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Polyphasic Characterization of Poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(HB-co-HV)) Metabolizing and Denitrifying *Acidovorax* sp. Strains

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Summary

For the purpose of denitrification in small drinking water plants, a bacterial mixed population was isolated from a packed bed column bioreactor with poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(HB-co-HV)) as a substrate for the denitrification of ground water (10 °C). Isolates 2nIII from the mixed culture, with the ability to denitrify and metabolize P(HB-co-HV), were used as starter cultures for the elimination of nitrate in ground water. The strains were characterized by diverse techniques. Classical phenotypic studies lead to rRNA group III of the genus *Pseudomonas*. Results obtained by molecular techniques demonstrated that the 2nIII strains are members of the *Comamonadaceae* and shows similarities to the genus *Acidovorax*. However, an integration of the 2nIII isolates within one of the known *Acidovorax* species is not possible for the moment. The 2nIII starter cultures clustered close to *Av. temperans* according to their whole cell proteins and fatty acids, whereas in DNA/DNA hybridization no significant DNA binding (< 25%) was found. In contrast a significant but low degree of DNA/DNA hybridization was found between the 2nIII strains and *Av. facilis* and *Av. delafieldii*. Our polyphasic results lead to the conclusion that the 2nIII strains may constitute a separate *Acicdovorax* species.

Key words: DNA/DNA Hybridization – Genus *Acidovorax* – Drinking Water – Denitrification – Poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(HB-co-HV))

Introduction

Various methods (ion-exchange, heterotrophic denitrification, autotrophic denitrification a.o.) can be applied to reduce the nitrate concentration in drinking water. The technology presented in this study has a fundamental advantage to other alternative heterotrophic ways of drinking water denitrification by using the biopolymer – Poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(HB-co-HV)) as an alternative carbon source instead of e.g. ethanol, methanol or other carbon sources. The advantage of P(HB-co-HV) is that it can be used as carbon source and matrix for microorganisms. P(HB-co-HV) should increase the efficiency of the denitrification process in drinking water. The crucial point was to find the appropriate microorganisms to simultanously reduce nitrate at a high rate and utilize P(HB-co-HV) as a carbon source. The required properties were found in the 2nIII isolates. The phenotypic properties of the 2nIII strains point to rRNA group III of the former genus *Pseudomonas*. But the isolates' key characteristics – P(HB-co-HV) hydrolysis and denitrification – did not seem to match with any of the described species in this group. The rRNA group III is now recognized as the *Comamonadaceae* and contains at least eight heterogeneous genera: *Comamonas, Xylophilus, Hydrogenophaga, Delftia, Polaromonas, Variovorax, Rhodoferax* and *Acidovorax* [DE LEY 1992, WILLEMS et al. 1991, WEN et al. 1999]. The genus *Acidovorax* was described by WILLEMS et al. [1990]. This genus consists of at least four species on two separate rRNA subbranches: *Acidovorax facilis*, *Av. delafieldii*, *Av. temperans* constitute one subbranch separate from *Acidovorax -Acidovorax konjaci subbranch*.

The purpose of this work was to examine the taxonomic status of the 2nIII isolates. For this reason a polyphasic taxonomic study was carried out. This ap-

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proach included the classical morphological, physiological and biochemical tests, fatty acid analysis, polyacrylamide gel electrophoresis of whole cell protein extracts, DNA/DNA hybridization, DNA base composition and rRNA/DNA hybridization.

Materials and Methods

Isolation of the 2nIII strains

A poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(HB-co-HV)) packed bed column was fluidized with ground water. To this ground water KNO_3 was added in order to increase the nitrate concentration in the water to approximately 200 mg/l. In a screening process many isolates were examined until several isolates with the key characteristics – P(HB-co-HV) hydrolysis and a high denitrification rate were found [BIEDERMANN et al. 1992]. Several denitrifying and P(HB-co-HV) metabolizing cultures were isolated. The most suitable microorganisms (seven 2nIII isolates) were further characterized. Application of flow cytometry and cell sorting was used to check for the purity of the isolates.

