Biodegradation **1**: 31–41, 1990.

Isolation and growth of a bacterium able to degrade nitrilotriacetic acid under denitrifying conditions

Ursula Wanner,[1] Judith Kemmler,[1] Hans-Ulrich Weilenmann,[1] Thomas Egli,[1]* Tarek El-Banna[2, 3] & Georg Auling[2]

[1] *Swiss Federal Institute for Water Resources and Water Pollution Control (EAWAG), Swiss Federal Institutes of Technology Zürich, CH-8600 Dübendorf, Switzerland;* [2] *Institute of Microbiology, University of Hannover, D-3000 Hannover, FRG* ([3] *present address: Faculty of Pharmaceutical Microbiology, Tanta University, Tanta, Arab Republic of Egypt) (* requests for offprints)*

Received 5 February 1990; revised and accepted 21 June 1990

Key words: biodegradation, denitrification, isolation of NTA-degrading bacterium, nitrilotriacetic acid (NTA), taxonomy, wastewater treatment

Abstract

A Gram-negative bacterium was isolated from river sediment which was able to grow with nitrilotriacetic acid as a combined carbon, nitrogen and energy source in the absence of molecular oxygen using nitrate as the terminal electron acceptor. Batch growth parameters and mass balances are reported for growth under both aerobic and denitrifying conditions.

The strain was characterized with respect to its substrate spectrum and other physiological properties. This denitrifying isolate is serologically unrelated to the comprehensively described Gram-negative obligately aerobic NTA-degrading bacteria all of which belong to the α-subclass of *Proteobacteria*. Chemotaxonomic characterization, which revealed the presence of spermidine as the main polyamine and ubiquinone Q-8, excludes the new isolate from the phylogenetically redefined genus *Pseudomonas* and indicates a possible location within the γ-subclass of *Proteobacteria* close to, but separate from the genus *Xanthomonas*.

Introduction

The wide-spread eutrophication of lakes and rivers is mainly due to the extensive use of polyphosphates in household detergents, to extensive application of fertilizers and to the overloading of soils with liquid manure from intensive animal husbandry (Vollenweider 1968; Stumm & Morgan 1981). In order to reduce the load of phosphate introduced into surface waters, polyphosphates have been banned from washing powders in Switzerland. One of the chelating agents used in large amounts to substitute the polyphosphates is nitrilotriacetate (NTA). Due to its increasing use in household detergents, NTA enters the aquatic environment in large quantities (Egli 1988).

Under oxic conditions NTA is known to be readily degraded in sewage treatment plants and surface waters (Bernhardt et al. 1984; Anderson et al. 1985), but its fate in the anoxic compartments of both technical and natural systems has been little investigated (Ward 1986). In spite of the fact that NTA has been used in Canada since 1970 without apparent negative effects (Shapiro et al. 1978), there is still considerable concern about potential problems under anoxic conditions. Due to the complexing capacity of NTA, incomplete degradation could lead to remobilization of heavy metals in

anoxic sediments and the subsequent transport of NTA/heavy metal complexes through the several process steps employed for drinking water production (Garnett et al. 1987). Even though some data are available on the degradation of NTA in anoxic environments (Tabatabai & Bremner 1975; Moore & Barth 1976; Kuhn et al. 1987), little is known about the microorganisms involved or their biochemistry (Enfors & Molin 1973a, b).

Recently, a denitrifying mixed culture was enriched from sediment of the River Glatt which was able to grow on NTA as a combined carbon and nitrogen source using nitrate as the terminal electron acceptor (Egli & Weilenmann 1986). Here the isolation, characterization and some growth characteristics of the Gram-negative bacterium responsible for NTA biodegradation in this enrichment culture are described.

Materials and methods

Isolation and cultivation

Isolation and maintenance of the enrichment culture has been described previously (Egli & Weilenmann 1986). For isolation, samples from the enrichment culture were diluted with sterile distilled water and plated out on plate count agar (PCA) 2/10 strength (Difco) or NTA/acetate agar plates and incubated aerobically at 30° C. NTA/acetate plate medium was prepared according to Egli & Weilenmann (1986) but modified by the addition of 0.5 g l^{-1} acetate. Well separated colonies from the plates were transferred into serum flasks and incubated under oxygen-free conditions in a synthetic medium (SM) described by Egli & Weilenmann (1986) containing 0.5 g l^{-1} NTA as the combined carbon and nitrogen source and 0.5 g l^{-1} nitrate as the terminal electron acceptor. Isolates that were able to grow through three successive transfers in this medium were rechecked for purity using PCA plates and selective agar plates containing either NTA/acetate or NTA. The ten pure cultures of denitrifying NTA-degrading bacteria obtained in this way were subjected to further characterization.

Characterization

Initially, purified cultures of denitrifying NTA-degraders were characterized using API20B test strips, a test system for classification of facultatively aerobic, heterotrophic bacteria (API bioMérieux, France). Isolate TE 11 was selected for further detailed characterization.

DNA base ratio. DNA was extracted from cells grown on SM containing 4 g l^{-1} NTA as the sole source of carbon, nitrogen and energy. For extraction of high quality DNA and the determination of the DNA G+C base ratio the methods described by Egli et al. (1988) were employed.

Quinones. Extraction, purification and identification of isoprenoid quinones was performed on both, fresh and freeze-dried cells as reported by Kroppenstedt (1982) and Auling et al. (1986). Identical results were obtained from both SM/NTA and PYEA medium (peptone and yeast extract 1 g l^{-1} each, plus 2.0 g l^{-1} acetate, pH 7.0, 27° C) grown cells.

