Deoxyribonucleic Acid Relationships Among Hydrogen-Oxidizing Strains of the Genera *Pseudomonas*, *Alcaligenes*, and *Paracoccus*

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Optical determination of deoxyribonucleic acid (DNA)-DNA reassociation kinetics was applied to the classification of 32 selected strains of hydrogen-oxidizing bacteria belonging to the genera *Pseudomonas*, *Alcaligenes*, and *Paracoccus*. The renaturation studies revealed a high intraspecies DNA homology for some strains of the species *Pseudomonas palleronii*, *Pseudomonas pseudoflava*, and *Alcaligenes paradoxus*, supporting former taxonomic concepts of different authors. The 12 denitrifying strains belonging to the genus *Paracoccus* were shown to be interrelated at various levels of percent degree of binding, and at least two clusters of very high genome-DNA relatedness have been found. DNA-DNA reassociation kinetics were also used for the calculation of the average molecular weight of genome DNA. The genome molecular weight of the hydrogen-oxidizing bacteria investigated in this study ranged from 3×10^9 to 5×10^9 .

Defined as a physiological group, the "hydrogen bacteria" are aerobic chemolithotrophs that can use the oxidation of molecular hydrogen as an energy source. To accommodate these organisms, the genus *Hydrogenomonas* was proposed by Orla-Jensen in 1909. Davis et al. (5) rejected the genus *Hydrogenomonas* and proposed the assignment of all gram-negative strains of hydrogen-oxidizing bacteria to *Pseudomonas*, *Alcaligenes*, or *Paracoccus*. By this and later investigations of gram-negative, polarly flagellated, hydrogen-oxidizing bacteria and related strains (1, 2, 5, 6), the following nine hydrogen-oxidizing species of the genera *Pseudomonas*, *Alcaligenes*, and *Paracoccus* were established: *Pseudomonas facilis*, *P. flava*, *P. pseudoflava*, *P. palleronii*, *P. saccharophila*, *Alcaligenes eutrophus*, *A. paradoxus*, *A. ruhl-andii*, and *Paracoccus denitrificans*. Serological methods have been used to show relatedness between strains of these species (2, 8). In spite of the intensive application of nucleic acid hybridization techniques for the classification of the pseudomonads (14, 16, 17), only very few deoxyribonucleic acid (DNA)-DNA reassociations have been reported for hydrogen-oxidizing strains of the genera *Pseudomonas* and *Alcaligenes* (2, 18).

The present investigation was undertaken to confirm, by DNA-DNA reassociation studies, the establishment of new genera and species for

the gram-negative, hydrogen-oxidizing bacteria. Nine new denitrifying strains, isolated by T. Nokhal from our institute, were included in this study. Their characterization and comparison with known strains of *Paracoccus denitrificans* will be described in a separate paper (Nokhal and Schlegel, manuscript in preparation).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. In addition, *Xanthomonas pelargonii* ICPB P121 was used as a reference in molecular weight determinations and was grown in nutrient broth (Difco). The culture conditions and the media for growth and maintenance of the hydrogen-oxidizing strains have been described elsewhere (2). The purity of each strain was checked by microscopic observation and by plating on nutrient broth agar.

Preparation of DNA. Only cells from cultures grown to the stationary phase of the growth curve were harvested for isolation of DNA. The DNA was prepared by a light modification of the procedure of Marmur (12), which included repeated digestion of proteins by proteinase K (Merck) and digestion of ribonucleic acid by ribonuclease A and T1 (both Boehringer). The DNA solutions were considered as sufficiently purified if the extinction ratios E_{230}/E_{280} , E_{260}/E_{280} , E_{290}/E_{260} were about 0.450:1.00:0.515. Crude DNA from four of the newly isolated denitrifying hydrogen-oxidizing *Paracoccus* strains was kindly provided by T. Nokhal from this institute.