Bacterial strains and growth media

Unless otherwise indicated all strains were grown aerobically in the dark at 30 °C on nutrient agar (**NA**, 0.1% beef extract, 0.2% yeast extract, 0.5% peptone, 0.5% NaCl and 2% agar, pH 7.0). The 2nIII isolates were maintained on P(HB-co-HV) mineral salt medium at 4 °C or at room temperature. **P(HB-co-HV)** mineral salt media contains 0.2% P(HB-co-5.6% HV) suspension (ZENECA, Billingham, UK), 0.35% K_2HPO_4, 0.15% KH_2PO_4, 0.3% KNO_3, 0.014% $MgSO_4 \cdot 7\ H_2O$, and 2% agar, pH 7.0. Several *Acidovorax* strains representing the named species and several polyhydroxyacid (PHA) degrading strains [MERGAERT and SWINGS 1996] were included for comparison (Table 1).

Morphological, physiological, biochemical and toxicological characterization

The classical tests involve morphological, physiological and biochemical parameters. Unless otherwise indicated, incubation for these tests was done at 30 °C. Tests for motility, spores, swarming, cell morphology, pigments, reserve materials, growth at different temperatures, pH range, nitrate reduction, catalase activity, oxidase, salt tolerance, antibiotic tolerances, VOGES-

Table1. Strains and isolates studied.

name/taxon	strain	source
unidentified	2nIII VK	ground water
unidentified	2nIII 23	ground water
unidentified	2nIII 184	ground water
unidentified	2nIII 398	ground water
unidentified	2nIII 5/92	ground water
unidentified	2nIII 5/93, DSM 9387	
unidentified	2nII 6/95	ground water
unidentified	PHA 313	soil
unidentified	PHA 63	compost
unidentified	PHA 179	soil
unidentified	PHA 204	soil
unidentified	PHA 553	surface water
unidentified	PHA 570	surface water
unidentified	PHA 662	surface water
Acidovorax facilis	LMG 2193 (T)	ATCC 11228
Av. facilis	LMG 2194	ATCC 15376
Av. facilis	LMG 6598	CCUG 15919 DSM 550
Av. facilis	LMG 6599_{t2}	CCUG 15920
Av. delafieldii	LMG 5943 (T)	CCUG 1779^T ATCC 17505
Av. delafieldii	LMG 1792_{t2}	STANIER 134_{t2}
Av. delafieldii	LMG 7168	CCUG 11062
Av. delafieldii	LMG 7166_{t1}	CCUG 11056_{t1}
Av. delafieldii	LMG 5944	CCUG 14478
Av. delafieldii	LMG 7164	CCUG 3746A
Av. temperans	LMG 7169 (T)	CCUG 11779
Av. temperans	LMG 6436	CCUG 9943Aa
Av. temperans	LMG 8452	CCUG 22215
Av. temperans	LMG 3332	CIP 239.74 21717
Av. temperans	LMG 6439	CCUG 17140
E. coli K12	DSM 498	ATCC 23716

BCCM™/**LMG** – Bacterial Collection, Laboratorium voor Microbiologie, State University Ghent, Belgium; **PHA** – Poly(3-hydroxybutyrate) degrading isolates of J. Mergaert, Laboratorium voor Microbiologie, State University Ghent, Belgium; **CCUG** – Culture Collection of the University Göteborg, Sweden; **DSM** – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; **ATCC** – American Type Culture Collection, USA; **HSM** – Hohenheimer Sammlung von Mikroorganismen, Stuttgart, Germany; **T** – type strain, $_{t1}$ and $_{t2}$ two stable colony types were isolated from the original culture and labeled t1 and t2. Since both types have almost identical protein electrophoretic patterns, we only used one of both types in some techniques.

PROSKAUER reaction (VP), lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase (ADH), o-nitrophenyl-β-D-galactopyranoside (ONPG), indole production, H_2S production, polyhydroxybutyrate (PHB) hydrolysis, Tween 80 hydrolysis, amylase, urease activity, citrate utilization, DNase activity, gelatin liquefaction, casein hydrolysis and oxidation-fermentation (OF-Test) were performed using standard methods [DREWS 1983, SÜSSMUTH et al. 1987] and as a control API-galleries (20E and 20NE, BIO-MÈRIEUX, Marcy-l'Etoile, France) were used.

