The Journal of Microbiology, June 2002, p.109-118 Vol. 40, No. 2

Isolation, Characterization and Numerical Taxonomy of Novel Oxalate-oxidizing Bacteria

Nurettin Sahin[1]*, Isa Gökler[2] and Abdurrahman Ü. Tamer[3]

[1]Mugla Üniversitesi, Fen Edebiyat Fakültesi, Biyoloji Bölümü, 48187 Kötekli, Mugla, Turkey
[2]Dokuz Eylül Üniversitesi, Egitim Fakültesi, Biyoloji Bölümü, 35100 Buca, Izmir, Turkey
[3]Celal Bayar Üniversitesi Fen Edebiyat Fakültesi, Biyoloji Bölümü, 45000 Manisa, Turkey.

(Received March 11, 2002 / Accepted May 30, 2002)

The present work is aimed at providing additional new pure cultures of oxalate utilizing bacteria and its preliminary characterization for further work in the field of oxalate-metabolism and taxonomic studies. The taxonomy of 14 mesophilic, aerobic oxalotrophic bacteria isolated by an enrichment culture technique from soil, rhizospheres, and the juice of the petiole/stem tissue of plants was investigated. Isolates were characterized with 95 morphological, biochemical and physiological tests. Cellular lipid components and carotenoids of isolates were also studied as an aid to taxonomic characterization. All isolates were Gram-negative, oxidase and catalase positive and no growth factors were required. In addition to oxalates, some of the strains grow on methanol and/or formate. The taxonomic similarities among isolates, reference strains or previously reported oxalotrophic bacteria were analysed by using the Simple Matching (S_{SM}) and Jaccard (S_J) Coefficients. Clustering was performed by using the unweighted pair group method with arithmetic averages (UPGMA) algorithm. The oxalotrophic strains formed five major and two single-member clusters at the 70-86% similarity level. Based on the numerical taxonomy, isolates were separated into three phenotypic groups. Pink-pigmented strains belonged to *Methylobacterium extorquens*, yellow-pigmented strains were most similar to *Pseudomonas* sp. YOx and *Xanthobacter autotrophicus*, and heterogeneous non-pigmented strains were closely related to genera *Azospirillum*, *Ancylobacter*, *Burkholderia* and *Pseudomonas*. New strains belonged to the genera *Pseudomonas*, *Azospirillum* and *Ancylobacter* that differ taxonomically from other known oxalate oxidizers were obtained. Numerical analysis indicated that some strains of the yellow-pigmented and non-pigmented clusters might represent new species.

Key words: oxalate-bacteria, oxalotrophic, oxalate, numerical taxonomy

Oxalates are widespread in the animal and plant kingdoms and among a wide variety of microorganisms. Oxalic acid is often accumulated as a metabolic end-product in plant cells either as a free acid, as sodium and potassium oxalate, or precipitated as an insoluble salt, most commonly calcium oxalate. Through interactions with Al and Fe, oxalate plays a major role in plant nutrition by increasing the availability of P, K, Mg and Ca in soils (Allison *et al.*, 1995). On the death and decay of plants, oxalate is released into the soil where it's powerful chelating properties very prove are toxic and interfere with plant growth (Horner and Wagner, 1995). Through interactions with Al and Fe, oxalate plays a major role in plant nutrition by increasing the availability of P, K, Mg and Ca in soils (Allison *et al.*, 1995). Therefore microbes that mineralise and/or convert oxalates to biomass may be important modulators in the biological carbon cycle.

Several species of aerobic bacteria are known to be able to grow with oxalate (Chandra and Shethna, 1975; Tamer, 1982). Among the aerobic bacteria *Ralstonia oxalatica* DSM 1105 (Khambata and Bhat, 1953; Sahin *et al.*, 2000), *Ralstonia eutropha* (Jenni *et al.*, 1988), *Methylobacterium extorquens* DSM 1337 (Bassalik 1913; Bousfield and Green, 1985), *Starkeya novella* (Starkey, 1935; Kelly *et al.*, 2000) and among the anaerobic bacteria *Oxalobacter formigenes* (Allison *et al.*, 1985) and *Oxalophagus oxalicus* (Dehning and Schink, 1989) have been described most completely from the taxonomic and physiological points of view. Recently some aerobic, obligately oxalotrophic strains have been described under the new genus *Ammoniphilus* (Zaitsev *et al.*, 1998).

This research describes the selective isolation and characterization of novel oxalotrophic bacteria from different habitats (soil, rhizosphere, plant tissue, wastewater etc.) for further work in the field of oxalate-metabolism and taxonomic studies.

* To whom correspondence should be addressed.
(Tel) 90-252-223-8002; (Fax) 90-252-223-8656
(E-mail) nsahin@mu.edu.tr

Materials and Methods

The bacterial strains used, source, collection numbers and references are listed in Table 1. Strains *Methylobacterium extorquens* DSM 1337^T, *Ralstonia oxalatica* DSM 1105^T and *Pseudomonas* sp. NEU98 were included for comparisons as representatives of oxalotrophic bacteria.

Enrichment and isolation

Selective enrichment cultures were used to obtain mixed bacterial populations that were capable of oxalate utilization. Enrichment was performed in 250 ml flasks containing 50 ml of Schlegel's basal mineral medium (Aragno and Schlegel, 1992). For enrichment in liquid cultures, 4 g l^{-1} potassium oxalate was added. Flasks were inoculated with about 1 g l^{-1} sample, and incubated with agitation (105 rpm) at 28°C. All enrichment cultures were transferred three times on the same medium at 5-day intervals. Suspensions of the enrichment culture were plated on mineral medium supplemented with 4 g potassium oxalate (KOx) and 15 g agar per liter. Before pouring the plates, 80 ml sterile $CaCl_2$ solution (0.1 mol l^{-1}) were added in 920 ml l^{-1} medium to convert part of the oxalate present to calcium oxalate (CaOx). The plates were incubated at 28-30°C for a week. Morphologically distinct colonies that developed clear zones on the CaOx medium were selected. Clear zones around colonies could be due to the oxalate being degraded and/or transformed and/or mineralised. Strains were purified by streaking and checked microscopically. The purified cultures were maintained on ammonium oxalate agar slants containing (g l^{-1}); $(NH_4)_2C_2O_4$, 2; glycerol, 10; yeast extract, 2; $Na_2HPO_4 \cdot 12H_2O$, 9; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 5; $CaCl_2 \cdot 2H_2O$, 0.01; ferric ammonium citrate, 0.001; agar, 15; pH 7.0 ± 0.2 and stored at 4°C or as glycerol suspensions (20%, v/v) of cells at -25°C.