DNA-DNA renaturation. Five-milliliter quantities of DNA in $1 \times$ SSC (standard saline citrate) buffer were dialyzed at room temperature against 0.667 M sodium-phosphate buffer, pH 7.0. After 3 days of dialysis with several buffer changes, the DNA solutions

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TABLE 1. *List of strains studied*^a

Species	Strain no.			Received from:	Reference/remark
	Authors'	DSM	ATCC		
<i>Alcaligenes</i>		531	17697	ICC	(5)
<i>A. eutrophus</i>	H16	428	17699	ICC	(5)
<i>A. faecalis</i>		30030	8750	DSM	Type species of the genus <i>Alcaligenes</i> ; emended description by Hendrie et al. (10)
<i>A. paradoxus</i>	351	30034	17713	ICC	(5)
<i>A. paradoxus</i>	353	646	17715	DSM	(5)
<i>A. paradoxus</i>	12x			ICC	Identified by Wilde as <i>A. paradoxus</i>
<i>Paracoccus denitrificans</i>	S	65	17741	ICC	(5)
<i>P. denitrificans</i>	V	415		ICC	(20)
<i>P. denitrificans</i>	M	413		Nokhal	(4)
<i>Paracoccus</i> sp.	N1			Nokhal	Isolated and identified by Nokhal as <i>P. denitrificans</i> or regarded as similar to <i>P. denitrificans</i>
<i>Paracoccus</i> sp.	N2			Nokhal	
<i>Paracoccus</i> sp.	N3			Nokhal	
<i>Paracoccus</i> sp.	N4			Nokhal	
<i>Paracoccus</i> sp.	N5			Nokhal	
<i>Paracoccus</i> sp.	N6			Nokhal	
<i>Paracoccus</i> sp.	N7			Nokhal	
<i>Paracoccus</i> sp.	N9			Nokhal	
<i>Paracoccus</i> sp.	N10			Nokhal	
<i>Pseudomonas facilis</i>	332		17695	ICC	(5)
<i>P. flava</i>		619		DSM	(11)
<i>P. pseudoflava</i>	GA3	1034			(2)
<i>P. pseudoflava</i>	9B32			Lalucat	Identified by Lalucat as <i>P. pseudoflava</i>
<i>Pseudomonas</i> sp.	9B31			Lalucat	Related to <i>P. pseudoflava</i>
<i>Pseudomonas</i> sp.	GA6				
<i>Pseudomonas</i> sp.	MA30			Aragno	May be related to <i>P. pseudoflava</i>
<i>P. palleronii</i>	362	63	17724	ICC	(6)
<i>P. palleronii</i>	366	650	17728	DSM	(6)
<i>P. palleronii</i>	RH2			Reh	Identified by Reh as <i>P. palleronii</i>
<i>P. palleronii</i>	Z32			Savel'eva	Identified as <i>Hydrogenomonas pantotropha</i> by Savel'eva and Zhilina (19)
<i>Pseudomonas</i> sp.	Z17			Sevel'eva	
<i>P. saccharophila</i>		654	15946	DSM	(5)

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany; ICC, Institute's (Göttingen) Culture Collection.

were sheared by repeated (2×) passage through a French pressure cell at about 1.5×10^6 Pa. The fragmented samples were centrifuged for 30 min at 4,000 rpm and adjusted to E_{260} of 1.25.

A combination of the methods of De Ley et al. (7) and of Gillis et al. (9) as well as of the procedure of Bradley (3) was developed for optical determination of the reassociation kinetics of DNA in sodium phosphate buffer. Formamide was added to the adjusted DNA samples, giving a final solvent concentration of 25% (vol/vol) formamide and 0.5 M sodium phosphate, pH 7.0. Three DNA solutions (DNA of A; DNA of B; a mixture of equal volumes of DNA of A and of DNA of B) and a blank with adenine were heat denatured in a recording spectrophotometer with an attached temperature programmer (Gilford Instruments Laboratories, Inc.). The denaturing temperature (95°C) was maintained for 15 min, and then the temperature was rapidly adjusted to 25°C below the midpoint of thermal denaturation of the DNA (T_m). Because the mid-

points of the hyperchromic shifts in the employed phosphate-formamide buffer system were about 85°C for most of the strains examined, we decided to use 60°C as the optimal temperature for renaturation throughout the study. The mean guanine plus cytosine (G+C) content of all strains tested was about 60 to 70 mol% (2, 6; unpublished data of T. Nokhal and of our laboratory). The absorbance at 270 nm was monitored for 30 min unless otherwise stated. This denaturation-renaturation procedure was repeated five or six times for each sample.