To study the morphology transmission electron microscopy (TEM; PHILIPS EM201, Eindhoven, NL) was used. The cells were prepared by negative staining in 1% (v/v) uranylacetate-solution. The degradation of carbon sources was performed in the following liquid medium: 0.35% K_2HPO_4, 0.15% KH_2PO_4, 0.1% KNO_3, 0.01% $MgSO_4 \cdot 7\ H_2O$, trace elements [SCHLEGEL 1993] and 9,4 mmol carbon source. The nutritional screening was performed in 3-hydroxybutyrate (**3-HBA**) liquid media (0.2% 3-HBA, 0.35% K_2HPO_4, 0.15% KH_2PO_4, 0.3% KNO_3, and 0.01% $MgSO_4 \cdot 7\ H_2O$, pH 7.0). Vitamins and trace elements were added in concentrations as described by SCHLEGEL [1993] and DREWS [1983]. The experiments were set up in threefold determinations with a greek-latin square [RUTTLOF et al. 1979]. The growth rates μ for the single vitamins and trace elements were calculated from absorbance at 610 nm measured with a LP2W spectrophotometer (Lange, Germany).

Autotrophic growth with hydrogen was assayed on the basal medium described by ARAGNO and SCHLEGEL [1992] supplemented with 0.01% (w/v) yeast extract [WILLEMS et al. 1990]. To obtain a suitable atmosphere, the method of SLY [1984] was modified. Anaerobic incubators (GA 630, GÖSSNER, Germany) and gas generating kits (BBL, GasPak Plus, Cockeysville, MD, USA) were used without a catalyst. In each jar six to ten strains were tested, including two hydrogen-oxidizing reference strains (positive controls: *Acidovorax facilis* LMG 6599 and *Av. facilis* LMG 2194) and one non hydrogen-oxidizing reference strain (negative control: *E. coli* K12). The jars were incubated at 30 °C for one week. A single strain was streaked out on one basal agar petri dish. Controls for each strain on basal medium were incubated at 30 °C under normal aerobic atmosphere. Autotrophic growth was regarded as positive if growth on autotrophic medium in the jar was clearly visible and more abundant than growth on the same medium incubated in air. All assays were repeated at least three times.

Fatty acid analysis

Cells were grown for 24 h at 28 °C on trypticase soy broth agar (TSBA) containing: 3.0% trypticase soy broth (BBL, Cockeysville, MD, USA) and 1.5% agar (BBL). After incubation, cells were harvested, saponified, and methylated as previously described [REINHOLD-HUREK et al. 1993]. The resulting fatty acid methyl esters were separated gas chromatographically on a fixed silver capillary column (HEWLETT-PACKARD model 5890A, Palo Alto, CA, USA). Data analysis was carried out by the microbial identification system (MIS) software version 3.8 (Microbial ID, Inc., Newark, Delaware, USA). Clustering of fatty acid methyl ester profiles was achieved by the unweighted-pair group method of averages (UPGMA) of the EUCLIDEAN distances calculated between each pair of profiles [SNEATH and SOKAL 1973]. The fatty acid profiles were also compared by principal component analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All strains were grown on nutrient agar (NA) at 28 °C in ROUX flasks. Whole cell protein extracts were prepared and SDS-PAGE was performed by using the procedure as described by KIREDJIAN et al. [1986]. The normalized densitometric traces of the protein patterns of 24 *Acidovorax* strains or possible *Acidovorax* strains were grouped by numerical analysis, using the PEARSON product moment correlation coefficient [*r*] as described by POT et al. [1989].

DNA base composition

The guanine plus cytosine contents (%G+C) were determined by using the bacterial DNA buoyant density centrifugation [FLOSSDORF 1983] and the thermal denaturation method [DE LEY and VAN MUYLEM 1963, DE LEY 1970].

For buoyant density centrifugation we used the BECKMAN Ultracentrifuge Model E (Palo Alto, CA, USA) and the AN-HT1 rotor. The relationship between%G+C and buoyant density (δ) was expressed by DE LEY (1970) in the following equation:%G+C = 1038.47 (δ – 1.6616). The DNA was prepared according to MARMUR's procedure modified by FLOSSDORF [1983]. The buoyant density (δ) and the%G+C-content of the sample DNA was determined by comparison with a known standard DNA from *Escherichia coli* K12 (δ = 1.710 g/cm^3, %G+C = 51.2%) and *Proteus vulgaris* (δ = 1.698 g/cm^3, %G+C = 39.3%). After 24 to 48 h scans were made at 260 nm. The radial positions of standard peaks and unknown DNA were measured and from these differences the%G+C could be calculated from the unknown sample as described by FLOSSDORF [1983].

For the thermal denaturation point method cells were grown in 1000 ml flasks for 16 h and DNA was isolated by using a modified method of MARMUR [1961]. To separate nucleic acid and proteins after lysis of the cells 5 M of NaCl was used instead of 5 M of sodium perchlorate. The%G+C was measured and calculated according to the method of DE LEY [1970].