The capacity of strains to utilize oxalate was tested in pure culture. Three repeats of each culture were done for each isolate. A fresh cell suspension (OD 0.5 at 600 nm) was incubated aerobically at 28°C with agitation (105 rpm) in a sterile 250 ml flasks containing basal medium with 4 g l^{-1} oxalate. After 24-48 h of incubation oxalate remaining in the culture medium was estimated by titration. Samples of the culture (3 ml in triplicate) were heated to 60°C with 1 ml of 2 mol l^{-1} sulfuric acid and titrated with 0.1 mol l^{-1} potassium permanganate solution (Blackmore and Quayle, 1968).

Utilization of carbon sources

Schlegel's basal mineral medium was used throughout the study (Aragno and Schlegel, 1992). Substrate concentrations were 2 g l^{-1} for sugars, 0.2 g l^{-1} for formate, methanol, formaldehyde and phenol, and 1 g l^{-1} for all other compounds. Each strain was spotted on the agar surface; a plate without carbon source served as a control. The plates were incubated for 1 and 2 weeks; in case of doubt, the test was repeated using static or agitated liquid medium. Growth with volatile carbon substrates was tested in 50 ml screw cap filled with 10 ml basal medium. All thermolabile substrates were neutralized to pH 7 and sterilized by filtration or tyndalization.

Table 1. Isolates and reference strains used in this study

Sources	Willcox ID (most likely taxa)	Strain	Collection no.	References
Rhizosphere/rhizoplane				
Oxalis sp.	*Azospirillum brasilense*	NS01	NEU 1208	This study
Oxalis sp.	*Burkholderia* sp.	NS02	NEU 1209	This study
Liquidambar orientalis	*Pseudomonas* sp.	NS13	NEU 1220	This study
Paper mill effluent	*Ancylobacter* sp.	NS03	NEU 1210	This study
	Ancylobacter sp.	NS04	NEU 1211	This study
Stem/petiole maceration				
Pelargonium zonale	*Methylobacterium extorquens*	NS06	NEU 1213	This study
Ficus elastica	*M. extorquens*	NS07	NEU 1214	This study
Begonia sp.	*M. extorquens*	NS08	NEU 1215	This study
Bryophyllum sp.	*M. extorquens*	NS09	NEU 1216	This study
Rumex sp.	*Pseudomonas* sp.	NS12	NEU 1219	This study
Soil	*Xanthobacter* sp.	NS14	NEU 1221	This study
	Pseudomonas sp.	TA17	NEU 98	Tamer, 1982
	Agrobacterium sp.	NS05	NEU 1212	This study
	M. extorquens		DSM 1337^T	Bassalik, 1913
Air contaminant	*Pseudomonas* sp.	NS10	NEU 1217	This study
Lichen colonized rock	*Pseudomonas* sp.	NS11	NEU 1218	This study
Earthworm intestine	*Ralstonia oxalatica*	Ox1	DSM 1105^T	Khambata and Bhat, 1953

NEU: Laboratoire de Microbiologie de l'Univeristé, Neuchatel, Switzerland. DSM (DSMZ): Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany

Growth experiments with oxalate, formate, methanol, and ethanol as sole carbon source, different pH, and oxalate concentrations were performed in 250 ml flasks with agitation (105 rpm). An aseptically washed suspension of previously grown cells was used as an inoculum in growth studies. Turbidity was determined at 546 nm for yellow-pigmented strains and at 436 nm for the rest using a spectrophotometer. Growth rates were obtained by absorbance or cell density (μg ml^{-1}) against time.

Pigment analysis

Approximately 150 μl cell mass was scraped from the agar surface and placed in a test tube. Methanol or acetone-methanol mixture in a 3:1 ratio (v/v, 3 ml) was added and incubated at 50°C in a water-bath for 5 min to extract pigments. After centrifugation and filtration, the absorbance of the supernatant was examined between 350 and 600 nm using Shimadzu 1601 spectrophotometer with spectroscopic grade methanol or acetone-methanol mixture (3:1, v/v) as a blank. Spectral characteristics of the extracts were compared with similar extracts from *M. extorquens* DSM 1337^T and *Pseudomonas* sp. NEU98.

Resistance to antibiotics

Resistance to antibiotics was determined on Mueller-Hinton agar (Difco 0252) using standard antibiotic disks (Oxoid). Inhibition zone was noted after 48 h incubation; resistance was recorded as positive. Strains were considered susceptible when the inhibition zone was 12 mm or more in diameter. Tests were performed in triplicate. The following antimicrobial agents were tested: erythromycin (15 μg), ampicillin (10 μg), bacitracin (10 μg), chloramphenicol (30 μg), and streptomycin (10 μg).

Heavy metal ion resistance

The resistance to heavy metal ions (Zn^{2+}, Ni^{2+}, Pb^{2+}, CrO_4^{2-}, Co^{2+}, Cu^{2+}, and Hg^{2+}) was evaluated by the agar-diffusion method (Thompson and Watling, 1983). Metal salts (100 μg ml^{-1}; $ZnSO_4 \cdot 7H_2O$, $NiCl_2 \cdot 6H_2O$, Pb-acetate, $K_2Cr_2O_7$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$ and $HgCl_2$) were dissolved in dimethyl sulphoxide. Paper disks (6 mm diameter) were impregnated with 25 μl metal solution and placed on half strength nutrient agar (NA, Oxoid CM3) surface. After incubation at 30°C for 48 h, the diameter of the zone of inhibition was measured. If the area surrounding a disk was free from bacterial growth, it was recorded that the strain was sensitive to that metal.

