Taxonomic Study of *Paracoccus denitrificans*

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Isolates of *Paracoccus denitrificans* were obtained from various habitats by enrichment in a mineral medium, using molecular hydrogen as the hydrogen donor and nitrate as the hydrogen acceptor. A total of 11 strains were compared with the following three reference strains: *P. denitrificans* Stanier 381^T (type strain) (= DSM 65^T = ATCC 17741^T), Morris (= DSM 413 = ATCC 19367), and Vogt (= DSM 415). A computer analysis based on 235 characters indicated that the strains clustered into subgroups. Deoxyribonucleic acid-deoxyribonucleic acid homology determinations confirmed this suggestion. A formal description of the species is presented, and the taxonomic position of *P. denitrificans* is discussed.

Paracoccus denitrificans (formerly known as *Micrococcus denitrificans*) was first isolated by Beijerinck and Minkman (5) and was reisolated as a bacterium that was capable of using molecular hydrogen in denitrification by Verhoeven et al. (56) and Vogt (57). Although this species has a guanine-plus-cytosine (G+C) content similar to the G+C contents of some members of *Micrococcus*, it differs from other species of *Micrococcus* as follows (40): (i) *P. denitrificans* forms rod-shaped cells in young cultures (34, 39, 55, 57); (ii) it is gram negative (9, 34, 56, 57); (iii) it has a cell wall peptidoglycan which contains a wide range of amino acids characteristic of gram-negative genera, and the cell wall contains diaminopimelic acid in place of the lysine characteristic of micrococci (4); and (iv) it contains ubiquinone as an electron carrier (46), which is characteristic of gram-negative bacteria. For these reasons a new genus, *Paracoccus* was created (9). The electron transport chain of *P. denitrificans* resembles the electron transport chain of the inner mitochondrial membrane more closely than the electron transport chain of any other bacterium (21). The hydrogenase of *P. denitrificans* strain Stanier 381^T (type strain) has been isolated and characterized (49, 50), and the regulation of hydrogenase formation as a diagnostic character of the reference strains of *P. denitrificans* and of newly isolated strains has been studied (35).

Although *P. denitrificans* was isolated more than 70 years ago (5) and has been used in many investigations, its taxonomic position is still ill defined; the genus was placed with "genera of uncertain affiliation" (11) because only two strains were available for study and taxonomic data were lacking.

The objectives of this study were to isolate many new strains of *P. denitrificans*, to deter-

mine their main features, to compare them with the strains kept in culture collections, to evaluate their similarities and delineate them from other related bacteria, and to determine their taxonomic niche.

MATERIALS AND METHODS

Organisms. The bacterial strains which we studied are listed in Table 1. A total of 11 strains, designated N1 through N11, were isolated after specific enrichment culture (24). Three strains of *P. denitrificans* (DSM 65^T, DSM 413, and DSM 415) were obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, Federal Republic of Germany (7). Strains of other species (Table 2) were compared with respect to deoxyribonucleic acid (DNA) base composition and biochemical and physiological characteristics (Table 2). These reference strains were grown in nutrient broth (Difco Laboratories, Detroit, Mich.).

Growth conditions. Unless otherwise stated, media were prepared from a standard mineral base (SM) which contained (per liter of distilled water) 9.0 g of NaH₂PO₄ · 12H₂O, 1.5 g of KH₂PO₄, 1.0 g of NH₄Cl, 0.2 g of MgSO₄ · 7H₂O, 0.0012 g of Fe(III)NH₄-citrate, and 0.0265 g of CaCl₂ · 2H₂O (43); SM also contained 10 ml of trace element solution SL6 per liter of distilled water (37). For autotrophic growth the medium was supplemented with 0.05% (wt/vol) NaHCO₃ (separately sterilized), and for heterotrophic growth the medium was supplemented with organic compounds as carbon and energy sources in concentrations ranging from 0.1% (wt/vol) for organic acids and bases to 0.2% (wt/vol) for other compounds, unless otherwise indicated. For anaerobic growth, either autotrophically or heterotrophically, 1.0% (wt/vol) KNO₃ (or KNO₂) was used as a terminal oxidant to substitute for free oxygen. Nutrient broth (Difco) was used as a nutritionally complex medium. If required, 1.7% (wt/vol) agar (Difco) was added to solidify the media used. The final pH was adjusted to 7.1, and the growth temperature was 30°C. For heterotrophic growth, the liquid cultures were stirred magnetically or shaken under a continuous air stream; for autotrophic growth, gas

TABLE 1. List of *P. denitrificans* strains studied^a

Strain	Isolated from:	Isolated by:		Other designation(s) ^b
		Worker(s)	Reference	
Stanier 381 ^T	Garden soil	Beijerinck and Minkman	5	DSM 65 ^T , ATCC 17741 ^T ^c
Morris 5	Garden soil	Verhoeven et al.	56	DSM 413, ATCC 19367, CCM 982, NCIB 8944
Vogt 11	Arable soil	Vogt	57	DSM 415, CCM 1396, NCIB 9722
N1	Sewage	Nokhal	This study	DSM 1403
N2	Sewage	Nokhal	This study	
N3	Sewage	Nokhal	This study	
N4	Sludge	Nokhal	This study	DSM 1404
N5	Meadow soil	Nokhal	This study	DSM 1405
N6	Meadow soil	Nokhal	This study	
N7	Horse manure	Nokhal	This study	DSM 1406
N8	Canal mud	Nokhal	This study	
N9	Well mud	Nokhal	This study	
N10	Cow dung	Nokhal	This study	DSM 1407
N11	Field soil	Nokhal	This study	DSM 1408

^a The 11 new isolates (strains N1 through N11) were identified as *P. denitrificans* on the basis of an enrichment procedure, as well as their morphological, nutritional, physiological, and biochemical characteristics (this study).