DNA/DNA hybridization

To prepare high molecular weight DNA cells were grown in ROUX flasks for three days, and high molecular weight DNA was isolated as described above. For DNA hybridization the concentration of DNA was determined by using the method of BURTON [1956].

The degree of binding was determined spectrophotometrically (UVIKON 932, KONTRON, Neufahrn, GERMANY) by using the initial renaturation rate method [DE LEY et al. 1970]. Renaturation was performed in 2 · SSC buffer (0.3 M NaCl and 0.03 M sodium citrate, pH 7.0) at the optimal renaturation temperature of 79.7 °C, with a total DNA concentration of 0.097 mM. For the renaturation temperature a thermostatically controlled cuvette chamber was used. Degrees of 25% of binding or less indicated no significant DNA hybridization.

rRNA/DNA hybridization

High molecular weight DNA was fixed on cellulose nitrate filters (type SM11358, SARTORIUS, Göttingen, Germany) as described by DE LEY and DE SMEDT [1975]. 16S [^{3}H]rRNA from *Acidovorax facilis* ATCC11228 (LMG 2193^T) was used [WILLEMS et al 1989]. Each hybrid was characterized by its $T_{m(e)}$, which is the temperature at which 50% of a DNA/rRNA duplex is denatured under standard conditions.

Results

Morphological, physiological and biochemical description

The major characteristics of 2nIII isolates are given in Table 2. The table is limited to those results of which the reliability was confirmed by the corresponding API – gal-

Table 2. Correspondent characters of the 2nIII isolates (n = 7).

cell	cell shape	curved rods
	cell size	0.7–0.9 by 1.5–2 µm
	motility	+
	swarming, spores, capsules	–
	flagellation type	≥ 1 polar flagella
	gram-stain	negative
	%G+C	63.0 ± 0.7 (T_m)
	reserve materials	PHB
colonies	color of colonies	colorless
physiology	optimum growth temperature	30 °C
	pH optimum	7
	catalase, oxidase, Tween 80 hydrolysis, PHB hydrolysis, nitrate and nitrite reduction, autotrophic growth, protocatechuate cleavage, *meta* cleavage	+
	gelatin hydrolysis, citrate utilization, urease, H_2S from peptone, arginine dihydrolase (ADH), lysine decarboxylase, ornithine decarboxylase	–
C - source utilization (30 °C)	acetate, pyruvate, propionate, hydroxypyruvate, succinate, n-valerate, 2-ketovalerate, xylose, D-mannose, lactose	+ under aerobic and anaerobic conditions
	4-hydroxybutyrate, 2-ketobutyrate, 2-hydroxyvalerate, 4-ketovalerate, 2-ketoisovalerate, D-glucose, D-fructose, sucrose	+ under aerobic and – under anaerobic conditions
	n-hexanedioic acid, 2-hydroxybutyrate, hydroxybutyrate, heptanoic acid octanoic acid, 2-hydroxyoctanoic acid, n-decanoic acid	– under aerobic and anaerobic conditions
ecology	natural habitat	ground water
	pathogenicity on rats and mice[1]	no pathogenic potential
nutrient requirement	vitamins	no requirements
	trace elements	Fe^{2+} promotes, Co^{2+} inhibits
antibiotics	antibiotics tolerances	E10, FD10, S10
	antibiotics susceptibilities	AML25, AMP2, MY15, P10

+ positive reaction, – negative reaction; µ – growth rate; AML25 – amoxicillin (25 µg); AMP2 – ampicillin (2 µg); MY15 – lincomycin (15 µg); P10 – penicillin (10 international units); E10 – erythromycin (10 µg); FD10 – fusidic acid (10 µg); S10 – streptomycin (10 µg); [1]STANISZEWSKI et al. 1994.

leries test and for which all 2nIII isolates gave the same reaction. Results that vary among the 2nIII strains are not shown. The 2nIII isolates are gram-negative, curved rods and motile by means of one or more polar flagella. 2nIII isolates grow readily on P(HB-co-HV) solid media and on NA. On NA media transparent colonies developed within 24 h at 30 °C. On P(HB-co-HV) mineral salt medium transparent colonies developed and hydrolysis was observed after 72 h at 30 °C. Swarming could not be observed on any of the employed media (swarming, P(HB-co-HV) and NA media). No acid was produced from any tested carbon source. 2nIII isolates require no additional organic growth factor. Iron ions increased growth rate (µ) whereas cobalt ions significantly inhibited growth rate. Growth rates were not significantly affected when vitamins were employed.