Plasmid DNA detection

The hot alkaline method of Kado and Liu (1981) was used. The crude lysates were incubated at 75°C for 20 min for chromosomal DNA elimination. Electrophoresis was performed in a Tris-acetate (TAE) buffer at 100 V and 60 mA for 2 h. Gels were stained with 0.5 μg of ethidium bromide ml^{-1}.

Extraction and analysis of the Cellular lipids

Strains were grown at 30°C on nutrient agar plates until good growth was observed. A 4 mm loop was used to harvest about 50 mg of bacterial cells from the third quadrant of the quadrant-streaked plate. The cells were placed in an Eppendorf tube. The cellular lipids were extracted overnight at 37°C with 250 μl chloroform /methanol (2:1, v/v) and freed from the bulk of water-soluble, non-lipid substances by centrifugation at 5000 g for 5 min. To avoid possible loss of polar lipids, no attempt was made to remove non-lipid contaminants from the extracts. Lipid solutions were stored in the dark at -20°C until analysed. The total extractable lipids were separated by Silica gel 60 TLC plates (Merck) with chloroform/methanol/acetic acid/water (100:20:12:5, v/v) as the developing solvent. A 50% sulfuric acid solution followed by heating at 110°C was used to detect spots of all kinds of lipids.

Phenotypic tests

Phenotypic tests, morphological and physiological characteristics were performed as described previously (Smibert and Krieg, 1994). The API 20NE and API ZYM micro test systems were used according to the recommendations of the manufacturer (bioMerieux).

Numerical taxonomy

Numerical analysis of the data obtained from morphological, biochemical, substrate utilization and antibiotic susceptibility tests was performed by using the Simple Matching Coefficient (S_{SM}; Sokal and Michener, 1958) and Jaccard Coefficient (S_J; Jaccard, 1908). The results of tests that were positive or negative for all of the strains were eliminated from subsequent calculations. The data matrix was used to estimate the strain similarities, with calculation of S_{SM}, which includes both positive and negative matches, and S_J, which does not take negative matches into account, were calculated (Jenni *et al.*, 1987; Logan, 1994). Cluster analysis was computed into the phenogram by using average linkage (the unweighted pair group method with arithmetic averages; UPGMA) clustering method (Sneath and Sokal, 1973). The Willcox probability matrix was used to assign and identify isolates where scores of 0.8 and above indicated a positive identification (Willcox *et al.*, 1973). Strains were determined following the directions given in the identification matrix of Holmes *et al.*, (1986), API 20NE database (v6.0) and Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

Procedures for objective character selection have been described in detail by Sneath (1979). Sneath recommends selection of characters with a VSP (variance of separation potential) of >25% where possible. The minimum number of characters was selected from the 95 characters used in numerical taxonomy. From the several separation indi-

ces provided by the CHARSEP program (Sneath, 1979), the VSP index was chosen to find the best differentiating characters. A few tests with high values were excluded due to practical difficulties in their determination.

Results

Oxalate-enrichment cultures from soil and plant environments yielded two yellow-pigmented, four pink-pigmented and eight non-pigmented strains.

Potassium oxalate enrichment cultures from the rhizosphere of *Oxalis* sp., petiole and stem tissue of several plants, waste water, and from soil yielded aerobic, facultatively methylotrophic or nitrogen fixing oxalotrophic strains, which differ markedly from the some oxalate-utilizers reported earlier (Table 1).

Morphology and cellular properties

All of the isolates were non-endospore forming, regular (NS02, NS05, NS10, NS11, NS13) pleomorphic (NS01, NS06, NS07, NS08, NS09, NS12, NS14) or curved rods (NS03, NS04) with mean dimensions of 0.5×3 μm. With oxalate as the sole carbon and energy source, three types of bacterial colonies, pink (NS06, NS07, NS08, NS09), yellow (NS13, NS14) and non-pigmented (NS01, NS02,

Table 2. Characteristics of the clusters defined in numerical taxonomy.

Clusters	A	B	C	D	E	F	G
Number of strains (*n*)	2	3	2	3	DSM1105^T	5	NS14
Pigmentation	-	-	y	-	-	p	y
Motility	+	-	+	+	+	+	-
Assimilation of :							
Glucose	+	67	-	-	-	60	+
Arabinose	+	67	-	-	-	-	+
Mannose	+	33	-	-	-	-	+
Maltose	+	-	50	-	-	-	+
Fructose	+	+	-	33	+	80	+
Xylose	+	+	-	33	-	80	+
Lactose	-	33	-	+	-	-	-
Galactose	+	+	-	+	-	-	-
Sucrose	50	-	-	33	-	20	-
Glycine	-	33	-	33	-	-	-
Gluconate	+	33	50	-	+	-	+
Caprate	50	-	-	-	+	-	-
Adipate	+	-	50	-	+	-	-
Malate	+	+	+	67	+	+	+
Citrate	50	-	-	-	+	-	-
Phenyl-acetate	50	-	-	-	+	-	-
Malonate	-	33	-	33	+	+	-
L-tartrate	+	+	-	-	-	+	-
Acetate	+	+	-	33	+	+	-
Benzoate	50	-	-	-	+	80	-
Formate	50	+	-	-	+	+	-
Methanol	-	+	-	-	-	+	-
Ethanol	50	+	-	-	+	+	-
Propanol	+	+	-	33	-	80	-
Mannitol	+	+	-	-	-	-	+
Sorbitol	50	+	-	-	-	20	+
Gylcerol	+	+	50	-	+	+	+
Phenol	50	-	-	33	+	-	-
Formaldehyde	+	-	-	-	-	-	+
Hydrolysis of :							
Casein	-	-	-	-	-	-	+
Gelatine	50	-	50	33	-	-	-
Esculine	+	+	-	-	-	-	+
Fermentation of :							
Arabinose	-	-	-	-	-	+	+
Glucose	-	-	-	-	-	-	+

Table 2. Continued.