^b ATCC, American Type Culture Collection, Rockville, Md.; CCM, Czechoslovak Collection of Microorganisms, J. E. Purkyne University, Brno, Czechoslovakia; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany; NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland.

^c See reference 9.

mixtures containing H₂, CO₂, and O₂ (80:10:10, vol/vol) and H₂ and CO₂ (85:15, vol/vol) were used for aerobic growth and anaerobic growth, respectively.

Growth and cell yield. Turbidity was determined at 436 nm (light path, 1 cm) with a Bausch & Lomb Spectronic 70 photometer. The protein contents of

TABLE 2. List of bacterial strains used as test organisms

Species	Strain ^a	Controlled tests
<i>Bacillus subtilis</i>	ATCC 6051 ^T (DSM 10 ^T)	Catalase activity, Voges-Proskauer reaction, citrate utilization, starch hydrolysis, casein digestion, alkalization and reduction of litmus milk with casein digestion
<i>Enterobacter aerogenes</i>	ATCC 13048 ^T (DSM 30053 ^T)	Malonate utilization, esculin hydrolysis, lysine and ornithine decarboxylases, oxidative-versus-fermentative test (aerogenic fermentation of glucose, lactose, and sucrose).
<i>Escherichia coli</i>	ATCC 11775 ^T (DSM 30083 ^T)	Acid reaction in litmus milk, methyl red reaction, indole production
<i>Proteus vulgaris</i>	ATCC 13315 ^T (DSM 30118 ^T)	Urease activity, gelatin liquefaction, H ₂ S production, growth in KCN, phenylalanine deamination, no change of litmus milk
<i>Pseudomonas aeruginosa</i>	ATCC 10145 ^T (DSM 50071 ^T , ICPB 2523 ^T)	Arginine dehydrolase, oxidase reaction, oxidative-versus-fermentative test (oxidizer), Tween 80 hydrolysis
<i>Xanthomonas pelargonii</i>	DSM 50857 (ICPB P121)	Determination of both DNA base composition and molecular weight of bacterial genome DNA

^a ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany; ICPB, International Collection of Phytopathogenic Bacteria, Davis, Calif.

whole-cell suspensions were estimated by the biuret method after alkaline hydrolysis of cells, as described by Schmidt et al. (44). Determination of cell dry weight was carried out on membrane filters (pore size, 0.2 μ m; Sartorius, Göttingen, Federal Republic of Germany) after drying at 80°C for 2 days. Samples from various stages of growth were centrifuged at $10,000 \times g$ for 10 min, washed twice in 0.067 M potassium phosphate buffer (pH 7.0), resuspended in the same buffer to give final protein concentrations of about 10 to 20 mg/ml, and stored at -20°C for enzyme assays.

Enrichment, isolation, and purification of new strains. A total of 30 samples of soil, river mud, humus, sewage, and sludge collected from various places in and around Göttingen, Federal Republic of Germany, were used as inocula. Enrichment cultures were started by inoculating individual 100-ml Erlenmeyer flasks containing 10 ml of SM medium supplemented with nitrate with a sample (about 0.5 g). Cultures were incubated under an atmosphere containing H₂ and CO₂ (85:15, vol/vol) in anaerobic jars without agitation. After 10 serial subcultures samples were plated onto agar, and pure cultures were isolated.

Maintenance of strains. Stock cultures were maintained as slants at 4°C under air. Autotrophically growing cultures were transferred every 3 to 6 months. In addition, all strains were kept on nutrient agar and transferred monthly. Lyophilized samples in 20% (wt/vol) skim milk were prepared for long-term storage.

Colony properties and growth appearance in liquid media. Colonies were described from 3-day aerobic growth on nutrient agar and autotrophic growth under H₂-CO₂-O₂ at 30°C. Growth responses in liquid media (nutrient broth, glucose, fructose, glycerol, acetate, pyruvate, lactate, succinate, gluconate) agitated on a rotary shaker at 30°C were determined after 48 h.

Cytological observations. Cells from both exponential and stationary growth phases in lactate broth were used to determine morphological properties. Motility was studied by light microscopic examination of hanging drop preparations of 24-h cultures in nutrient broth. All staining was performed by the methods of Cowan (8). Poly- β -hydroxybutyric acid granules were recognized by their typical appearance and by their solubility in chloroform and insolubility in ether. Quantitative determinations of poly- β -hydroxybutyric acid were made by the procedure of Jüttner et al. (22). Polyphosphate granules were detected by electron microscopy and confirmed by Neisser stain (8). Pigment production was tested by cultivation of slants on both nutrient agar and lactate agar slopes illuminated for 10 days by a 40-W incandescent lamp from a distance of 50 cm.

Growth and nutritional screening. For assays of utilization of organic compounds as sole carbon and energy sources, each strain was tested for its ability to grow in the presence of 100 different compounds by using the replica technique described by Stanier et al. (54); we used the basal medium supplemented with 0.2% (wt/vol) carbohydrate, 0.025% (wt/vol) phenol, or one of the other compounds at a concentration of 0.1% (wt/vol) and solidified by 1.7% agar (Difco). Each test series included a control plate lacking the carbon source. Inoculated test plates were incubated under air and scored visually after 2, 4, and 7 days. Strains with questionable growth on plates were fur-

ther examined in liquid media, where the growth yield was determined turbidimetrically. Utilization of water-insoluble hydrocarbons was determined in liquid basal medium as described by Stanier et al. (54). The utilization of nitrogen sources was determined in the same manner as the utilization of carbon sources. Growth requirements were examined in basal medium supplemented with fructose, and growth was followed turbidimetrically.