Polyamines. In order to compare the polyamine pattern of strain TE 11 to those published for 50 reference species of *Proteobacteria* (Busse & Auling 1988) and those reported for obligately aerobic NTA-utilizing strains (Egli et al. 1988; El-Banna 1989) cells were grown in PYEA medium and harvested when the culture reached 70% of the final optical density. Extraction, analysis and quantification of polyamines was done according to Busse & Auling (1988).

Pattern of soluble proteins. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) of soluble proteins was carried out as described by Auling et al. (1986). Cells were grown on PYEA medium and harvested as described in the previous section on polyamines.

Serological methods. Cross-reaction of isolate TE 11 with antisera raised against the three obligately aerobic NTA-degrading strains ATCC 29600, TE 2 and TE 6 was tested using both an immunodiffu-

sion and an indirect immunofluorescence test. Preparation of antisera in rabbits and Ouchterlony double diffusion assays with bacterial extracts were carried out as described by Auling et al. (1976). Details on the preparation of antisera and the indirect immunofluorescence test have been described by Wilberg (1990).

Antibiotic susceptibility tests. The susceptibility towards antibiotics was tested with impregnated discs (API bioMérieux, France) by the Kirby-Bauer method. Inhibition zones were assessed on Müller-Hinton agar and evaluated after 18 and 24 hours of incubation at 30° C according to the manufacturer's instructions.

Substrate utilization spectrum. The ability of strain TE 11 to grow with different carbon substrates under oxic conditions was investigated in test tubes containing liquid SM supplemented with NH_4Cl (0.54 g l^{-1}) and the carbon source (0.20 g l^{-1}) to be tested. The ability to grow with a selection of primary, secondary and tertiary amines structurally related to NTA was tested in liquid SM containing 0.5 g l^{-1} of amine, but no NH_4Cl, under both aerobic and denitrifying conditions. To test for growth under denitrifying conditions the SM was supplemented with 0.5 g l^{-1} nitrate and the tubes were flushed with helium after inoculation and sealed. Only if the cells were able to grow with either a particular carbon source or an amine during three successive transfers into fresh medium was growth considered positive.

Other tests. All other tests used for the taxonomical characterization of isolate TE 11 were performed as described previously (Egli et al. 1988).

Electron microscopy. The methods described by Wehrli & Egli (1988) have been used for electron microscopy of the isolated strain.

Stoichiometry

Yield coefficients and mass balances for aerobically growing cultures were obtained by growth experiments carried out in a MBR Mini Bioreactor (MBR Bioreactor AG., Switzerland) of 3 liter operating volume using the synthetic medium supplemented with a mixture of vitamins as described by Egli et al. (1988). NTA (1 g l^{-1}) was supplied as the source of carbon, nitrogen and energy and nitrate (1 g l^{-1}) as the terminal electron acceptor. Temperature and pH were kept constant at 30° C and 6.8, respectively. For anaerobic cultivation, the culture was flushed continuously with oxygen-free helium (approximately 5 ml min^{-1}). Helium was rendered free of oxygen by passing it through a reduced chromium(II) solution (prepared by reduction of a Cr(III) solution in the presence of Zn^0 and HCl) before it entered the bioreactor. Mass balances for anaerobically growing cultures were carried out in sealed 1 liter flasks containing 500 ml of growth medium. pH was regulated discontinuously between 6.8 and 7.0 and the temperature was maintained at 30° C. For experiments under denitrifying conditions flasks were flushed with helium after inoculation.

Growth was measured spectrophotometrically at 546 nm using an UVIKON 860 spectrophotometer (Kontron Instruments, Switzerland). NTA and bicarbonate were determined by ion exclusion HPLC according to the method described by Schneider et al. (1988). Nitrate and ammonium were both measured using an automated ion analyzer (Skalar, The Netherlands). Molecular nitrogen and nitrous oxide were determined gas chromatographically using a Shimadzu GC 8A fitted with a thermal conductivity detector (column: molecular sieve 5Å, 80/100 mesh, 2.5 m; oven temperature, 70° C; carrier gas helium, 40 ml min^{-1}). Dissolved organic carbon was measured with a TOCOR2 type TOC/DOC analyzer (Mayhak, Federal Republic of Germany). A C,N,S-analyzer model 1500 (Carlo Erba, Italy) was used to measure the carbon and nitrogen content of cells. All chemicals were purchased from Fluka (Switzerland) and were of pure grade or better.

Results

Composition of enrichment culture and isolation of the bacterium responsible for NTA degradation

In our laboratory a culture able to degrade NTA as the sole source of carbon, nitrogen and energy was enriched under anoxic conditions in a chemostat ($D = 0.02\,h^{-1}$) using a synthetic medium with nitrate as the potential electron acceptor (Egli & Weilenmann 1986). Initial attempts to isolate the bacterium responsible for NTA degradation in this mixed culture enriched from sediment of the River Glatt by plating out on NTA/nitrate agar plates and incubation under anoxic conditions were not successful. Colonies developed slowly under such conditions and invariably failed to grow with NTA/nitrate in liquid medium. Therefore, on the assumption that the bacterium would be a facultatively anaerobic heterotroph, samples from the enrichment culture were diluted 10^6 to 10^8 times and were plated out on both PCA and NTA/acetate agar plates and incubated aerobically. On PCA plates four different types of colonies were recognizable. Three comprized fast growing bacteria that were unable to grow on NTA under any of the conditions tested; the fourth type was a slow growing bacterium that formed small, white, round colonies which became visible only after 3–4 days of incubation. If samples of the enrichment culture diluted less than 10^8 times were plated, the non-NTA-degraders completely overgrew the slowly growing NTA-utilizing bacterium and isolation was impossible. As could be estimated from colony numbers on PCA plates more than 99% of the enrichment culture consisted of this NTA-degrading strain and less than 1% were non-NTA-degrading contaminants, probably growing on either lysis or excretion products of the NTA-degrading strain. From NTA/acetate agar plates ten well separated colonies were picked and, after their ability to grow in liquid culture with NTA/nitrate under anoxic conditions was confirmed, were selected for further characterization.