Clusters	A	B	C	D	E	F	G
Number of strains (*n*)	2	3	2	3	DSM1105^T	5	NS14
Growth on/at :							
MacConkey agar	50	67	+	-	+	-	+
Cetrimide agar	+	+	+	33	+	60	-
42 °C	+	67	-	67	+	-	-
3% NaCl	+	+	+	33	-	20	+
4% NaCl	50	-	+	-	-	-	+
2% Oxalate	50	67	+	-	-	-	+
Antibiotic resistance							
Chroramphenicol	-	33	50	-	-	80	-
Erythromycine	-	33	-	33	-	80	-
Streptomycine	50	-	-	67	-	80	-
Ampicilline	+	33	+	67	-	+	-
Bacitracine	+	+	+	+	+	+	-
Enzyme activity							
Nitrate reduction	+	-	-	67	+	-	+
Denitrificiation	-	-	-	-	+	-	-
Urease	+	33	-	33	+	+	-
DNase	+	+	-	33	+	20	-
ONPG	50	+	-	-	-	-	+
N-acetly-glucosamine	+	-	-	-	-	-	+
Lipase (C14)	-	67	50	-	-	-	-
Valine arylamidase	+	+	+	33	-	80	+
Cystine arylamidase	+	+	50	67	-	80	-
Trypsine	-	+	-	+	-	+	-
Acid phosphatase	+	+	50	67	+	+	-
α-galactosidase	50	-	-	-	-	-	-
β-glucosidase	50	67	-	-	-	-	+
β-galactosidase	-	67	-	-	-	-	+
α-chymotrypsin	-	67	-	-	-	20	-
Alkaline phosphatase	+	+	+	+	+	+	-
α-glucosidase	-	-	-	-	-	-	+
N-acetly-β-glucosaminidase	-	-	-	-	-	-	+

The values given in the table are the percentage of positive strains; [+]: all strains positive, [−]: all strains negative, y: yellow, p: pink.

NS03, NS04, NS05, NS10, NS11, NS12) were isolated on CaOx agar plates. Generally, pinpoint colonies (except NS01 and NS14) appeared between 48-72 h on CaOx and KOx agar plates, but no colonies were visible up to 48 h. After ≥ 72 h the colonies were about ≤ 1 mm diameter. All the three types were strictly aerobic. All isolates grew on NA, CaOx and KOx agar plates.

Biochemical and phenotypic characterization

All isolates were Gram-negative, catalase and cytochrome oxidase positive. The following tests were negative for all strains: acetoin production, methyl red, indole production, hydrogen sulphide production, growth with 7% NaCl, arginine dihydrolase, lysine- and ornithine decarboxylase, hemolysin, β-glucuronidase, α-mannosidase and α-fucosidase. The following tests were positive for all strains: Poly-β-hydroxybutyrate production, growth at 37°C, alkali reaction in litmus milk, C_4-esterase, C_8-esterase lipase, leucine arylamidase, and naphthol-AS-BI-phosphatase. The results are shown in Table 2.

Growth characteristics

The optimum conditions for growth in liquid medium with oxalate as the sole source of carbon and energy were investigated. Strains NS01, NS03, NS04, NS13 and NEU 98 tolerated oxalate in concentrations up to 2.5% (w/v) with an optimal value of 0.4 to 1.5% (w/v) in the mineral salts medium. Four g l^{-1} of oxalate was optimum for growth of strain NS13 as it permitted rapid growth with a reasonably short lag period. Higher concentrations inhibited growth. In concentrations of over 1.5% (w/v) there was little or no growth. Permanganate titrations showed that over 50% of oxalate was utilized within 24 h of growth by strains NS02, NS04, NS06 and NS13 at 30°C.

All of the isolates grew well at neutral pH ranging from pH 7 to 7.5. A majority of strains were able to grow with

slightly alkaline pH. During the growth with oxalate the medium pH rose to nearly 9. Pink-pigmented strains grew faster on methanol than the other tested substrates. But on the other hand non-pigmented facultatively methylotrophic strain NS03 grew better on ethanol than methanol (Table 5).

Absorption spectra of the pigments

Bacterial pigments are from secondary metabolites, and most of them are called as carotenoids. The basic characteristics of each carotenoid are the position and number of the peaks of maximum absorption. In bacterial systematic, carotenoids serve as important chemotaxonomic markers in genera such as *Micrococcus, Kocuria, Flavobacterium, Xanthomonas, Sphingobacterium* (Krieg and Holt, 1984; Kocur, 1986 Balows *et al.*, 1992). Pigment spectra are a simplifying and accelerating factor for the identification and characterization of the pigmented bacteria, if it used with other distinctive criteria (Aragno and Schlegel, 1992). Therefore, the pigments present in oxalotrophic isolates were investigated in more detail. The absorption spectra of pink and yellow-pigmented isolates were determined and compared with those obtained by other workers (Jayasuriya, 1955; Anthony and Zatman, 1964; Mehta, 1973; Chandra and Shethna, 1975; Tamer, 1982).

Isolates NS06, NS07, NS08, NS09 contained pink, and NS13, NS14 yellow water-insoluble pigments. Absorbance spectra of the pigment extracts in acetone: methanol mixture (3:1, v/v) from pink pigmented oxalotrophic strains had absorbance maxima in the region 525 to 527 nm and 495 to 497 nm, with a slight infection at 464 to 466 nm (Fig. 1).

Absorbance spectra of the pigment extracts in methanol from yellow-pigmented isolate NS13 had absorption maxima in the region 403 and 426 nm, with a slight infection at 445 nm. Similar absorption spectra were observed for reference strain NEU 98. Strain NS14 had absorption maxima in the region 413, 436, and 465 nm, with a slight infection at 390 nm (Fig. 1). These pigment spectra indicated that the yellow and pink pigmentation of the strains is chiefly be due to carotenoids and there was a similarity of the pigment spectrums of close-related strains.