Biochemical and physiological tests. Growth parameters were determined as described below. (i) The temperature range for growth was determined on nutrient agar. The optimum temperature was estimated in SM medium containing lactate. (ii) The pH growth range and the pH optimum were determined in SM medium containing lactate. (iii) Relationships to oxygen were estimated by the method of Hugh and Leifson (18), and tolerance to oxygen tension was evaluated as indicated below (see Table 7). (iv) Tolerance to sodium chloride was estimated as described below (see Table 7). Indole production from tryptophan was tested by using the modification of Gore (51). Urea hydrolysis was tested by the National Collection of Type Cultures micromethod (8), and decarboxylation of L-amino acids (arginine, lysine, ornithine) was estimated by both the Moeller method and the Falkow method (8). The hydrolysis of arginine was estimated as described by Holding and Collee (17). The production of acid, acetoin, or 2,3-butanediol was investigated as described by Skerman (51). The type of aromatic ring cleavage was tested by the method of Hosokawa, as described by Stanier et al. (54), using *p*- or *m*-hydroxybenzoate as the growth substrate. Accumulated β -keto acid (β -ketoadipate) was detected by the Rothera reaction (17). The type of nitrate reduction under anaerobic growth conditions was investigated in both autotrophic and heterotrophic cultures in media containing 1.0% (wt/vol) KNO₃. For autotrophic denitrification, 20-ml portions of SM medium containing bicarbonate and nitrate in 100-ml Erlenmeyer flasks were inoculated and incubated for 4 days under H₂-CO₂ (85:15, vol/vol). Heterotrophic denitrification tests were carried out as described by Stanier et al. (54). The presence of nitrite was estimated by using sulfanilic acid and α -naphthylamine. Residual nitrate was determined by adding zinc to nitrite-negative cultures.

Extracellular enzymes. All tests were performed and interpreted by using the methods of Holding and Collee (17), except that the test for poly- β -hydroxybutyrate depolymerase was carried out as described by Stanier et al. (54). Phospholipases were estimated by using egg yolk agar. Lipases were estimated by determining the decomposition of Tween 80, Tween 60, and Tween 40, using the method of Sierra (48). Proteinases were estimated by their proteolytic activities, which caused clear zones around colonies grown on milk agar or casein agar; gelatinase activity was determined by the method of Frazier, using 30% trichloroacetic acid as a substitute for acid mercuric chloride and the method of Pitt and Dey (38). Amylase activity on starch agar was detected with iodine solution. Cellulase activity was tested by using the method of Skinner (52).

Enzymes characteristic of major metabolic pathways.

(i) Preparation of cell-free extracts. Thawed cell suspensions were disintegrated by single passages

through a precooled French press (American Instruments Inc., Silver Spring, Md.) at 147 MPa, and unbroken cells and cell debris were removed by centrifugation at $10,000 \times g$ for 20 min at 4°C. The resulting supernatant was referred to as the crude extract. The extract was then centrifuged at $140,000 \times g$ for 90 min at 4°C to obtain the soluble fraction and membrane sediment. The membrane sediment was suspended in approximately 5 volumes of 50 mM potassium phosphate buffer (pH 7.0) and homogenized with a Potter-Elvehjem homogenizer. All extracts were kept at 0°C.

(ii) **Determination of protein.** The protein contents of cell-free extracts were determined by the method of Lowry et al. (31).

(iii) **Enzyme assays.** All enzyme assays were carried out in cuvettes (light path, 1 cm) at 25°C by measuring the change in absorbance with a Zeiss model PM4 spectrophotometer linked to a transmission converter and recorder. The specific enzyme activities are expressed as milliunits per milligram of protein (units per gram of protein); 1 U of enzyme activity was defined as the amount which converted 1 μ mol of substrate or formed 1 μ mol of product(s) per min.

Ribulosebiphosphate carboxylase (EC 4.1.1.39) activity was measured by ribulosebiphosphate-dependent incorporation of $^{14}\text{CO}_2$ into an acid-stable product, as described by Bowien et al. (6), and phosphoribulokinase (EC 2.7.1.19) activity was determined by the method of Abdelal and Schlegel (1), with slight modifications (the final concentration of reduced nicotinamide adenine dinucleotide was 1.0 mM and the final concentration of ribulose 5-phosphate was 1.5 mM). Membrane-bound hydrogenase activity was determined by reduction of methylene blue in the presence of molecular hydrogen, as described by Schink and Schlegel (42), and the reduction of oxidized nicotinamide adenine dinucleotide by soluble hydrogenase (EC 1.12.1.2) was determined by the method of Schneider and Schlegel (45). The activity of the Entner-Doudoroff system (phosphogluconate dehydratase [EC 4.2.1.12] plus phospho-2-keto-3-deoxygluconate aldolase [EC 4.1.2.14]) was estimated by the method of Gottschalk et al. (16). Malate dehydrogenase (EC 1.1.1.37) and reduced nicotinamide adenine dinucleotide oxidase (EC 1.6.99.3) were measured as described by Aggag and Schlegel (2).

Respiratory enzymes. Cytochrome difference spectra were determined with a Zeiss model DM 21 spectrophotometer. Samples were reduced and oxidized by solid dithionite and ferricyanide, respectively. Extraction and determination of quinones were done by the method of Kröger et al. (28). The activities of the terminal respiratory enzymes cytochrome oxidase (EC 1.9.3.1) and catalase (EC 1.11.1.6) were estimated as described by Kovacs (27) and Cowan (8), respectively.

Other tests. Growth with thiosulfate as the energy source was conducted in SM medium containing bicarbonate and 1.0% (wt/vol) $\text{Na}_2\text{S}_2\text{O}_3$, and the inoculated cultures were incubated under a gas mixture containing CO_2 , O_2 , and N_2 (10:10:80, vol/vol) for up to 1 month. Hydrogen sulfide production was tested by cultivating cells on slopes of triple sugar iron agar, using the method of Cowan (8). The litmus milk reaction was carried out and interpreted by the method of Holding and Collee (17).

Susceptibility to inhibitors and antibiotics. The ability

to grow in Moeller KCN broth was examined as described by Skerman (51). Antibiotic susceptibilities were determined by using a modified agar diffusion test; fructose agar plates were layered with 3 ml of soft agar inoculated with 0.5 ml of a 12-h-old culture. Four different susceptibility disks (Oxoid Ltd., London, England) were placed on each plate, and the diameters of the inhibition zones were measured after 48 h of incubation at 30°C.