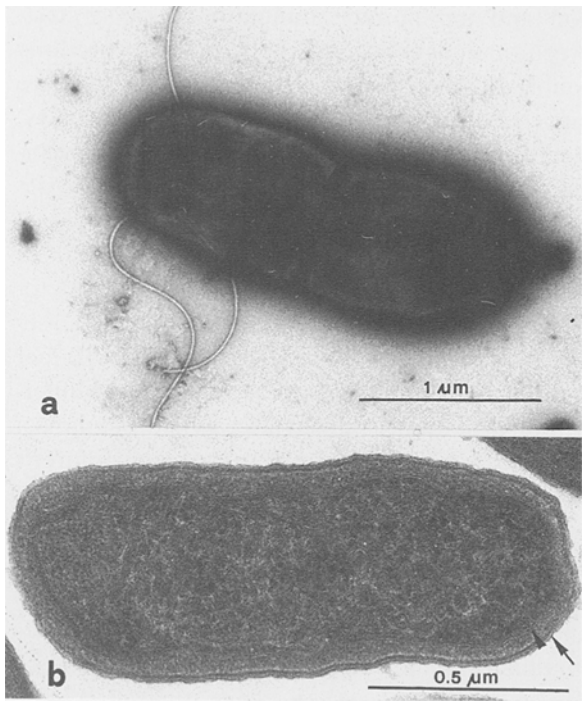

Fig. 1. Electron micrographs of the denitrifying, NTA-degrading isolate TE 11. A) Negative-staining; B) Thin-section, arrow: outer membrane, arrow head: cytoplasmic membrane.

Characterization

Characterization with API20B revealed that all the ten isolates selected exhibited identical properties in this test system. Therefore, they were assumed to be identical and only the strain that exhibited fastest growth was chosen for further work and was designated strain TE 11, in analogy to the novel obligately aerobic NTA-degrading bacterial strains TE 1–TE 10 isolated recently (Egli et al. 1988).

Morphology. In the electron microsocpe cells of isolate TE 11 were always rod-shaped (approximately $1.2–1.4 \times 0.5\,\mu m$) and exhibited the typical Gram-negative cell wall arrangement (Fig. 1B). Motile cells usually exhibited one to three subpolarly inserted flagella (Fig. 1A). Formation of pleomorphic cells or the presence of an S-layer, as in the case of the obligately aerobic Gram-negative NTA-degrading bacteria (Wehrli & Egli 1988), was never observed.

Nutritional properties. The ability of isolate TE 11 to grow with various organic carbon compounds as sole source of carbon and energy, including a range of primary, secondary and tertiary amines structurally similar to NTA, was tested in liquid culture.

Carbon sources which supported growth were: *Sugars and sugar alcohols:* Arabinose, D-(+)-cellobiose, D-(−)-fructose, galactose, gentobiose, D-(+)-glucose, glycerol, inositol, D-(+)-maltose, mannitol, D-(+)-mannose, rhamnose, D-(+)-ribose, saccharose, D-(−)-sorbitol, xylitol and D-(+)-xylose. *Amino acids:* Arginine, alanine, aspartate, glutamate, glycine, glycyl-glycine, lysine and serine. *Acids and alcohols:* acetate, n-butanol, butyrate, citrate, gluconate, glycollate, glyoxylate, lactate, malate, propionate, pyruvate. *Amines:* dimethylamine, methylamine, N-(2-acetamido)iminodiacetate, N-acetylglucosamine, N-methylaminodiacetate, N,N-dimethylglycine, trimethylamine, sarcosine, Tris-(hydroxymethyl)-amino methane.

No growth was recorded with the following substrates: *Sugars and sugar alcohols:* Adonitol, arabitol, erythritol, esculine, fucose, lactose, D-(+)-raffinose. *Amino acids and amines:* Acetamide, dimethylformamide, methylacetamide, methyldiethylamine, iminodiacetate, phenylalanine, triethanolamine, urea. *Acids and alcohols:* Ethanol, formate, iso-propanol, malonate, methanol, succinate. *Others:* Aniline, benzoate, dimethylsulfoxide, H_2/CO_2, methane, n-decane, phenol, xylene.

In addition, growth under denitrifying conditions was observed with N,N-dimethylglycine, N-methylaminodiacetate, sarcosine and trimethylamine but not with dimethylamine. In the absence of oxygen isolate TE 11 grew on various substrates (acetate, glyoxylate, glycollate) when nitrate was supplied as terminal electron acceptor and, in the absence of any other nitrogen source, nitrate was used as source of nitrogen for growth as well as serving as the terminal electron acceptor.