Fatty acid analysis

Table 3 shows the average cellular fatty acid compositions of the 2nIII strains examined, including the composition of the reference taxa belonging to the *Acidovorax* genus. Only small differences among the individual strains were found. The fatty acids contain unbranched fatty acids with chain lengths ranging from 12 to 18 carbon atoms. Palmitoleic acid (16:1 ω7c), palmitic acid (16:0) and cis-vaccenic acids (Δ11-18:1) made up for approximately 85% of the total fatty acid content. The two hydroxylated acids, 3-hydroxyoctanoic (3-OH-8:0), and 3-hydroxydecanoic acid (3-OH-10:0), were present in quantities of approximately 5% in the genus *Acidovorax* and in the 2nIII isolates.

The fatty acid data of the test and reference strains were also analyzed by using soft modeling of class analogy principal component analysis and UPGMA clustering of EUCLIDEAN distances. The principal component analysis technique transforms similarities and dissimilarities among the individual strains into distances in a two dimensional plot. The separation along the first and second principal components accounted for 66% and 13% of the variation among the strains (results not shown). The strains of *Av. facilis* and *Av. delafieldii* formed one single cluster. The *Av. temperans* strains clustered separately. Six of the 2nIII isolates formed the third cluster. The strain 2nIII 5/93 was found between the three clusters.

Table 3. Mean values of major cellular fatty acid compositions of *Acidovorax delafieldii*, *Av. facilis*, *Av. temperans* and 2nIII isolates.

fatty acid	average% fatty acid of total fatty acid content standard deviation			
	Av. facilis n = 4	*Av. delafieldii* n = 3	*Av. temperans* n = 4	2nIII n = 7
12:0	3.6 ± 0.3	3.2 ± 0.3	4.7 ± 0.7	4.5 ± 0.4
14:0	3.1 ± 0.1	3.4 ± 0.2	1.1 ± 0.7	3.4 ± 0.1
15:0	< 0.5	< 0.5	2.2 ± 1.3	< 0.5
16:1 ω7c	41.9 ± 1.5	41.1 ± 1.7	46.0 ± 1.4	45.4 ± 0.8
16:0	25.8 ± 2.3	26.4 ± 0.3	25.0 ± 0.6	23.5 ± 1.4
17:0	< 0.5	< 0.5	1.2 ± 0.6	< 0.5
3-OH-8:0	1.1 ± 0.2	1.0 ± 0.1	1.0 ± 0.02	1.3 ± 0.3
3-OH-10:0	4.5 ± 0.8	4.1 ± 0.3	3.6 ± 0.2	6.0 ± 0.8
Δ11–18:1	19.2 ± 3.6	20.5 ± 1.9	14.3 ± 1.5	15.7 ± 1.2

n – the number of strains investigated; 12:0 = dodecanoic acid; 14:0 = tetradecanoic acid; 15:0 = pentadecanoic acid; 16:1 ω7c = palmitoleic acid; 16:0 = palmitic acid; 17:0 = 3-OH-8:0 = 3-hydroxyoctanoic acid; 3-OH-10:0 = 3-hydroxydecanoic acid, Δ11-18:1 = cis-vaccenic acid.

The clusters delineated with the principal component analysis could also be separated from each other by UPGMA of EUCLIDEAN distances. In the dendrogram (Figure 1) two main clusters can be distinguished: a first one containing the *Av. temperans* strains cluster together with the 2nIII strains and the PHA isolates and a second one containing the *Av. facilis* and the *Av. delafieldii* strains. The clustering of the fatty acid analysis showed a homogeneous group. On the one hand it was possible to delineate two clusters of organisms, but on the other hand the EUCLIDEAN distances between these organisms were very narrow. The biggest EUCLIDEAN distance between a test strain and a reference strain was 8.0 whereas EUCLIDEAN distance below 10 will normally not delineate a species.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