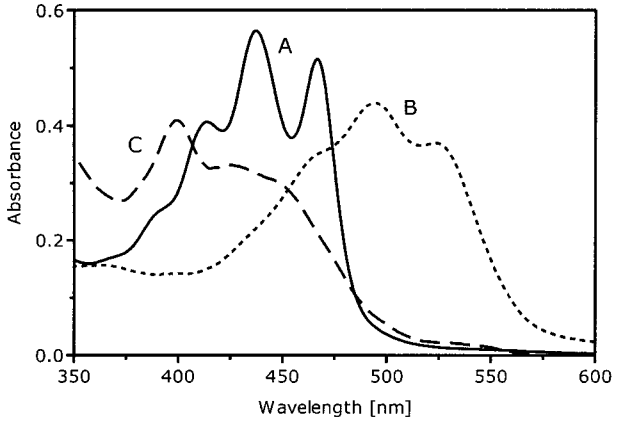

Fig. 1. Absorption spectra of main yellow and red pigment from some oxalate oxidizer and related reference strains. **A**: strain NS14, **B**: *Methylobacterium extorquens* DSM 1337^T and strains NS06, NS07 NS08, NS09; **C**: strains NS13 and NEU 98.

Table 3. Heavy metal ion tolerance of selected strains

		Metal ion						
	Pigment	Hg^{2+}	Cu^{2+}	CrO_4^{2-}	Ni^{2+}	Zn^{2+}	Co^{2+}	Pb^{+2}
NS01	np	+++	-	-	+	-	-	-
NS02	np	++	+	-	-	-	-	-
NS03	np	++	-	+	-	-	++	+
NS10	np	+++	-	+++	-	-	-	-
NS11	np	+++	-	-	-	-	-	-
NS12	np	++	-	-	-	-	-	-
NS05	np	++	-	-	-	-	-	-
NS06	p	+	-	+	-	+	-	+
DSMZ 1337^T	p	++	+	+	-	+	-	-
NS13	y	++	-	+++	-	-	-	-

Signs indicate degrees of inhibition zone : [+, ≤ 10 mm; ++, 11-20 mm; +++, ≥ 21 mm], [–], no inhibition zone was observed and colonies appeared around the metal containing disk in 7d incubation at 30°C. p: pink, y: yellow, np: non-pigmented.

Heavy metal tolerance and antibiotic resistance of isolates

The selected isolates showed varied degrees of tolerance to the seven metals tested (Table 3). Mercury was found to be the most toxic metal to all the three types of (yellow pigmented, pink pigmented and non-pigmented) bacteria. The order of metal toxicity observed was Hg>Cr>Cu=Zn=Pb>Co>Ni.

Comparison of tolerance to mercury and chromium indicated that several of the isolates were sensitive to these metal ions, but each isolate exhibited a characteristic level of sensitivity to each metal. Pink-pigmented strains were the most susceptible to toxic effects of metals. In most of the cases non-pigmented strains were the most resistant to metal toxicity.

A high proportion (87.5%) of the tested isolates were resistant to two or more of the studied antibiotics. Their extreme sensitivity towards chloramphenicol allows a reliable differentiation between strains (Table 4).

Plasmids and cellular lipids

Strains harbouring plasmids size of 7 and 10 to12 MDa (NS05, NS06, NS07, NS08) and 24 to 29 MDa (NS01, NS03, NS04, NS10) were detected. Similar or higher size of plasmids designated as pHG1, pTA19 and pTA07 have been reported by Jenni *et al.* (1988).

Several major spots of phospholipids and minor spots of unidentified lipids were observed on the TLC plate. Analysis of the lipids demonstrated that pink-pigmented strains consisted of at least two similar components, and strain

Table 4. Antibiotic resistance of the isolates

	AMP	B	E	CN	S	CH
NS01	+	+	-	-	-	-
NS02	+	+	-	+	+	-
NS03	-	+	-	nt	-	-
NS05	+	+	+	-	-	+
NS06	+	+	+	+	+	+
NS07	+	+	+	+	+	+
NS08	+	+	+	+	+	+
NS09	+	+	+	+	+	+
DSMZ 1337^T	+	+	-	-	-	-
NS10	+	+	-	nt	+	-
NS11	+	+	-	-	+	-
NS12	+	+	+	-	-	-
NS13	+	+	-	-	-	+
NEU98	+	+	-	-	-	-
NS14	-	-	-	-	nt	-
DSMZ 1105^T	+	nt	+	-	-	-

AMP; Ampicillin, **B**; Bacitracin, **E**; Erythromycin, **CN**; Gentamycin, **S**; Streptomycin, **CH**; Chloramphenicol. [+], no inhibition zone or an inhibition zone ≤ 12 mm in diameter was observed in 48 h incubation at 30 °C. [−], an inhibition zone of ≥ 12 mm was observed, nt: not tested.

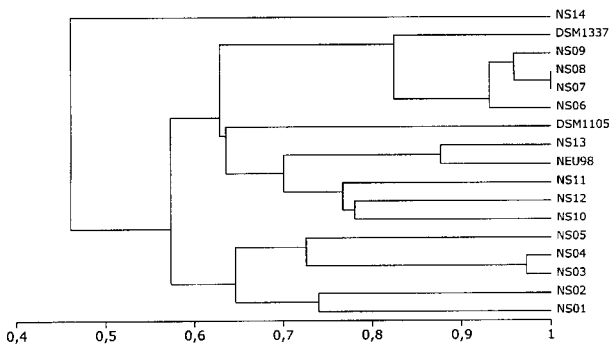

Fig 2. Dendrogram showing phenotypic similarities among the isolates and reference strains. Scale shows the similarities (S_{SM}).

NS13 and NS04 consists of at least three components. A number of spots more than three were observed in strain NS02 pattern. According to calculated R_f values major and ubiquitous cellular lipids of all 11 strains tested were phosphatidylethanolamine [PE], phosphatidylcholine [PC], phosphatidylinositol [PI], and phosphatidylserine [PS].

Numerical taxonomy

Ninety-five features were analysed to determine phenotypic characteristics of isolates. The results of the cluster analysis with similarity coefficients (S_{SM}) calculated from the 73 morphological and biochemical characteristics of the 17 strains studied are shown in Fig. 2.

To provide a benchmark for assessing homogeneity of characteristics among isolates, three reference strains (DSM 1105^T, DSM 1337^T, NEU98) were included in this study. A cluster analysis of the similarity coefficient calculated by using the S_{SM} and S_J equations resulted in identical relative relationships, but S_{SM} showed a higher level of similarity.