Biochemical and physiological properties. Isolate TE 11 was characterized chemotaxonomically and with respect to a range of physiological properties. The DNA base ratio was found to be 65.3 ± 0.3 mol% guanine plus cytosine. Ubiquinone with eight isoprenoid units in the side chain (Q-8) was the dominating quinone component. In addition, minor amounts of Q-10 (< 15% of total Q) were present. Characteristic for the intracellular polyamine pattern of isolate TE 11 was the high content of spermidine (41.7 μmol/mg dry cell weight) and the much lower concentrations of spermine (5.7), cadaverine (6.4) and putrescine (2.9), whereas *sym*-homospermidine was not detectable.

Strain TE 11 was found to be resistant towards the following antibiotics (concentration given in ugor units): penicillin G. (10), ampicillin (10), carbenicillin (100), cephaloridine (30), cephalexin (30), streptomycin (10), neomycin (30), kanamycin (30), gentamycin (10), tobramycin (10), tetracycline (30), doxycycline (30), erythromycin (15), rifampicin (30), novobiocine (30), vancomycin (30), nalidixic acid (30) and sensitive against chloramphenicol (30), polymyxin B (300) and trimethoprim/sulphamethoxazole (1.25/23.75). Such multiresistant characteristics clearly discriminate the denitrifying strain TE 11 from the obligately aerobic NTA-utilizing Gram-negative bacteria of the proposed new genera *'Chelatobacter'* and *'Chelatococcus'* (Egli et al. 1988; El-Banna 1989; El-Banna, Auling, Wilberg & Egli, manuscript in preparation).

In immunological studies, cell homogenates or whole cells of the denitrifying isolate TE 11 did not cross-react in either immunodiffusion (Fig. 2) or immunofluorescence tests with polyclonal antisera raised against the central strains ATCC 29600, TE 6 (*'Chelatobacter'*) and TE 2 (*'Chelatococcus'*) of the recently isolated obligately aerobic NTA-degrading bacteria (Egli et al. 1988; El-Banna 1989; Wilberg 1990).

Electrophoretic separation of cellular proteins represents a further tool recommended for taxonomic evaluation of the relationship between bacterial isolates (Norris 1980; Kersters 1985). The results in Fig. 3 show that the PAGE soluble protein pattern of isolate TE 11 is completely different from that of the obligately aerobic NTA-utilizing bacteria (Egli et al. 1988). Because the chemotaxonomic results indicated a possible relationship of the denitrifying strain TE 11 to the genus *Xan-*

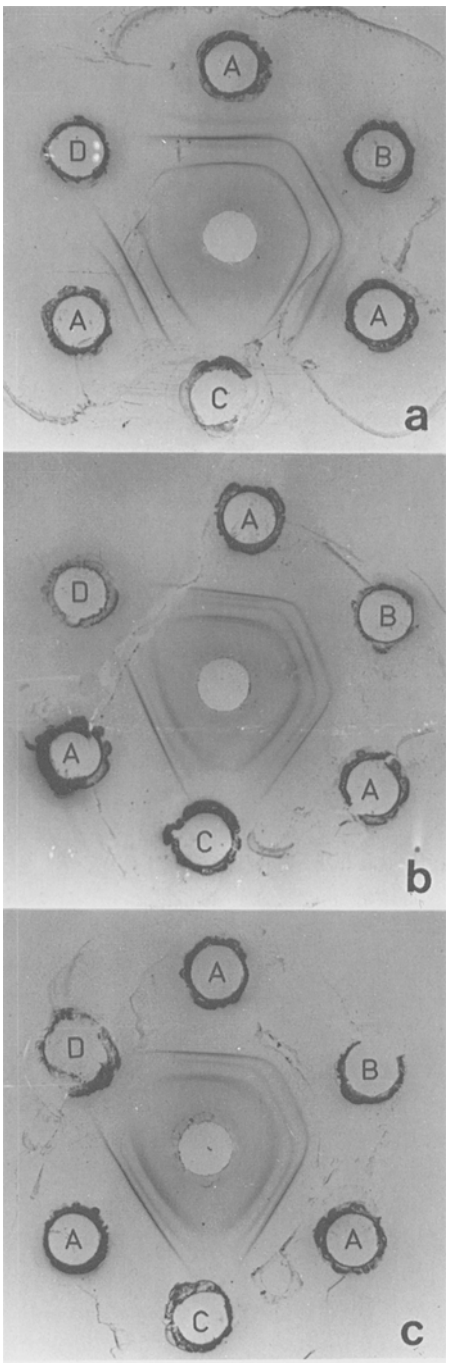

Fig. 2. Cross-reactivity of anti-TE 2, anti-TE 6 and anti-ATCC 29600 antiserum with bacterial homogenates of NTA-utilizing bacteria.
a) central well: anti-TE 2 antiserum; A: TE 2; B: TE 1; C: TE 11; D: ATCC 29600.
b) central well: anti-TE 6 antiserum; A: TE 6; B: ATCC 29600; C: TE 2; D: TE 11.
c) central well: anti-ATCC 29600 antiserum; A: ATCC 29600; B: TE 6; C: TE 2; D: TE 11.