RESULTS

Calculations of the degree of binding and of genome size are based on comparison of renaturation rate constants, which are only reliable when reassociation, as monitored optically, proceeds as a second-order reaction (9, 22). Initial linearity of the recorded renaturation lines due

to second-order kinetics was obtained in every case for some 40 min after the onset of the reassociation conditions. The degree of binding between the denatured DNA samples was calculated by the method of De Ley et al. (7). Attempts to monitor the reassociation reactions for longer times to obtain $C_0T_{1/2}$ values as described by Bradley (3) did not result in stringent second-order kinetics. Under our test conditions the plots always showed a slight deviation from the needed linearity. We nevertheless tried to calculate the degree of nucleotide sequence homology for some reassociations by the method of Bradley (3). The results thus found proved to be 10 to 20% lower than those obtained from the initial linear renaturation lines of De Ley et al. (7).

For our DNA-DNA renaturation studies, we selected *P. palleronii* 362, *P. pseudoflava* GA3, *P. flava* DSM 619, *A. paradoxus* 351, and *P. denitrificans* S as references because they were type strains. In addition, *P. denitrificans* M and *Paracoccus* sp. N4 were chosen because they seemed to be "centrally" located in a group of phenetically studied, denitrifying, hydrogen-oxidizing strains (T. Nokhal, unpublished data). The resulting DNA homologies are presented in Tables 2 and 3.

In the *P. palleronii* homology group, *P. palleronii* 362 and *P. palleronii* 366, the only strains used by Davis et al. (6) in their proposal of a new species, showed identical polynucleotide sequences. Strain Z32, identified as *Pseudomonas pantotropha* by Savel'eva and Zhilina (19) and strain RH2, previously identified as *P. palleronii* (Reh, unpublished data), also exhibited close relatedness to *P. palleronii* 362.

Several strains of the *P. pseudoflava* homology group included in this study (strains GA2, GA3, and GA4) have already been subjected to an extensive characterization which resulted in the proposal of *P. pseudoflava* (2). Further strains, aside from 9B32, subsequently isolated by different authors showed only moderate or remote relatedness to *P. pseudoflava* GA3.

Of the strains tested for degree of binding to *P. flava* DSM 619, only a few showed moderate relatedness. In the *A. paradoxus* homology group, strains 351 and 353 had nearly identical polynucleotide sequences, and strain 12X was closely related to the reference (type) strain *A. paradoxus* 351. Absolutely no relatedness was found between *A. paradoxus* 351 and strain DSM 30030 of *A. faecalis*, the type species of the genus *Alcaligenes*.

The data from the optical reassociation of 12

TABLE 2. Degree of binding (%D) among various strains of hydrogen-oxidizing bacteria

Species	Strain ^a	%D with			
		<i>P. palleronii</i> 362	<i>P. pseudoflava</i> GA3	<i>P. flava</i> DSM 619	<i>A. paradoxus</i> 351
<i>Pseudomonas palleronii</i>	362	100			
<i>P. palleronii</i>	366	99			
<i>P. palleronii</i>	Z32	84			
<i>P. palleronii</i>	RH2	76			
<i>Pseudomonas</i> sp.	Z17	64			
<i>Pseudomonas pseudoflava</i>	GA3	25	100		
<i>P. pseudoflava</i>	GA4		99 ^b	62	
<i>P. pseudoflava</i>	GA2		85 ^b	52	
<i>P. pseudoflava</i>	9B32		100		
<i>Pseudomonas</i> sp.	9B31		69		
<i>Pseudomonas</i> sp.	GA6		62	43	
<i>Pseudomonas</i> sp.	MA30		41		
<i>Pseudomonas flava</i>	DSM 619	34	65	100	
<i>P. facilis</i>	332	15	37	18	
<i>P. saccharophila</i>	DSM 654	16	12	18	
<i>Alcaligenes paradoxus</i>	351	17	34	29	100
<i>A. paradoxus</i>	353		20		96
<i>A. paradoxus</i>	12X				76
<i>A. eutrophus</i>	H16		6	24	39
<i>A. eutrophus</i>	DSM 531				18
<i>A. faecalis</i>	DSM 30030				6

^a Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany.

^b Data from Auling et al. (2).

