, lysine and ornithine decarboxylase as well as arginine dihydrolase are negative. Tween 80 and starch hydrolysis may give a positive or negative result depending on the strain. The spectrum of substrates utilized is listed in Table 1; it may fluctuate from strain to strain. Members of this species can be distinguished from the other mesophilic species by their inability to use D-galactose, D-glucosamine, lactulose and D-xylose. Susceptible to most classes of antibiotics except aminoglycosides. Predominant fatty acids are, in decreasing abundance, 15:0 iso, 17:1 iso *cis*7, 16:0 iso, 11:0 iso 3-OH and 11:0 iso. Growth is possible with 0–40 g NaCl l⁻¹, at 10–37 °C and pH 5.8–9.75 and is optimal at 30–37 °C, pH 7–8 and without NaCl. The DNA G+C content is 67.8 ± 2 mol%.

The type strain, AMX 26B^T (=ATCC 700999^T=CIP

106674^T=JCM 11524^T), was isolated from the sludge of an anaerobic reactor treating the wastewater of a cheese factory. Strain UR374_02 (=DSM 15133) was isolated from the urine of a child.

Description of *Pseudoxanthomonas japonensis* sp. nov.

Pseudoxanthomonas japonensis (ja.po.nen'sis. N.L. fem. adj. *japonensis* pertaining to Japan, where the type strain was isolated).

Exhibits all of the characteristics of the mesophilic members of the genus. Tests for the hydrolysis of ONPG/PNPG, aesculin, gelatin, DNA and Tween 80 are positive, while those for starch and urea hydrolysis, catalase, indole production, lysine and ornithine decarboxylase but also arginine dihydrolase are negative. The spectrum of substrates used is listed in Table 1. Can be distinguished from the other mesophilic species by its ability to use fumarate and L-malate but not L-proline. Susceptible to most classes of antibiotics except aminoglycosides, fusidic acid and pipemidic acid. Predominant fatty acids are, in decreasing abundance, 15:0 iso, 17:1 iso *cis*7, 16:0 iso and 11:0 iso 3-OH. Growth is possible with 0–30 g NaCl l⁻¹, at 10–37 °C and pH 6.2–9.5 and is optimal at 30–37 °C, pH 8 and without NaCl. The DNA G+C content is 65.2 ± 1 mol%.

The type strain, 12-3^T (=CCUG 48231^T=CIP 107388^T=JCM 11525^T), was isolated from polluted urban soil.

Acknowledgements

Financial support provided by IRD to S.T. and A.S.O. and by the Secretaria de Relaciones Exteriores (Foreign Affairs) of Mexico to S.T. is gratefully acknowledged. Many thanks are due to Anne Le Flèche (Pasteur Institute; biochemical tests), Florence Megglé and Marie Christine Bray (Alphabio; antibiogram), Karen Dohrman (Microbial ID; CFA analysis), John Bartell (MIDI Labs; 16S rDNA sequencing) and Peter Schumann (DSMZ; G+C and DNA–DNA hybridization) for their professional and fine suggestions on the techniques that they performed for us on a commercial basis. Thanks are also due to Jean-Louis Garcia, Bernard Ollivier, Yannick Combet-Blanc and Jean Lemer for fruitful discussions on taxonomy, phylogeny and many other topics, to Bernard La Scola for advice on antibiogram interpretation and to Jean-Luc Cayol for the preliminary analysis of the 16S rRNA gene sequence of strain AMX 26B^T and for its submission to GenBank. All our gratitude goes to Monica Bonilla, Marie-Laure Fardeau, Ashish Jhina and Nicolas Bernet for their decisive contributions to the identification of the final products of nitrite reduction. We also appreciated very much the help of André Lipski and Guang-Huey Lin, who always kindly answered our questions about the strains of *P. broegbernensis* and *P. taiwanensis* that they respectively described. Finally, we are indebted with Paulino Rivas, Omar Reyes and Abelardo Villareal of Quesos Caperucita who provided the sludge and wastewater used to operate the reactor from which strain AMX 26B^T was isolated.

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