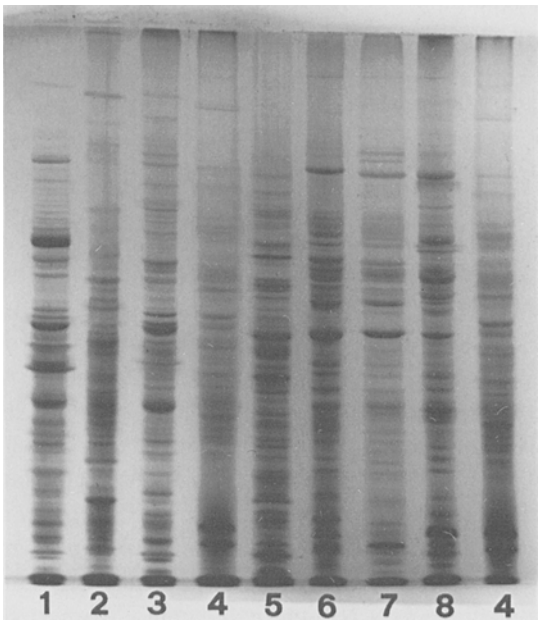

Fig. 3. Discrimination of isolate TE 11 from other NTA-degrading bacteria and from some reference strains of *Xanthomonas* by PAGE of soluble proteins. The lanes contain 1, *'Chelatobacter'* sp. TE 6; 2, *'Chelatococcus'* sp. TE 2; 3, *'Chelatobacter'* sp. ATCC 29600; 4, Isolate TE 11; 5, *Xanthomonas fragariae* DSM 3587; 6, *Xanthomonas pelargonii* DSM 1350; 7. *Xanthomonas axonopodis* DSM 3585; 8, *Xanthomonas albilineans* DSM 3583.

thomonas extracts of four reference strains of this genus were included in the PAGE fingerprinting. However, no similarity of the protein patterns was observed (Fig. 3).

Additional data on various physiological properties of strain TE 11 are given in Table 1. Although acid formation from glucose was positive if tested with API20B no significant accumulation of short chain volatile organic acids was detected during growth either with glucose or ethanol under oxic and anoxic conditions when acids were tested for gaschromatographically (< 1 mg l^{-1} propionate, n-butyrate, iso-butyrate, 2-methyl butyrate, 3-methyl butyrate and valerate). Cells of TE 11 were not acid-fast and degradation of protein was found to be weak. Also it should be noted that the ability to produce acetoine was strongly dependent on the medium used for growth and that aerobically grown cells were not able to reduce nitrate to nitrite when tested with API20B.

Growth of isolate TE 11 and mass balances

During batch cultivation in the bioreactor using NTA as a combined source of carbon, energy and nitrogen and nitrate as the terminal electron acceptor strain TE 11 grew under anoxic conditions with a doubling time of 22.8 h. The typical growth pattern for growth under denitrifying conditions is shown in Fig. 4. Although the ratio of N_2/N_2O produced during denitrification was not constant for different experiments, N_2O was mostly produced in the late exponential phase by pure cultures of TE 11. When oxygen was present the doubling time was reduced to 8.6 h. As expected, the growth yield coefficient ($Y_{X/S}$) was lower during growth with NTA under denitrifying conditions when compared to oxic growth (Table 2). With glucose as the supplementary carbon source, growth rates were doubled under both oxic and anoxic conditions (Table 2). The surplus nitrogen from NTA was excreted as ammonium ions in a medium where NTA was the only carbon source, but the NTA-nitrogen was completely incorporated into biomass in the presence of glucose. Nitrate was assimilated only in the absence of ammonium and NTA in a glucose-containing medium. When using NTA as the sole source of carbon, energy and nitrogen a change from oxic to anoxic growth conditions always resulted in a lag phase of 3–4 days before growth resumed, whereas no lag phase was observ-

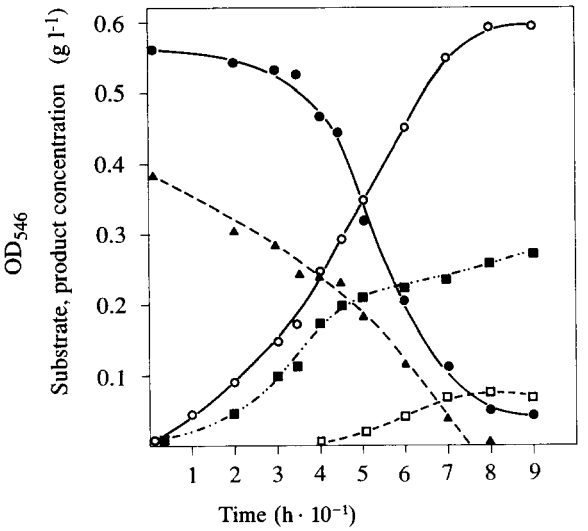

Fig. 4. Growth of isolate TE 11 in batch culture with NTA as the only source of carbon, energy and nitrogen and nitrate as the terminal electron acceptor. pH = 6.8–7.0; Temperature = 30° C. ●—●, NTA-carbon; ○—○, OD_{546}; ▲---▲, Nitrate-N; ■-·-■, N_2; □---□, N_2O-N.

Table 1. Physiological characterization of NTA-utilizing strain TE 11.

Gelatin liquification[1]	−	Acid from saccharose[1]	±
β-Galactosidase[1]	−	Acid from arabinose[1]	+
Cytochrome oxidase[1]	+	Acid from mannitol[1]	+
Catalase[1]	+	Acid from fructose[1]	+
Urease[1]	+	Acid from glucose[1]	+
H_2S formation[1]	−	Acid from maltose[1]	+
Acetoin formation[2]	+[2]	Acid from amidone[1]	−
Indole formation[1]	−	Acid from rhamnose[1]	+
Nitrate reduction[1]	−	Acid from galactose[1]	+
Nitrite reduction[1]	−	Acid from mannose[1]	+
Gas from glucose	−	Hydrolysis of DNA	−
Glucose fermentation	−	Hydrolysis of starch	−
Vitamin requirement	−	Hydrolysis of Tween 80	−
Growth with 1% NaCl	+	Nitrate as N source	+
Growth with 10% NaCl	−	N_2 as N source	−
Pigment formation[3]	−	Tellurite reduction[4]	+
PHB inclusions[5]	+	KCN tolerance	−