Table 5. Growth properties of selected strains in oxalate, ethanol, formate and methanol as a substrate

Substrates	Concn. (%)	NS07		NS09		NS03	
		OD_{436}	wt	OD_{436}	wt	OD_{436}	wt
Oxalate	0.4	0.266	85.79	0.283	83.56	0.139	44.93
Formate	0.2	0.201	43.45	0.290	55.71	0.078	14.85
Methanol	0.2	1.172	257.40	0.923	193.89	0.295	50.14
Ethanol	0.5	0.165	34.91	0.153	32.68	0.388	65.37

The absorbance was measured after 67-72 h of incubation on a shaking water bath. The turbidity has been recorded without substracting the control values. The purity of the cultures when growth had occurred was checked by streaking on nutrient agar plates. *wt*: dry weight concentration ($\mu g\ ml^{-1}$).

Five major and two singe-member clusters could be distinguished at a simple-matching similarity level of 74%-90% (Fig. 2) using the strains of previously described species as reference. A strain of *Xanthobacter* sp. NS14 and *Ralstonia oxalatica* DSMZ 1105^T could not be included in any cluster at a 70% S_{SM} similarity level.

Cluster A formed at an S_{SM} value of ≥74% (S_J≥62%) and contained two rhizospheric isolates (NS01, NS02); all strains in this cluster were able to utilize formaldehyde as a carbon source.

Cluster B contained three [S_{SM} ≥97%, (S_J≥94%) 2 isolates and S_{SM}≥74%, 1 isolate] facultatively methylotrophic, non-motile, non-pigmented strains (NS03, NS04 and NS05) isolated from waste water and soil. This cluster did not contain any reference strains.

Cluster C (S_{SM} ≥87%; S_J≥60%) consisted of yellow-pigmented isolates including reference strain NEU98. These slow or moderately slow-growing isolates formed colonies of less than 1 mm in diameter on NA plates within 7 days. All isolates produced water-insoluble yellow pigments on NA medium. These strains were able to grow with 4% NaCl and 2% oxalate.

Cluster D is more heterogeneous, and contained three non-pigmented (NS10, NS11 and NS12), lactose positive strains at an S_{SM} value of ≥76%. These strains were unable to growth with formate as the sole carbon source.

Cluster F [S_{SM}≥90%, (S_J≥80%) 4 isolates] included strains isolated from plant tissues. They were pink pigmented and facultatively methylotrophic. Type strain DSMZ 1337^T was included in this cluster with an S_{SM} value of ≥83%.

There was some variability in the biochemical characteristics of the isolates grouped under same clusters. Only six strains NS01, NS03, NS04, NS13, NEU98 and NS14 that grouped under the cluster A, B, C and G were grown on 2% oxalate. Formaldehyde (NS01, NS02 and NS14) and phenol (NS02, NS10 and DSM1105^T) utilization were observed limited number of strains belonging to cluster A D, E and G.

Discussion

One of the important goals of this study was to establish a physiological and phenotypic framework to enable characterization of oxalotrophic bacteria. The present work was also aimed at providing new isolates for further work in the field of oxalate-metabolism.

Eight non-pigmented isolates were obtained. Strains were grouped under three more heterogeneous clusters (cluster A, B and D) according to results of numerical taxonomy. Strain NS02 was more closely related to strain NS01 (62% S_J equation and 74% S_{SM} equation) and is able to use a wide range of compounds tested. These strains were assigned to the genus *Burkholderia* (NS02) and *Azospirillum* (NS01) based on the results obtained from phenotypic characterization and Willcox probability matrix with the previously published data of the Gram-negative bacteria in the Bergey's Manual of Systematic Bacteriology (Palleroni, 1984) and The Prokaryotes (Balows *et al.*, 1992).

In the previous study done by Weber *et al.* (1999), some *Azospirillum brasilense* and *Burkholderia cepacia* strains, which were isolated from roots, stems, and leaves of banana and pineapple plants were able to utilize oxalate in liquid media containing 0.1 g l^{-1} ammonium chloride. Our new strains were the most closely related to these species (Willcox *P* 0.98).

Two facultatively methylotrophic nonmotile, bean-shaped strains (NS03 and NS04) were obtained from a paper plant wastewater unit. The strains are able to grow at temperatures ranging from 25 to 42°C, with an optimum growth temperature of 30-35°C and at pH 6.0 to 8.5, with an optimum pH value of 7.0-7.5. The strains are highly homologous (94% S_J equation and 97% S_{SM} equation) to each other and based on the results obtained, both strains are assigned to the genus *Ancylobacter*. The genus *Ancylobacter* (formerly *Microcyclus*; Raj, 1989) represents nonmotile curved or bean shaped cells, which form circular structures before fission. Like strains NS03 and NS04, a number of strains have also been isolated from pulp mill oxidation lagoons (Staley and Konopka, 1984). According to our knowledge and literature survey this is the first report on the oxalate utilization of the strains belonging to genus *Ancylobacter*.

Methylotrophic strain NS05 resembles the non-pigmented methylotrophic isolates. According to the Willcox probability results, strain NS05 was closely related to genus *Agrobacterium* and most likely species is *A. rhizogenes* (Willcox *P* 0.86). In a previous study, oxalate-utilization has been reported in some *Agrobacterium* bv.1 strains isolated from nodules of tropical legumes (Lajudie *et al.*, 1999).

The phenotypic properties and probabilistic identifications of other non-pigmented oxalotrophic isolates NS10, NS11 and NS12 placed them into the genus *Pseudomonas*. An important feature and distinctive characteristic of these strains is their inability to grow on formate as the growth substrate. But they did not resemble any of the species previously reported as oxalate utilizers or other described species in the main literature such as Bergey's Manual of Systematic Bacteriology (Palleroni, 1984) or The Prokaryotes (Balows *et al.*, 1992).

Out of the 14 isolates obtained by enrichment technique, 4 were identically pink-pigmented and facultatively methylotrophic, and appeared most closely related to *Methylobacterium extorquens* DSM 1337^T. They were found to be practically identical in their phenotypic characteristics and pigment spectra. Two strains (NS06 and NS09), however, were slightly different from the other two strains in their tested features. The pink pigment showed a maximum absorbance at 464, 495 and 525 nm in spectroscopic acetone: methanol (3:1, v/v), which suggests that they are carotenoids (Fig. 1), and was similar to those reports from previous studies (Anthony and Zatman, 1964; Mehta, 1973; Tamer, 1982).