DNA relationships. Isolation and purification of bacterial DNAs and determinations of both DNA-DNA homology and molecular weights of bacterial genome DNAs were performed as described by Auling et al. (3). DNA base composition (G+C content) was estimated by the thermal denaturation method of Mandel and Marmur (32), using $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate). The midpoint of thermal denaturation of DNA (T_m) was determined graphically after correction of absorbance values for thermal solvent expansion. The G+C content (in moles percent) was calculated from the following equation: $\text{G+C} = 2.08T_m - 106.4$, based on thermal denaturation in $0.1 \times \text{SSC}$ (36).

Computer analysis. For calculations of similarities among the strains studied, the similarity coefficient of Jaccard (19, 53) and the product-moment correlation coefficient, as modified for binary data (41), were used. A similarity matrix cluster was formed by complete linkage (30). Computer analysis was performed by using the algorithms of Wishart (41) and a Univac model 1108 computer (Gesellschaft für wissenschaftliche Datenverarbeitung, Göttingen, Federal Republic of Germany).

Chemicals. The polymer granules of poly- β -hydroxybutyric acid were extracted from *Alcaligenes eutrophus* H16. All other chemicals were of analytical grade and were commercially available.

RESULTS

About 400 bacterial isolates were obtained by specific enrichment to select strains of *P. denitrificans* (i.e., only those strains which grew anaerobically with molecular hydrogen as the energy source, CO_2 as the carbon source, and nitrate or nitrite as the electron acceptor). Robust strains, which survived for 1 year at 4°C as autotrophic slants under air, were characterized and compared with the strains of *P. denitrificans* that had been kept in culture collections for many years (Table 1).

All strains studied showed considerable conformity in cultural, morphological, ultrastructural, biochemical, and physiological properties.

Cytology. All strains were short rods (coccobacilli), and generally the cells were shorter in stationary growth phases than during exponential growth. The average dimensions were 0.6 by 0.85 μm and 0.8 by 1.2 μm in stationary and exponential growth phases, respectively (Fig. 1a and b). Cell division was by binary transverse fission. Flagella or pili were not found in any strain, and light microscopic preparations confirmed that the cells were immobile. The cells were covered with a fibrous material which

FIG. 1. Light microscopic phase-contrast micrographs of strain N4 (= DSM 1404) cells. (a) Cells in exponential growth phase. (b) Cells in stationary growth phase. Bar = 10 μ m. S.381, Stanier 381^T; Vogt, Vogt 11; Morris, Morris 5.

showed a positive reaction with Ruthenium red, indicating the presence of large amounts of extracellular polyanions and acidic mucosubstances (34). All strains were gram-negative, and the cell wall fine structures were typical for gram-negative bacteria. Poly- β -hydroxybutyric acid was the main storage material, and its level ranged between 3 and 30% of the cell dry weight, depending on the growth substrate and conditions; in addition, polyphosphate and glycogen-like material were detected (34). All strains failed to produce pigment or endospores under various cultural growth conditions, and none was acid fast.

Cultural properties. Surface colonies of *P. denitrificans* on nutrient agar were circular, 2 to 4 mm in diameter, smooth or finely granulated, entire or undulate, convex or umbonate, creamy or faintly brown, and translucent with a soft consistency. On nitrogen-limited media the colonies were white and opaque. Liquid cultures were turbid with no pellicle. Old cultures tended to become brownish, but cultures of strain Stanier 381^T turned pinkish, especially under low oxygen tensions.

Growth and nutritional screening. Of 100 organic compounds tested as sole carbon and energy sources for heterotrophic growth, only 41 were utilized universally, and 28 substrates could not be used by any strain (Table 3). The remaining 31 compounds were utilized by 1 or more of the 14 strains tested (Table 4). Cultures from mutant clones grew on some additional substrates, presumably due to altered permeability (15, 23, 47). Molecular hydrogen but not carbon monoxide, methane, propane, ferrous iron, elemental sulfur, or reduced sulfur compounds served as an electron donor for growth and CO₂ fixation at pH 7.0.

Of 14 nitrogenous compounds and molecular nitrogen tested as nitrogen sources in defined media, L-alanine, L-arginine, L-phenylalanine, L-proline, urea, ammonium chloride, and potassium nitrate were universal nitrogen sources. None of the 14 strains tested could grow with molecular nitrogen. Seven other compounds were used by one or more of the strains (Table 5).

None of the strains needed additional growth factors when they were grown heterotrophically or autotrophically with oxygen, although L-arginine, L-phenylalanine, and L-proline improved growth. This effect suggests that the nitrogen in these compounds is more readily available than the nitrogen in ammonium chloride. Yeast autolysate stimulated the growth of some strains (Table 6).

Biochemical and physiological features. The rate of growth in mineral medium containing lactate was maximum at 36°C. Growth occurred at 10 and 40°C but not at 5 or 41°C. Growth occurred at pH values ranging from 6.0 to 10 but not below pH 5.0 or above pH 11; growth was optimal at pH 7.6. Metabolism was always respiratory and never fermentative. All strains failed to grow anaerobically unless nitrate or nitrite was added. Table 7 shows tolerance to oxygen tension under autotrophic growth conditions. All strains could grow well in nutrient broth containing from 0 to 3% (wt/vol) sodium chloride; some strains exhibited a higher salt tolerance (Table 7). The methyl red test was negative throughout. All strains failed to grow on benzoate or L-tryptophan, but all could cleave either *m*- or *p*-hydroxybenzoate via protocatechuate (ortho cleavage). The enzyme system was inducible; cells grown on nutrient broth gave negative reactions. Only strain N9 failed to grow on any of the aromatic compounds tested. Under anaerobic conditions nitrate was reduced to N₂. Indole was produced from tryptophan. Urease activity, decarboxylation of L-amino acids (arginine, lysine, ornithine), and arginine dihydrolase tests were negative. No strain emulsified phospholipid agents in egg yolk agar, decomposed