[1] Tested with API20B with cells grown aerobically on NTA agar or PCA plates; [2] PCA-grown cells were negative; [3] Tested on King's medium A and B; [4] Formation of black colonies on Tellurite agar; [5] From EM observations. Details on the methods used are given in Egli et al. (1988).

ed after a change from anoxic to oxic conditions. During aerobic growth cultures of TE 11 grown on glucose and ammonium alone always required a period of adaptation after inoculation into medium containing NTA only, but cells growing exponentially on peptone started to grow immediately after transfer to a medium containing NTA as the combined carbon and nitrogen source. This suggests that either peptone contains a factor which induces the synthesis of NTA-degrading enzymes or that these enzymes are derepressed during growth in a medium containing peptone.

Pure cultures of isolate TE11 were able to completely degrade NTA to carbon dioxide, ammonium and bacterial biomass. No significant amounts of byproducts were formed. For batch growth, aerobic (Eq. 1) and anaerobic (Eq. 2) NTA degradation by the pure culture and anaerobic NTA degradation by the enrichment culture (Eq. 3, from Egli & Weilenmann 1986) can be described by the following mass balance equations:

$$C_6N(NTA) \rightarrow C_{1.8}N_{0.4}(cells) + 0.6\,NH_4^+\text{-}N + 4.2\,CO_2\text{-}C \quad (1)$$

$$C_6N(NTA) + 3.5\,NO_3^-\text{-}N \rightarrow C_{1.0}N_{0.2}(cells) + 0.75\,NH_4^+\text{-}N + 5\,CO_2\text{-}C + 1.4\,N_2\text{-}N + 2.1\,N_2O\text{-}N \quad (2)$$

$$C_6N(NTA) + 2.8\,NO_3^-\text{-}N \rightarrow C_{1.0}N_{0.2}(cells) + 0.81\,NH_4^+\text{-}N + 5\,CO_2\text{-}C + 2.8\,N_2\text{-}N \quad (3)$$

The nitrogen balances from both enrichment and pure culture indicate that under denitrifying growth conditions nitrate served exclusively as the terminal electron acceptor and was not assimilated. This again implies that the ammonium excreted was derived from the surplus nitrogen contained in NTA.

Discussion

Taxonomical aspects

A number of NTA-degrading bacterial isolates have been described in the literature and most of them are Gram-negative obligate aerobes (Bernhardt et al. 1984; Egli 1988). So far, only two bacteria able to degrade NTA in the absence of oxygen, both of them denitrifiers, have been isolated in pure culture (Enfors & Molin 1973a, b; Kakii et al. 1986). All of these strains have consistently been assigned to the genus *Pseudomonas*. However, this allocation was based on a limited number of physiological and biochemical properties. Only recently, a number of obligately aerobic isolates, including the two reference strains *'Pseudomonas'* spp. ATCC 27109 and 29600, have been investigated in more detail (Egli et al. 1988; Wehrli & Egli 1988; El-Banna 1989). It was found that neither of these bacteria belonged to the genus *Pseudomonas* but should be included in the α-subclass of *Proteobacteria* where they form the basis of two new genera, the proposed names of which are *'Chelatobacter'* and *'Chelatococcus'* (El-Banna, Auling, Wilberg & Egli, manuscript in preparation).

Apart from its inability to grow on some of the carbon sources tested (phenylalanine, benzoate, ethanol, isopropanol and succinate), its considerably higher DNA base ratio, its remarkable resistance to antibiotics and its ability to use nitrate as a terminal electron acceptor, there are few differences in the nutritional and physiological proper-

Table 2. Growth parameters of isolate TE 11 determined from aerobic and denitrifying batch growth in synthetic medium. NTA (1 g l^{-1}) or NTA/glucose (1 g l^{-1}/1 g l^{-1}) served as sources of carbon, energy and nitrogen.

	maximum specific growth rate constant (h^{-1})	growth yield (g $gNTA^{-1}$)
Aerobic growth on NTA	0.08	0.27
Aerobic growth on glucose/NTA	0.13	–
Denitrifying growth on NTA	0.03	0.17
Denitrifying growth on glucose/NTA	0.06	–

ties exhibited by isolate TE 11 when compared with the obligately aerobic *'Chelatobacter'* spp. described previously. However, the tests for cross reactivity reported in this study revealed no serological relationship between the denitrifying isolate TE 11 and the Gram-negative obligately aerobic NTA-degrading bacteria. Quinone and polyamine patterns also discriminate TE 11 from the latter (El-Banna 1989; El-Banna, Auling, Wilberg & Egli, manuscript in preparation), as do protein fingerprints and antibiotic susceptibility. The presence of ubiquinone Q-8 and spermidine as the main polyamine strongly suggest that isolate TE 11 does not belong to the α- and the β-subclass of *Proteobacteria* but should be allocated to the γ-subclass where it can be excluded from the phylogenetically redefined genus *Pseudomonas*. These two taxonomic parameters suggest allocation to the genus *Xanthomonas* (Ikemoto et al. 1980; Busse & Auling 1988; Busse et al. 1989; Auling & Kneifel, unpublished results). The G+C ratio of 65.3 ± 0.3 does not contradict this allocation. However, protein fingerprints – although not all reference strains of the presently ill-defined genus *Xanthomonas* (Vauterin et al. 1990) have been included – and the physiological and nutritional characteristics of *Xanthomonas* spp. shown in Table 3 (Bradbury 1984) indicate that it will be highly unlikely that the NTA-degrading isolate TE 11 can be included into the genus *Xanthomonas*. Clearly, there is more work required to elucidate the exact phylogenetic position of this Gram-negative bacterium, e.g., by sequencing of 16S rRNA. The possibility remains that a new genus has to be established to allocate strain TE 11.