Strain NS13 showed a yellow water-insoluble pigment on NA agar and very limited carbon substrate utilization spectrum; only 10 out of 63 tested substrates were utilized. This strain resembles *Pseudomonas* YOx (Chandra and Shethna, 1975), *Pseudomonas* OD1 (Jayasuriya, 1955) and is most closely related to NEU98 (Tamer, 1982), but when compared with the published descriptions of these isolates, strain NS13 differs by some phenotypic characteristics (Table 2). A yellow-pigmented oxalotrophic strain isolated from petiole maceration of *Alocasia* sp. (Yara and Usami, 1968) shows a similar description to *Pseudomonas* OD1 of Jayasuriya (1955) except that the former is able to grow on a medium in which formate or acetate is the sole carbon source, while the latter is not. To our knowledge none of the above mentioned yellow-pigmented strains except NEU98 is available in culture collections. The poor description of these organisms does not allow comparison with our strains. But main differences can be observed on formate and/or acetate utilization and the absorption spectrum of the yellow pigments.

Strain NS14 isolated from soil litter close to *Oxalis* sp. showed completely different cell and colony morphology and substrate utilization patterns. Its pigment spectrum is also different from strains NS13 and NEU 98. The cell and colony morphology and biochemical features of strain NS14 were highly similar to those observed in *Xanthobacter autotrophicus* (Jenni *et al.*, 1987). The yellow pigment was soluble in methanol, where it showed distinct peaks at 413, 436 and 465 nm and a shoulder at 390 nm (Fig. 1). It was found to be identical to *Xanthobacter* spp., a mixture of zeaxanthin dirhamnoside and its monorhamnoside and free carotenoid derivatives (Wiegel and Schlegel, 1984; Jenni and Aragno, 1987).

We think therefore that strains in the clusters C and D might constitute novel taxa at the species level, charac-

terized by a particularly oxalate utilization, together with a limited substrate spectrum and the inability to grow with one-carbon compounds. Such organisms could therefore be considered as "specialized" aerobic oxalate utilizers.

Acknowledgments

The authors gratefully acknowledge Prof. Michel Aragno (University of Neuchatel, Switzerland) for valuable advice and providing strain NEU 98. We are particularly grateful to Nicole Jeanneret, who kindly freeze-dried the novel oxalotrophic strains. We are also deeply indebted to two anonymous reviewers for their many helpful comments and suggestions, which improved the manuscript.

References

Allison, M.J., K.A. Dawson, W.R. Mayberry, and J.G. Foss. 1985. *Oxalobacter formigenes* gen. nov., sp. nov.: oxalate-degrading anaerobes that inhabit the gastrointestinal tract. *Arch. Microbiol.* 141, 1-7.

Allison, M.J., S.L. Daniel, and N.A. Cornick. 1995. Oxalate-degrading bacteria. In *Calcium Oxalate in Biological Systems*. Ed. Khan, S.R. pp. 131-168. Boca Raton: CRC Press, Inc.

Anthony, C. and L.J. Zatman. 1964. The microbial oxidation of Methanol: I. isolation and properties of *Pseudomonas* sp. M27. *Biochem. J.* 92, 609-614.

Aragno, M. and H.G. Schlegel. 1992. The Mesophilic Hydrogen-Oxidizing (Knallgas) Bacteria. In *The Prokaryotes. A handbook on the biology of bacteria: Ecophysiology, isolation, identification, applications*. 2nd edition. Eds. Balows, A., Trüper, H.G., Dworkin,M. Harder, W., Schleifer, K-H. pp. 344-384. Berlin, Heidelberg, New York: Springer-Verlag.

Bassalik, K. 1913. Über die verarbeitung der oxalsäure durch *Bacillus extorquens* n. sp. *Jahrb. Wiss. Bot.* 53, 255-302.

Balows, A., H.G. Trüper, M. Dworkin, W. Harder, and K-H. Schleifer. 1992. The Prokaryotes. A handbook on the biology of bacteria: Ecophysiology, isolation, identification, applications. 2nd ed. Vol. I-IV. Berlin, Heidelberg, New York: Springer-Verlag.

Blackmore, M.A. and J.R. Quayle. 1968. Choice between autotrophy and heterotrophy in *Pseudomonas oxalaticus*. Growth in mixed substrates. *Biochem. J.* 107, 705-713.

Bousfield, I.J. and P.N. Green. 1985. Reclassification of bacteria of the genus *Protomonas* Urakami and Komagata 1984 in the genus *Methylobacterium*. (Patt, Cole and Hanson) emend. Green and Bousfield 1983. *Int. J. Syst. Bacteriol.* 35, 209.

Chandra, T.S. and Y.I. Shethna. 1975. Isolation and characterisation of some new oxalate-decomposing bacteria. *Antonie van Leeuwenhoek* 41, 101-111.

Dehning, I. and B. Schink. 1989. Two new species of anaerobic oxalate-fermenting bacteria, *Oxalobacter vibrioformis* sp. nov. and *Clostridium oxalicum* sp. nov., from sediment samples. *Arch. Microbiol.* 153, 79-84.

Holmes, B., C.A. Pinning, and C.A. Dawson. 1986. A probability matrix for the identification of Gram-negative, aerobic, non-fermentative bacteria that grow on nutrient agar. *J. Gen. Microbiol.* 132, 1827-1842.

Horner, H.T. and B.L. Wagner. 1995. Calcium oxalate formation in higher plants. In *Calcium Oxalate in Biological Systems*. ed Khan, S.R. pp. 53-71. Boca Raton: CRC Press, Inc.

Jaccard, P. 1908. Nouvelles recherches sur la distribution florale. *Bull. Soc. Vaud. Sci. Nat.* 44, 226-270.

Jayasuriya, G.C.N. 1955. The isolation and characteristics of an oxalate-decomposing organism. *J. Gen. Microbiol.* 12, 419-428.