TABLE 3. Organic substrates utilized by all or none of the strains studied as sole sources of carbon and energy^a

Class of compounds	Organic compounds utilized by:	
	All strains	No strain
Organic acids (salts)	Acetate, formate, fumarate, gluconate, 2-keto-D-gluconate, α -keto-glutarate, glucuronate, glycerate, 3-hydroxybutyrate, L-lactate, L-malate, pyruvate, succinate	Benzoate, citroconate, poly- β -hydroxybutyrate, DL-mandelate
Amino acids (salts)	L-Alanine, β -alanine, DL- α -alanine, 4-aminobutyrate, L-aspartate, L-glutamate, glycyl-glycine, L-histidine, L-isoleucine, L-leucine, L-phenylalanine, L-proline, L-serine	L-Arginine, L-lysine, L-methionine, L-threonine, L-tryptophan
Carbohydrates and sugar derivatives	D-Fructose, D-galactose, D-glucose, maltose, D-mannose, sucrose, trehalose	D-Arabinose, cellobiose, cellulose, L-fucose, D-fucose, 6-deoxy-D-glucose, glycogen, lactose, raffinose, starch, D-xylose
Alcohols	Glycerol, D-mannitol, <i>n</i> -propanol, D-sorbitol	
Amines and nitrogenous compounds	Creatin, L-asparagine, L-glutamine, sarcosine	Ethanolamine, triethanolamine, urea
Other compounds		Dodecan, hexadecan

^a Defined media containing NH₄Cl as the nitrogen source were used.

Tween 80, Tween 60, and Tween 40, peptonized milk, hydrolyzed casein, gelatin, starch, or cellulose, or depolymerized poly- β -hydroxybutyric acid granules.

Ribulosebiphosphate carboxylase and phosphoribulokinase were present in autotrophically grown cells and were absent or showed low activity in cells grown on fructose. All 14 strains studied contained only one type of hydrogenase bound to cell membranes that did not reduce pyridine nucleotides but did reduce methylene blue. In autotrophically grown cells average enzyme activities were 1,400 U/g of crude extract protein and 5,700 U/g of protein in membrane fractions. All strains but one (Stanier 381^T) formed hydrogenases constitutively, whereas strain Stanier 381^T was unique among the 14 strains studied; the hydrogenase of this strain behaved as an inducible enzyme and was only formed when hydrogen was present (35). Fructose-grown cells contained both 6-phosphogluconate dehydratase and phospho-2-keto-3-deoxygluconate aldolase (Entner-Doudoroff system) at elevated levels of 120 to 250 U/g of protein, and this activity decreased to about 10 U/g of protein in autotrophically grown cells.

Cells grown anaerobically with glucose and nitrate contained membrane-bound type *b* and type *c* cytochromes; no type *a* cytochrome was detected. The soluble fraction exhibited a reduced-minus-oxidized difference spectrum with peaks at 419, 520, 620, and 670 nm, indicative of cytochrome *cd*. In contrast, cells grown autotrophically with H₂ as the electron donor and O₂ as the electron acceptor had essentially the same cytochrome pattern; however, there was a de-

creased concentration of the soluble type *cd* cytochrome and a type *a* cytochrome in the membrane fraction. Membrane-bound ubiquinone could be demonstrated. These results are in agreement with data reported previously (29, 33, 46). Cytochrome oxidase and catalase activities were very high.

None of the strains exhibited an ability to grow on thiosulfate as the electron donor instead of hydrogen at pH 7.0. No hydrogen sulfide was produced from thiosulfate on triple sugar iron agar slants. Without exception, growth in litmus milk caused a slight alkaline reaction with no detectable proteolytic activity.

Only strain N7 grew in Moeller KCN broth. All strains were susceptible to 25 μ g of ampicillin per ml, 10 U of bacitracin per ml, 30 μ g of kanamycin per ml, 30 μ g of neomycin per ml, 30 μ g of novobiocin per ml, 300 U of polymyxin B per ml, and 50 μ g of tetracycline per ml and resistant to 300 μ g of sulfatriad per ml. Only a few of the antibiotics tested discriminated among the strains studied and, therefore, were of taxonomic value (Table 8).

DNA base compositions, DNA-DNA homology, and molecular weights of bacterial genome DNAs. The DNA base compositions of the strains tested ranged from 65 to 67 mol% G+C, as estimated by the thermal denaturation method. The strains were previously characterized by the degree of DNA-DNA binding (duplexing), which ranged from 40 to 100% binding to the three reference strains studied (Stanier 381^T, Morris 5, and N4) (3); the molecular weights of the genome DNAs ranged from 2.5×10^9 to 3.4×10^9 (3).

TABLE 4. Organic substrates utilized by one or more, but not all, of the strains studied^a