Growth characteristics

When comparing anaerobic growth of the pure culture with that of the enrichment culture (Egli & Weilenmann 1986), it becomes evident that for each mole of NTA degraded more nitrate was reduced by the pure culture than by the enrichment culture and that nitrate was partly reduced to both nitrous oxide and di-nitrogen by the pure culture. The smaller amount of nitrate reduced by the enrichment culture is consistent with the view that complete reduction of the terminal electron acceptor results in the generation of more biologically useful energy for biomass production. The accumulation of nitrous oxide by the pure culture cannot be fully explained yet. Important factors influencing N_2O accumulation include the presence of other nitrogen oxides and oxygen, the availability of carbon substrates, pH and temperature (Golterman 1985). However, in most respects the pure culture mass balance did not differ significantly from that of the enrichment culture. Therefore, the influence of contaminants on NTA degradation in the enrichment culture can be considered to be essentially negligible.

Biochemistry of NTA degradation

Isolate TE 11 can degrade NTA under both oxic and anoxic growth conditions. In all strains of obligately aerobic NTA-degrading bacteria studied so far NTA breakdown is catalyzed by an O_2/NADH-dependent mono-oxygenase (Cripps & Noble 1973; Firestone & Tiedje 1978). Since isolate TE 11 is able to grow on NTA under denitrifying condi-

Table 3. Comparison of physiological and biochemical characteristics of the denitrifying NTA-utilizing isolate TE 11 and *Xanthomonas* spp.[1]

	Strain TE 11	*Xanthomonas*
Plant pathogenicity	?	+
Denitrification	+	−
Colonies yellow/fluorescent	−	+
Oxidase test	+	− (or weak)
Growth factors required	−	+
PHB inclusions	+	−
Urease	+	−
Voges-Proskauer test	+	−
Xanthan production	−	+
Flagellation	1–3 sp[2]	1 p (rarely 2 p)[2]
Acid from rhamnose	+	−
Acid from sorbitol	+	−
Acid from mannitol	+	−

[1] Data for *Xanthomonas* spp. taken from Bradbury (1984); only properties listed which are the same for all species within the genus. [2] p = polar, sp = subpolar.

tions another enzyme which splits NTA in the absence of molecular oxygen must be present in this bacterium. Either removal of a carboxyl or of an acetyl group from NTA by either a decarboxylase or a dehydrogenase, respectively, can be hypothesized as possible enzymic steps in the pathway of NTA degradation. In preliminary studies using cell-free, membrane-free extracts of isolate TE 11 grown under denitrifying conditions the presence of a dehydrogenase was confirmed and the stoichiometrical formation of iminodiacetate and glyoxylate from NTA was observed (Kemmler et al. 1990). In aerobically grown cells this NTA dehydrogenase activity was absent and, instead, activity of an oxidase – that also produced iminodiacetate and glyoxylate from NTA – was detected. Isolation of both enzymes will be necessary to confirm these findings. Also, the influence of growth conditions on the regulation of the synthesis of both enzymes remains to be examined in detail.

Acknowledgements

The authors would like to thank I. Reupke for excellent technical assistance and M. Berg for help with the photographs. We are also indebted to E. Wehrli, ETH-Zürich, for preparing the electron micrographs and to G. Hamer for his support throughout the study and help during the preparation of the manuscript. The work was financed by the Swiss National Science Foundation (projects NF-3.204.-0.85 and 31-26326.89) and by EAWAG (projects 20-833/834). G. Auling was supported by the Gesellschaft für Biotechnologische Forschung mbH (GBF) for performing research of relevance for the German Collection of Microorganisms (DSM, Braunschweig).

References

Anderson RL, Bishop WE & Campbell RL (1985) A review of the environmental and mammalian toxicity of nitrilotriacetic acid. CRC Critical Reviews in Toxicology 15: 1–102

Auling G, Reh M, Lee CM & Schlegel HG (1976) *Pseudomonas pseudoflava*, a new species of hydrogen-oxidizing bacteria: Its differentiation from *Pseudomonas flava* and other yellow-pigmented, Gram-negative hydrogen-oxidizing bacteria. International Journal of Systematic Bacteriology 28: 82–95

Auling G, Probst A & Kroppenstedt RM (1986) Chemo- and molecular taxonomy of D(−)-tartrate utilizing Pseudomonads. Systematic and Applied Microbiology 8: 114–120

Bernhardt H, Berth W, Förster U, Hamm A, Janicke W, Kandler J, Kanowski S, Kleiser HH, Koppe P, Opgenorth HJ, Reichert JK & Stehfest H (1984) NTA: Studie über die aquatische Umweltverträglichkeit von Nitrilotriacetat (NTA). Verlag Hans Richarz, Sankt Augustin

Bradbury JF (1984) *Xanthomonas*. In: Krieg NL & Holt JF (Eds) Bergey's Manual of Systematic Bacteriology, Vol I (pp 199–210). Williams & Wilkins, Baltimore

Busse HJ & Auling G (1988) Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. Systematic and Applied Microbiology 11: 1–8

Busse HJ, El-Banna T & Auling G (1989) Evaluation of different approaches for identification of xenobiotic-degrading Pseudomonads. Applied and Environmental Microbiology 55: 1578–1583