Jenni, B., M. Aragno, and K.W. Wiegel. 1987. Numerical analysis and DNA-DNA hybridization studies on *Xanthobacter* and emendation of *Xanthobacter flavus*. *System. Appl. Microbiol.* 9, 247-253.

Jenni, B. and M. Aragno. 1987. *Xanthobacter agilis* sp.nov., a motile, dinitrogen-fixing, hydrogen-oxidizing bacterium. *System. Appl. Microbiol* 9, 254-257.

Jenni, B., M. Realini, M. Aragno, and A.Ü. Tamer. 1988. Taxonomy of non H_2-lithotrophic, oxalate-oxidizing bacteria related to *Alcaligenes eutrophus*. *System. Appl. Microbiol.* 10, 126-133.

Kado, C.I. and S.T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* 145, 1365-1373.

Kelly, D.P., I.R. McDonald, and A.P. Wood. 2000. Proposal for the reclassification of *Thiobacillus novellus* as *Starkeya novella* gen. nov., comb. nov., in the α-subclass of the proteobacteria. *Int. J. Syst. Evol. Microbiol.* 50, 1797-1802.

Khambata, S.R. and J.V. Bhat. 1953. Studies on a new oxalate-decomposing bacterium, *Pseudomonas oxalaticus*. *J. Bacteriol.* 66, 505-507.

Kocur, M. 1986. Genus I. *Micrococcus* Cohn 1872, 151^{AL}. In: Sneath PHA, Holt JG (eds) Bergey's Manual of Systematic Bacteriology, vol 2. Williams and Wilkins, Baltimore, pp 1004-1008.

Krieg, N.R. and J.G. Holt. 1984. Bergey's Manual of Systematic Bacteriology, vol. 1. Williams and Wilkins, Baltimore.

Lajudie, P., A. Willems, G. Nick, S.H. Mohamed, U. Torck, R. Coopman, A. Filali-Maltouf, K. Kersters, B. Dreyfus, K. Lindström, and M. Gillis. 1999. *Agrobacterium* bv.1 strains isolated from nodules of tropical legumes *System. Appl. Microbiol.* 22, 119-132.

Logan, N.A. 1994. Similarity and arrangement, In *Bacterial systematics*. pp 47-61. Oxford, London: Balckwell Sci.

Mehta, R.J. 1973. Studies on methanol-oxidizing bacteria I. Isolation and growth studies. *Antonie van Leeuwanhoek* 39, 295-302.

Palleroni, N.J. 1984. Genus I. *Pseudomonas* Migula 1894, 237^{AL} (Nom. cons. Opin. 5, Jud. Comm. 1952, 237). In *Bergey's Manual of Systematic Bacteriology*, vol. 1. eds N.R. Krieg and J.G. Holt. pp. 141-199. Baltimore: Williams and Wilkins.

Raj, H.D. 1989. Oligotrophic Methylotrophs: *Ancylobacter* (Basonym "Microcyclus" Orskov) Raj. gen.nov. *Crit. Rev. Microbiol.* 17, 89-106.

Sahin, N., K. Isik, A.Ü. Tamer, and M. Goodfellow. 2000. Taxonomic position of "*Pseudomonas oxalaticus*" strain $Ox1^T$ ($DSM\ 1105^T$) (Khambata and Bhat, 1953) and its description in the genus *Ralstonia* as *Ralstonia oxalatica* comb. nov. *System. Appl. Microbiol.* 23, 206-209.

Smibert, R.M. and Krieg, N.R. 1994. Phenotypic characterization. In *Methods for General and Molecular Bacteriology*. eds Gerhardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R. pp.607-654. American Society for Microbiology : Washington, D.C.

Sneath, P.H. and R.R. Sokal. 1973. Numerical taxonomy. The principles and practice of numerical classification. San Francisco: W.H. Freeman and Co.

Sneath, P.H.A. 1979. BASIC program for character seperation indices from an identification matrix of percent positive characters. *Comput. Geosci.* 6, 21-26.

Staley, J.T. and A.E. Konopka. 1984. Genus *Microcyclus*. In. Bergey's Manual of Systematic Bacteriology, vol. 1, pp. 133-135. eds N.R. Krieg and J.G. Holt. Baltimore: Williams and Wilkins.

Starkey, R.L. 1935. Isolation of some bacteria which oxidize thiosulfate. *Soil Sci.* 39, 197-219.

Sokal, R.R. and C.D. Michener. 1958. A statistical method for evaluating systematic relationships. *Univ. Kansas Sci. Bull.* 38, 1409-1438.

Tamer, A.Ü. 1982. Ph.D. thesis. Ege Üniversity, Izmir-Turkey.

Thompson, G.A. and R.J. Watling. 1983. A simple method for the determination of bacterial resistance to metals. *Bull. Environ. Contam. Toxicol.* 31. 705- 711.

Weber, O.B., V.L.D. Baldani, K.R.S. Teixeira, G. Kirchhof, J.I. Baldani, and J. Dobereiner. 1999. Isolation and characterization of diazotrophic bacteria from banana and pineapple plants. *Plant and Soil* 210, 103-113.

Wiegel, J.K.M. and H.G. Schlegel. 1984. Genus *Xanthobacter*. In. *Bergey's Manual of Systematic Bacteriology*, vol. 1. N.R. Krieg and J.G. Holt. pp. 325-333. Baltimore: Williams and Wilkins.

Willcox, W.R., S.P. Lapage, S. Bascomb, and M.A. Curtis. 1973. Identification of bacteria by computer: theory and programming. *J. Gen. Microbiol.* 77, 317-330.

Yara, K. and S. Usami. 1968. Studies on oxalate-decomposing bacteria isolated from *Alocasia* plants. *Bot. Mag. Tokyo* 81, 423-433.

Zaitsev, G.M., I.V. Tsitko, F.A. Rainey, Y.A. Trotsenko, J.S. Uotila, E. Stackebrandt, and M.S. Salkinoja-Salonen. 1998. New aerobic ammonium-dependent obligately oxalotrophic bacteria: description of *Ammoniphilus oxalaticus* gen. nov., sp. nov. and *Ammoniphilus oxalivorans* gen. nov. sp. nov. *Int. J. Syst. Bacteriol.* 48. 151-163.