Substrate	Utilization by the following strains: ^b											DSM 65 ^T	DSM 413	DSM 415
	New isolates													
	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11			
Organic acids (salts)														
L-Ascorbate	—	—	m	m	—	m	m	—	—	m	—	m	m	—
Citrate	—	m	m	m	—	m	—	—	—	—	—	—	—	—
D-Galacturonate	—	—	—	—	—	—	—	—	+	+	+	—	+	+
Glycolate	+	+	+	+	+	+	+	+	—	—	+	+	+	+
Glyoxylate	+	+	+	+	+	+	+	—	m	m	+	+	+	+
3-Hydroxybenzoate	+	+	+	+	m	m	+	+	—	—	—	—	+	+
4-Hydroxybenzoate	+	+	m	+	—	—	+	+	—	m	+	+	+	+
2-Hydroxybutyrate	—	—	m	—	—	—	m	—	—	—	—	m	—	m
Isobutyrate	+	m	+	+	+	+	+	+	m	m	+	+	+	+
DL-Iso-citrate	—	—	—	—	—	—	+	—	+	+	—	—	—	+
Malonate	—	—	—	—	—	—	—	—	+	+	—	+	+	+
Mesaconate	m	—	—	—	—	—	m	—	—	—	—	—	+	+
Oxalate	m	m	—	m	m	m	—	—	—	—	—	—	—	—
Propionate	+	m	m	m	m	m	m	m	—	—	m	m	m	m
Suberate	—	—	—	—	—	—	—	+	—	—	+	—	—	—
Tartrate	+	+	+	—	+	+	m	—	—	—	—	+	—	—
Amino acids (salts)														
L-Cysteine	—	—	m	m	m	m	—	—	—	—	—	—	—	—
Glycine	+	+	+	+	+	+	m	m	—	—	—	—	+	+
L-Ornithine	—	—	—	—	—	—	—	—	—	—	—	—	m	m
L-Tyrosine	+	+	—	—	—	—	+	—	+	—	—	+	+	+
Urate	+	+	+	+	+	+	+	—	—	—	+	+	+	+
L-Valine	—	—	—	m	—	—	m	m	—	—	—	—	+	+
Sugars														
L-Arabinose	+	+	+	+	+	+	+	—	+	+	—	m	+	+
D-Ribose	+	+	+	+	+	+	+	—	—	—	—	+	+	+
L-Rhamnose	—	—	+	—	—	—	+	—	+	+	—	—	—	—
L-Sorbose	m	m	—	m	—	—	—	—	—	—	—	—	—	—
D-Tagatose	+	+	+	+	+	+	+	—	—	—	—	—	+	+
Alcohols														
Ethanol	+	+	+	+	+	+	+	+	+	—	+	+	+	+
Methanol	—	+	—	—	—	—	+	—	—	—	—	+	+	+
Phenol	—	—	—	—	+	+	—	—	—	—	—	—	—	—
Casein hydrolysate	—	—	+	+	+	+	+	—	+	+	—	+	+	+

^a A defined medium containing NH₄Cl as the nitrogen source was used.^b +, Utilized; —, not utilized; m, mutational event.

Computer analysis. Figure 2 shows a dendro-grammatic plot of the relationships among the strains studied, which was drawn from a similarity matrix (Jaccard coefficient).

DISCUSSION

Isolation of numerous strains of denitrifying hydrogen-oxidizing bacteria from different localities and ecosystems indicated the great adaptability of these organisms. These strains possessed rather versatile metabolic systems and could satisfy their energy demands from (i) organic substrates and molecular oxygen, (ii) organic substrates and nitrate, nitrite, or nitrous oxide, (iii) molecular hydrogen and oxygen, and (iv) molecular hydrogen and nitrate, nitrite, or nitrous oxide. The last system is the basis of the specific epithet of *P. denitrificans*, which refers to the outstanding property of this organism, a

distinctive character not found in any other organism.

Our data showed that the strains which we studied are closely related and matched the definition of *P. denitrificans*. The close relationships are based on the DNA base composition, which ranges from 65 to 67 mol% G+C, and on high DNA homology values, as well as on the basic cultural, morphological, cytological, physiological, and biochemical data. The recognition of *P. denitrificans* as a well-delineated, homogeneous species (9) is thus confirmed.

In contrast to the finding of Davis et al. (9) that strains ATCC 13543, ATCC 17741^T (= Stanier 381^T), and ATCC 19367 (= Morris 5) may represent the same original isolate of *P. denitrificans*, our data provide several characters that differentiate these strains from one another and support the suggestion of Kocur and

TABLE 5. Nitrogenous compounds utilized by one or more, but not all, of the strains studied as a sole nitrogen source^a

Substrate	Utilization by the following strains: ^b											DSM 65 ^T	DSM 413	DSM 415
	New isolates													
	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11			
β-Alanine	+	+	+	+	+	+	+	+	+	+	—	+	+	+
L-Aspartate	+	+	+	+	+	+	+	+	—	—	+	+	+	+
L-Asparagine	±	±	±	±	±	±	+	+	—	—	—	—	—	—
L-Lysine	+	+	+	+	+	+	+	+	±	±	±	+	±	±
L-Ornithine	m	m	—	±	m	m	—	±	m	m	m	m	m	m
Urate	+	+	+	+	+	+	+	m	m	m	+	+	+	+
Potassium nitrite	+	+	+	+	+	+	+	—	±	±	+	+	+	+

^a A nitrogen-free defined medium containing fructose as the growth substrate and 1 g of the tested nitrogen compound per liter was used.

^b +, Good growth; ±, scanty growth; —, no growth; m, mutational event.

Martinec (25) that they should be dealt with as distinct strains. This notion received further support by the detection of plasmids in three reference strains of *P. denitrificans* (14a). Strains DSM 65^T, DSM 413, and DSM 415 contain large plasmids having molecular weights of more than 300×10^6 . These plasmids are apparently not identical. The type strain, strain DSM 65^T, harbors an additional small plasmid having a molecular weight of 50×10^6 .

On the basis of a computer analysis of 235 characters, the strains which we examined belong to four subgroups (Fig. 2). Subgroup I includes strains N1 through N6, with similarities of 74% or more; subgroup II includes strains N7, Morris 5, Vogt 11, and Stanier 381^T, with a

similarity of 75%; subgroup III includes strains N8 and N11, with a similarity of 76%; and subgroup VI includes strains N9 and N10, with a similarity of 88%. The degrees of similarity (Jaccard coefficients) for these subgroups were 68% for subgroups II and III, 65% for subgroups I, II, and III, and 62.5% for all subgroups simultaneously. This subgrouping is in agreement with that obtained by DNA-DNA reassociation kinetics (3).