Cripps RE & Noble AS (1973) The metabolism of nitrilotriacetate by a pseudomonad. Biochemical Journal 136: 1059–1068

Egli T (1988) (An)aerobic breakdown of chelating agents used in household detergents. Microbiological Sciences 5: 36–41

Egli T & Weilenmann H-U (1986) Biodegradation of nitrilotriacetic acid (NTA) in the absence of oxygen. Experientia 42: 1061–1062

Egli T, Weilenmann H-U, El-Banna T & Auling G (1988) Gram-negative, aerobic, nitrilotriacetate-utilizing bacteria from wastewater and soil. Systematic and Applied Microbiology 10: 297–305

El-Banna T (1989) Characterization of some unclassified *Pseudomonas* species. PhD thesis, University of Tanta, Egypt

Enfors S-O & Molin N (1973a) Biodegradation of nitrilotriacetate (NTA) by bacteria – I. Isolation of bacteria able to grow anaerobically with NTA as a sole carbon source. Water Research 7: 881–888

Enfors S-O & Molin N (1973b) Biodegradation of nitrilotriacetate (NTA) by bacteria – II. Cultivation of an NTA-degrading bacterium in anaerobic medium. Water Research 7: 889–893

Firestone MK & Tiedje JM (1978) Pathway of degradation of nitrilotriacetate by a *Pseudomonas* species. Applied and Environmental Microbiology 25: 955–961

Garnett K, Kirk PWW, Lester JN & Perry R (1987) Assessment of the interactions of metals and nitrilotriacetic acid in soil/sludge mixtures. Water Air and Soil Pollution 34: 55–69

Golterman HL (1985) Denitrification in the Nitrogen Cycle. Plenum Press, New York

Ikemoto S, Suzuki K, Kaneko T & Komagata K (1980) Characterization of strains of *Pseudomonas maltophila* which do not require methionine. International Journal of Systematic Bacteriology 30: 437–447

Kakii K, Yamaguchi H, Iguchi Y, Teshima M, Shirakashi T & Kuriyama M (1986) Isolation and growth characteristics of nitrilotriacetate-degrading bacteria. Journal of Fermentation Technology 64: 103–108

Kemmler J, Wanner U, Egli T, Snozzi M & Hamer G (1990) Degradation of nitrilotriacetic acid by two different enzymes in a denitrifying bacterium (strain TE 11)? Abstract, Symposium on Environmental Biotechnology, organised by the European Environmental Research Organisation, held at Braunschweig, FRG, April 23–27, 1990

Kesters K (1985) Numerical methods in the classification of bacteria by protein electrophoresis. In: Goodfellow M & Jones D (Eds) Computer-Assisted Bacterial Systematics (pp 337–368). Academic Press, London

Kroppenstedt RM (1982) Anwendung chromatographischer HP-Verfahren (HPTLC und HPLC) in der Bakterien-Taxonomie. GIT Labor und Medizin 5: 266–275

Kuhn E, van Loosdrecht M, Giger W & Schwarzenbach RP (1987) Microbial degradation of nitrilotriacetate (NTA) during river water/groundwater infiltration: Laboratory column studies. Water Research 21: 1237–1248

Moore L & Barth EF (1976) Degradation of NTA acid during anaerobic digestion. Journal of the Water Pollution Control Federation 48: 2406–2409

Norris JR (1980) Introduction. In: Goodfellow M & Board RG (Eds) Microbiological Classification and Identification (pp 1–10). Academic Press, London

Schneider RP, Zürcher F, Egli T & Hamer G (1988) Determination of nitrilotriacetate in biological matrices using ion exclusion chromatography. Analytical Biochemistry 173: 278–284

Shapiro J, Chapman PJ, Dick RJ, Dillon PJ, O'Melia CR, Spacie A & Leduc G (1978) Report to the Great Lakes Research Advisory Board on the effects of non-phosphate detergent builders: Final report on NTA

Stumm W & Morgan JJ (1981) Aquatic Chemistry, 2nd edition. John Wiley & Sons, New York

Tabatabai MR & Bremner JM (1975) Decomposition of nitrilotriacetate (NTA) in soils. Soil Biology and Biochemistry 7: 103–106

Vauterin L, Swings J, Kersters K, Gillis M, Mew TW, Schroth MN, Palleroni NJ, Hildebrand DC, Stead DE, Civerolo EL, Hayward AC, Maraite H, Stall RE, Vidaver AK & Bradbury JF (1990) Towards an improved taxonomy of *Xanthomonas*. International Journal of Systematic and Applied Microbiology (in press)

Vollenweider RA (1968) The scientific basis of lake and stream eutrophication. Technical report DAS/CSI/68, p. 27. The Organization for Economic Cooperation and Development, Paris

Ward TE (1986) Aerobic and anaerobic biodegradation of nitrilotriacetate in subsurface soils. Ecotoxicology and Environmental Safety 11: 112–120

Wehrli E & Egli T (1988) Morphology of nitrilotriacetate-utilizing bacteria. Systematic and Applied Microbiology 10: 306–213

Wilberg E (1990) Zur Physiologie und Ökologie Nitrilotriacetat (NTA) abbauender Bakterien. PhD thesis Nr. 9015, ETH Zürich

Yamada A, Takinama-Nakamura H, Tahara Y, Oyaizu H & Komagata K (1981) The ubiquinone systems in the strains of *Pseudomonas* species. Journal of General and Applied Microbiology 28: 7–12