For the analysis of the protein patterns of 24 strains all points of the interpolated traces were used to construct the dendrogram in Figure 2. The numerical analysis separated the 24 strains in 5 clusters and 2 strains with a separate protein profile (PHA 313 and PHA 662). All 2nIII isolates have nearly identical protein profiles and clustered together; they can easily be recognized by the high molecular weight band. When omitting the zone containing this band in the analysis, the 2nIII strains still clustered together (results not shown). Four *Av. temperans* strains formed a single cluster including PHA 553 which is separate from the *Av. facilis* and the *Av. delafieldii* cluster. PHA 179 and PHA 204 belonged in the *Av. facilis* protein cluster and

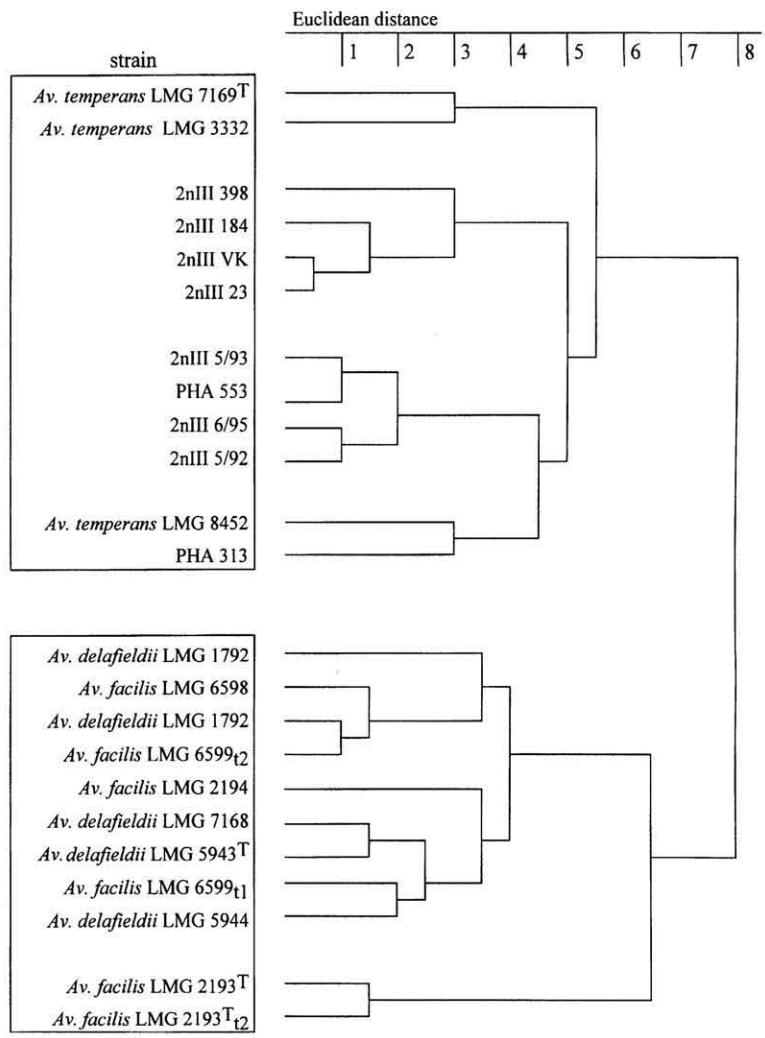

Fig. 1. Dendrogram obtained by unweighted average linkage cluster analysis (UPGMA) expressed as EUCLIDEAN distance of cellular fatty acid composition of *Acidovorax temperans*, *Acidovorax facilis*, *Acidovorax delafieldii*, and the 2nIII strains.