Taxonomic considerations. As stated above and as has been pointed out previously by both Baird-Parker (4) and Rosypal et al. (40), this gram-negative, nonsporeforming, nonpigmented, short, rod-shaped bacterium (coccobacillus) cannot be assigned to either the genus *Micrococ-*

TABLE 6. Effect of yeast autolysate on autotrophic growth of *P. denitrificans* strains^a

Strain	Doubling times (h) with the following electron acceptors:			
	Oxygen		Nitrate	
	With yeast extract ^b	Without yeast extract	With yeast extract ^b	Without yeast extract
N1	5.0	4.7	29	20
N2	5.3	4.5	22	17
N3	5.0	4.2	16	21
N4	4.3	3.9	42	8
N5	4.6	4.5	59	31
N6	4.6	3.9	36	24
N7	3.1	4.1	15	15
N8	50	40	26	21
N9	4.1	3.8	13	12
N10	10	16	15	20
N11	7.3	8.8	18	20
DSM 65 ^T	3.7	4.1	41	5
DSM 413	4.9	4.4	22	22
DSM 415	4.7	4.8	21	18

^a Cultures were grown in 300-ml Erlenmeyer flasks containing 40 ml of SM medium supplemented with bicarbonate, shaken in a 30°C water bath and flushed with a gas mixture containing H₂, CO₂, and O₂ (80:10:10, vol/vol) for aerobic growth. For anaerobic growth, the same medium was supplemented with 1% (wt/vol) KNO₃, and the gas mixture contained H₂ and CO₂ (90:10, vol/vol). Growth was measured turbidimetrically.

^b Yeast extract (0.005%, wt/vol) was added.

TABLE 7. Tolerance of *P. denitrificans* strains to oxygen under autotrophic conditions and to salt under heterotrophic conditions

Strain	Maximum oxygen concn (% vol/vol) ^a	Maximum salt concn (% wt/vol) ^b
N1	40	4
N2	40	6
N3	45	6
N4	65	4
N5	45	4
N6	45	5
N7	45	4
N8	25	4
N9	25	4
N10	25	7
N11	40	4
DSM 65 ^T	30	7
DSM 413	40	3
DSM 415	40	3

^a Cells were grown in SM medium containing bicarbonate under a gas mixture composed of 5% CO₂ and different concentrations of O₂ and H₂ (intervals of 5%). The values indicate the highest oxygen concentrations under which growth occurred within 5 days. H₂ served as a balance.

^b Cells were grown in nutrient broth supplemented with sodium chloride, and growth was followed turbidimetrically daily for 1 week. The values indicate the highest NaCl concentrations in which growth occurred within 7 days.

cus or the genus *Staphylococcus*, both of which are gram positive. It seems likely that the type species of the genus *Paracoccus* is closely related to some facultatively chemolithoautotrophic species in the genera *Alcaligenes* and *Pseudomonas* and resembles at least the facultatively organotrophic species of the genus *Thiobacillus* (11), as well as members of the genera *Neisseria* and *Moraxella* (9), "*Acinetobacter cerificans*" (12), and "*Methylococcus capsulatus*" (26); e.g., it shares the following characters with the peritrichous species *Alcaligenes eutrophus* Davis: gram negative, neither sporulation nor pigmentation, growth on hydrogen plus carbon dioxide, accumulation of poly-β-hydroxybutyric acid, growth and denitrification in organic media, inability to use arginine either aerobically or anaerobically as a carbon or energy source, strictly aerobic, and DNA base composition ranging from 66 to 67 mol% G+C; furthermore, the cells of *A. eutrophus* become almost spherical and nonflagellated in the stationary phase of growth. *Paracoccus denitrificans* shares with *Pseudomonas facilis*, *Pseudomonas flava*, *Pseudomonas pseudoflava*, *Pseudomonas paleronii*, and *Pseudomonas saccharophila* the following properties: gram negative, nonspore-forming, growth on hydrogen and carbon dioxide, accumulation of poly-β-hydroxybutyric

TABLE 8. Antibiotic susceptibility patterns of *P. denitrificans* strains

Strain	Inhibitor ^a						
	Ca	Ch	Cl	Er	Ox	Pe	St
DSM 65 ^T	+	+	—	±	+	+	—
DSM 413	+	+	+	±	+	—	±
DSM 415	+	+	+	±	+	—	—
N1	+	+	—	+	+	±	—
N2	+	+	—	—	+	+	—
N3	+	+	—	±	+	±	—
N4	+	+	—	±	+	±	±
N5	+	+	—	—	+	+	±
N6	+	+	—	—	+	+	—
N7	+	—	—	—	+	—	±
N8	—	+	—	—	—	—	±
N9	+	+	—	—	—	—	±
N10	+	+	—	—	—	—	±
N11	+	+	—	±	+	—	±

^a Abbreviations: Ca, carbenicillin (100 µg/disk); Ch, chloramphenicol (50 µg/disk); Cl, cloxacillin (5 µg/disk); Er, erythromycin (15 µg/disk); Ox, oxytetracycline (30 µg/disk); Pe, penicillin G (10 U/disk); St, streptomycin (25 µg/disk). The zones of inhibition were measured from the edges of susceptibility disks (Oxoid) used to the edges of clear zones. —, Resistance (inhibition zone, <2 mm); ±, weak susceptibility (inhibition zone, 2 to 5 mm); +, full susceptibility (inhibition zone, >5 mm).

acid, growth on a wide spectrum of organic substrates, inability to use arginine aerobically or anaerobically as a carbon or energy source, absence of arginine dihydrolase (10), obligately respiratory, inability to grow at 41°C, no growth factor requirements, and identical DNA base

FIG. 2. Dendrogram showing the arrangement of medium-sized strains studied after complete linkage clustering. Similarities were determined by using the Jaccard coefficient and the product-moment correlation coefficient, as modified for binary data (40).

compositions (64 to 69 mol% G+C). In many respects, *P. denitrificans* resembles *Thiobacillus novellus*, which is a gram-negative, nonmotile, nonpigmented, short, rod-shaped or coccoidal bacterium, is strictly respiratory, can grow autotrophically with CO₂ as the carbon source, and has a DNA base composition of 66 to 68 mol% G+C (20). A relationship with the thiobacilli is indicated by the fact that *P. denitrificans* can grow on thiosulfate at pH 8.0 under strictly lithoautotrophic conditions (14). *Neisseria* and *Moraxella* (9) differ from *P. denitrificans* in DNA base composition (40 to 46 mol% G+C in *Moraxella* and about 50 mol% G+C in *Neisseria*). Kocur et al. (26) assumed that both *P. denitrificans* and *Paracoccus halodenitrificans* are morphologically and biochemically similar to the gram-negative cocci described as "*Acinetobacter cerificans*" (12) and to *Methylococcus capsulatus* (13).