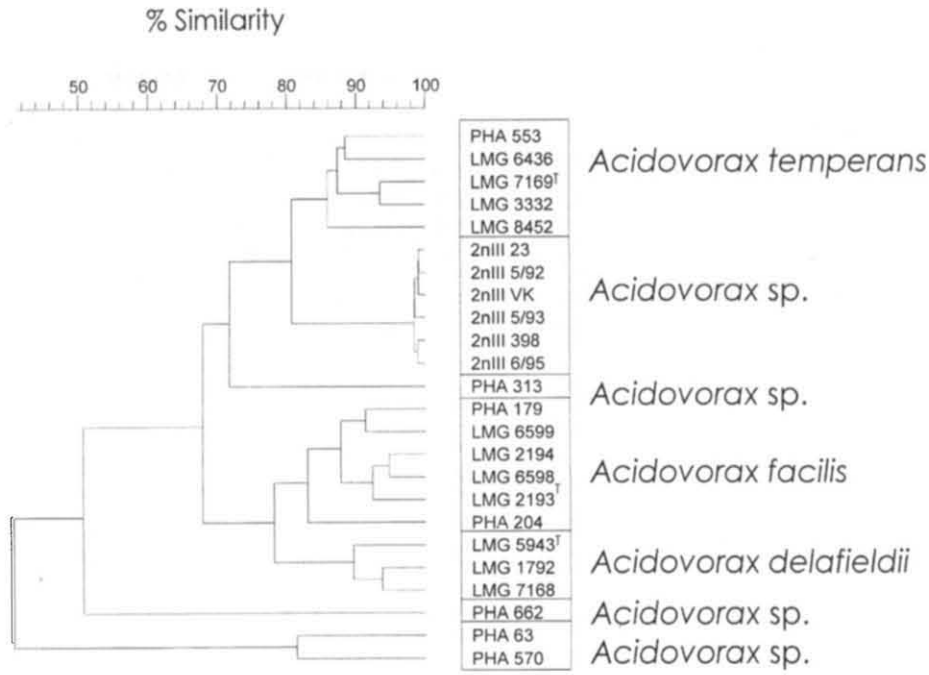

Fig. 2. Dendrogram showing the relationships between the electrophoretic protein patterns of *Acidovorax temperans*, *Acidovorax delafieldii*, *Acidovorax facilis* and test strains.

PHA 63 and PHA 570 are clearly separated from all the others and show (some) similarity to each other.

rRNA/DNA hybridization, DNA base composition and DNA/DNA hybridization

The results of the rRNA/DNA hybridization experiments are shown in Table 4. *Av. facilis*, *Av. delafieldii* and *Av. temperans* form DNA/rRNA hybrids with the labeled rRNA from the type strain of *Av. facilis* with $T_{m(e)}$ values between 78.7 and 79.8 °C. The corresponding $T_{m(e)}$ value for the 2nIII strain DSM 9837 is in the same range showing that this representative 2nIII strain belongs in the Acidovorax rRNA cluster.

The average%G+C value of the 2nIII isolates (n = 7) is 63 ± 1%.

The DNA binding values are shown in Table 5. The DNA/DNA hybridization values within and between the 3 known species belonging to the *Av. delafieldii* subbranch have been determined before (Willems et al. 1990) and it was shown that *Av. delafieldii* was genomically heterogeneous with DNA/DNA hybridization values between 40 and 100 and with at least 2 subclusters, one around strain LMG 1792 and another one around the former EF group 13 strains. We found comparable results namely three homogeneous clusters corresponding to *Av. temperans*, *Av. facilis* and the more heterogeneous *Av. delafieldii* DNA group. The representative 2nIII strain (5/93) had low hybridization values with *Av. temperans* but values between 40 and 50 with both other groups showing that it does not belong to any of the three DNA clusters.

Table 4. rRNA/DNA hybridization results with labeled rRNA from type strain of *Acidovorax facilis* ATCC 11228 (LMG 2193T).

DNA from	hybridized with *Av. facilis* LMG 2193T $T_{m(e)}$ [°C]
Av. facilis LMG 2194	79.8
Av. delafieldii LMG 1792	78.7
Av. temperans LMG 7169T	78.9
2nIII 5/93 (DSM 9387)	79.2

(*Av. delafieldii* LMG 1792 shares more than 70% of DNA/DNA hybridization with the type strain).

Discussion

Fatty acid analysis identified the 2nIII isolates as belonging to *Acidovorax*, a genus which was created and described by Willems et al. 1990. It is a member of the *Comamonadaceae*, belonging in the β-subclass of the Proteobacteria [De Ley 1992]. The fatty acid profiles of the 2nIII isolates have the highest similarity with those of *Av. temperans*, but a clear cut differentiation of the 2nIII group and members of *Acidovorax* was not possible, because the differences in the fatty acids patterns were too narrow.

The phenotypic results on the 2nIII strains are in accordance with the genus *Acidovorax*. The 2nIII strains grow on D-glucose, show various enzymatic activities and preferential growth on organic acids. They do grow autotrophically like *Av. facilis* and some strains of *Av. delafieldii* and they do reduce nitrite like *Av. temperans*. But as far as the results are available they can phenotypically not been identified as a member of one of the three species (*Av. delafieldii*, *Av. facilis* and *Av. temperans*).

A closer relationship between *Av. facilis* and *Av. delafieldii* and the more distant relationship to *Av. temperans* and the 2nIII isolates became apparent from the re-

Table 5. Average degrees of binding obtained from DNA/DNA hybridization between representative *Acidovorax* strains and possibly related strains.

2nIII 5/93		100									
Av. delafieldii	7166_{t1}	44	100								
Av. delafieldii	1792_{t2}	44		100							
Av. delafieldii	5943^T	40	50	54	100						
Av. delafieldii	7164	46	50	74	42	100					
Av. facilis	2194	39				23	100				
Av. facilis	6599_{t2}	50						100			
Av. facilis	2193^T	32			8		97	60	100		
Av. temperans	7169^T	18								100	
Av. temperans	6439	19									100
Av. temperans	8452	24								74	100

50% of binding or more
25% of binding or more
25% of binding or less (do not represent any significant DNA binding)

Table 6. Important differentiating characteristics of the investigated *Acidovorax* strains and the 2nIII test strains. Nitrite reduction, autotrophic growth and gelatinase from *Av. delafieldii*, *Av. temperans*, and *Av. facilis* were adopted from WILLEMS et al. [1990].

	Acidovorax facilis	*Acidovorax delafieldii*	*Acidovorax temperans*	2nIII (n = 7)
nitrite reduction	–	d	+	+
PHB hydrolysis	+	–	ND	+
gelatinase	+	d	–	–
chemoautotrophic growth	+	d	–	+
whole cell proteins (in average r values compared to 2nIII 5/93)	0.65	0.65	0.72	
DNA/DNA binding (in% average compared to 2nIII 5/93)	45	44	20	

+ is a positive reaction, present in 90% of the strains, – is a reaction present in 10% or less of the strains; d is a reaction present in 11 to 89% of the strains, ND is not determined.

sults of the protein gel electrophoresis as well. 2nIII isolates have nearly identical protein profiles indicating that they are similar to each other. The highest similarity was found with *Av. temperans* strains and one PHA strain (PHA 553). The other PHA strains had lower similarities to the 2nIII isolates, some of them clustering with *Av. facilis* and *Av. delafieldii* while others occupy separate positions. rRNA/DNA hybridization confirmed that the strains belong on the *Av. delafieldii* subbranch in the genus *Acidovorax*. The $T_{m(e)}$ values of the different *Acidovorax* strains ranged between 78.7 and 79.8 °C versus labeled rRNA from *Acidovorax facilis* LMG 2193^T. All other taxa belonging to the *Comamonadaceae* (*Comamonas*, *Hydrogenophaga* and *Xylophilus)* were located at a mean $T_{m(e)}$ level of 76 ± 1.1 °C [WILLEMS et al. 1990]. Comparison of full 16S rDNA sequences of the different members of the Comamonadaceae (WEN et al. 1999) confirmed that *Acidovorax* is subdivided into 2 subclusters one containing *Av. facilis*, *Av.temperans* and *Av. delafieldii*.

The division of members of *Acidovorax* belonging to the *Av. delafieldii* rRNA subbranch was based on the combined results of DNA/DNA hybridization and phenotypic results, since no clear cut differentiation was found on the basis of protein profiles and fatty acid profiles. However the three species could be differentiated phenotypically by eleven features [WILLEMS et al. 1990] and could be delineated by DNA/DNA hybridization values below 40%. Our new DNA/DNA hybridization results including the representative 2nIII strain made the interpretation more complicated, because the 2nIII isolate has low but significant binding values with representative strains of *Av. delafieldii* and *Av. facilis* and only low DNA/DNA hybridization %'s with members of *Av.*

temperans. Consequently we can conclude that the 2nIII isolates do not belong to *Av. temperans*. However our new results indicate that *Av. delafieldii* and *Av. facilis* have higher DNA/DNA hybridization values than reported before [WILLEMS et al. 1990]. More studies are definitely required to further unravel the relationships between the species belonging to this subbranch including more DNA:DNA hybridizations, the inclusion of more strains and the sequencing of the16S rDNA of a 2nIII strain. Because the 2nIII isolates have analogous low but significant degrees of binding with both species, we can suppose that the 2nIII isolates may constitute another species of *Acidovorax*, the more because the 2nIII isolates can phenotypically be differentiated from *Av. delafieldii* and *Av. facilis* (Table 5). We propose to classify the 2nIII strains as *Acidovorax sp.* until more isolates of the three involved species and more *Acidovorax spp.* have been included in a comprehensive polyphasic study.

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