On the basis of our data, we propose the following formal taxonomic description.

Amended description of *Paracoccus denitrificans*. *Paracoccus denitrificans* (Beijerinck) Davis 1969, 384. (*Micrococcus denitrificans* Beijerinck and Minkman 1910, 54; Pa. ra. coc'cus. Gr. prep. *para* like, *coccus* a grain, berry; M.L. masc. n. *Paracoccus* like a coccus. de. ni. tri'fi.cans. L. prep. *de* away from; L. n. *nitrum* soda; M. L. n. *nitrum* nitrate; M. L. v. *denitrifo* denitrify; M. L. part. adj. *denitrificans* denitrifying).

Cell morphology and cytological characters. Nonpigmented, immotile, nonflagellated, short, rod-shaped cells (coccobacilli) with average dimensions of 0.6 by 0.85 μ m in stationary phase and 0.8 by 1.2 μ m in exponential growth phase. No spores or resting bodies are formed. One-plane transverse fission produces equal daughter cells. Cells occur singly and in pairs but never in aggregates and accumulate granules of poly- β -hydroxybutyric acid, glycogen-like material, and poly-phosphate as intracellular reserve material. Gram negative and non-acid fast.

The G+C content of the DNA is 65 to 67 mol%. The molecular weight of the bacterial genome DNA ranges from 2.3×10^9 to 3.4×10^9 .

Cultural properties. Agar colonies are circular, smooth, glistening, semitranslucent, creamy with a soft consistency, and turn white and opaque in nitrogen-deficient media. Liquid cultures are turbid without pellicles.

Growth substrates. Of 100 organic compounds tested, 41 were utilized, 28 were not utilized, and the rest were utilized by one or more of the strains. L-Alanine, L-arginine, L-phenylalanine, L-proline, urea, ammonium chloride, and potassium nitrate serve as universal nitrogen sources, but nitrogen fixation does not occur. Growth

factors, activators, or vitamins are not required. Utilize molecular hydrogen, but not carbon monoxide, ferrous iron, elemental sulfur, or reduced sulfur compounds at pH 7.0 as electron donors for autotrophic growth.

Biochemical and physiological features. Good growth in media with pH values of 6 to 10 and at temperatures of 8 to 40°C. Optimum growth in media at pH 7.6 and 36°C. Metabolism respiratory, never fermentative. Tolerant to oxygen concentrations up to 65% (vol/vol) under autotrophic growth conditions. Tolerant to about 7% (wt/vol) NaCl. Free oxygen, nitrate, nitrite, or nitrous oxide serves as a terminal electron acceptor. Autotrophic denitrification, as well as organotrophic denitrification with several organic compounds. Dissimilation of aromatic compounds via protocatechuate by ortho cleavage. The following tests were negative: methyl red, Voges-Proskauer, indole formation, urease activity, decarboxylation of L-amino acids (arginine, lysine, ornithine), arginine dihydrolase activity, phospholipase (lecithinase) activity, lipase activity on Tween 80, Tween 60, and Tween 40, proteinase activity on both vitamin-free casein and milk casein, gelatin liquefaction, starch and cellulose hydrolysis, exogenous poly- β -hydroxybutyric acid depolymerase activity, as well as H₂S production. Hydrogenase is exclusively membrane bound and constitutive except in strain Stanier 381^T, where it is inducible. Phosphoribulokinase and ribulosebiphosphate carboxylase, the key enzymes of the Calvin cycle, are inducible. Fructose-grown cells contain both 6-phosphogluconate dehydratase and phospho-2-keto-3-deoxygluconate aldolase at elevated levels and lack 6-phosphofructokinase and fructose biphosphate aldolase. The membrane fraction of cells grown anaerobically with nitrate contains type *b* and *c* cytochromes but not type *a* cytochromes, and the soluble fraction includes a type *cd* cytochrome; the membrane fraction of aerobically grown cells contains type *a*, *b*, and *c* cytochromes, and the soluble fraction contains only a few type *cd* cytochromes. Membrane-bound ubiquinone. Terminal respiratory enzymes, as well as oxidase and catalase, are very active. Slight alkaline reaction in litmus milk. Only one strain, strain N7 (= DSM 1406), could grow in Moeller KCN broth.

Susceptibility to antibiotics. Tolerant to 300 μ g of sulfatriad per ml but not to 25 μ g of ampicillin per ml, 10 U of bacitracin per ml, 30 μ g of kanamycin per ml, 30 μ g of neomycin per ml, 30 μ g of novobiocin per ml, 300 U of polymyxin B per ml, or 50 μ g of tetracycline per ml.

Habitat. Presumably widely distributed in soil, mud, animal manure, sewage, and sludge.

Type strain. ATCC 17741^T (= DSM 65^T).

Type species of the genus *Paracoccus* (Beijer-

inck) Davis.

Six of the newly isolated strains have been deposited with the Deutsche Sammlung von Mikroorganismen under the following numbers: DSM 1403 (= strain N1), DSM 1404 (= strain N4), DSM 1405 (= strain N5), DSM 1406 (= strain N7), DSM 1407 (= strain N10), and DSM 1408 (= strain N11).

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