TABLE 3. Degree of binding (%D) of hydrogen-oxidizing bacteria of the genus *Paracoccus*

Species	Strain	%D with		
		S	M	N 4
<i>P. denitrificans</i>	S	100	54 ± 5	68 ± 10
<i>P. denitrificans</i>	M	54 ± 5	100	57 ± 4
<i>P. denitrificans</i>	V	51 ± 3	106 ± 4	57 ± 2
<i>Paracoccus</i> sp.	N4	51 ± 9	53 ± 3	100
<i>Paracoccus</i> sp.	N1	52 ± 5	52 ± 4	98 ± 5
<i>Paracoccus</i> sp.	N2	60 ± 6	52 ± 5	98 ± 7
<i>Paracoccus</i> sp.	N3	48 ± 1	64 ± 6	87 ± 2
<i>Paracoccus</i> sp.	N5	47 ± 3	68 ± 10	81 ± 3
<i>Paracoccus</i> sp.	N6	53 ± 2	58 ± 3	81 ± 4
<i>Paracoccus</i> sp.	N7	54 ± 2	62 ± 2	82 ± 2
<i>Paracoccus</i> sp.	N9	40 ± 4	63 ± 2	50 ± 2
<i>Paracoccus</i> sp.	N10	53 ± 2	61 ± 9	57 ± 5

FIG. 1. Diagrammatic representation of the relationship between 12 strains of the genus *Paracoccus*. The symbols mark the position of the strains in the diagram. Black symbols were chosen for the reference strains (▲, *P. denitrificans* S, ■, *P. denitrificans* M, ●, *Paracoccus* sp. N4). White symbols were chosen for strains with a very high degree of binding to any of the reference strains (□, strains of the *P. denitrificans* M-homology group, ○, strains of the *Paracoccus* sp. N4-homology group). The symbol T marks the position of those strains which could not be assigned to any homology group.

denitrifying strains of the genus *Paracoccus* revealed more than one DNA homology group. All the *Paracoccus* strains examined were characterized by a degree of binding ranging from at least 40 up to 100% to the three reference strains—*P. denitrificans* S, *P. denitrificans* M, and *Paracoccus* sp. N4 (Table 3). The clustering

of the *Paracoccus* strains on the basis of their percent degree of binding to the *Paracoccus* strains cited above has been illustrated graphically (Fig. 1) according to the suggestions of R. Lungershausen (Ph. D. thesis, Universität Göttingen, Göttingen, Federal Republic of Germany, 1978).

For genome size determinations, we compared the apparent renaturation rates of our DNA samples in relation to each other and in relation to *X. pelargonii* ICPB P121. Gillis et al. (9) described the haploid genome of *X. pelargonii* as having a mean G+C content of 66.5 mol% (similar to the value reported for most of the hydrogen-oxidizing strains examined) and a molecular weight of 2.4×10^9 . Therefore, no corrections for differences in the G+C content were necessary. The results of our genome size calculations, applying equation $M_1/M_2 = K'_2 K'_1$ of Gillis et al. (9), are summarized in Table 4. The average molecular weight of the genome DNA from the strains of the genus *Paracoccus* was about 3×10^9 and ranged from 3×10^9 to 4×10^9 for the strains of the genus *Pseudomonas*. The strains of the genus *Alcaligenes* were found to have a genome with the largest molecular weight (4×10^9 to 5×10^9).

DISCUSSION

The demonstration of isolated DNA homology groups within the gram-negative, hydrogen-oxidizing strains as revealed by the combined results of this and a recent study (2) gives further support to the taxonomic subdivision (5) of this group of physiologically related aerobic bacteria previously assigned to the genus *Hydrogenomonas*.

The first attempt to obtain DNA-DNA homology evidence for the abandonment of this genus was made by Ralston et al. (18), who used

TABLE 4. Average molecular weight of genome DNA from some hydrogen-oxidizing species^a

DNA source	No. of determinations	Mol wt ($\times 10^{-6}$) (Na-salt)
<i>Pseudomonas palleronii</i> 362	12	2.8
<i>P. palleronii</i> 366	6	3.1
<i>P. palleronii</i> Z32	6	2.5
<i>P. pseudoflava</i> GA3	24	3.7
<i>P. pseudoflava</i> 9B32	5	4.4
<i>P. flava</i> DSM 619	15	3.1
<i>P. facilis</i> 332	9	2.8
<i>P. saccharophila</i> DSM 654	9	3.5
<i>Alcaligenes paradoxus</i> 351	15	4.7
<i>A. paradoxus</i> 353	3	4.8
<i>A. eutrophus</i> H16	3	4.1
<i>A. eutrophus</i> DSM 531	3	5.1
<i>Paracoccus denitrificans</i> S	36	2.5
<i>P. denitrificans</i> M	36	3.0
<i>P. denitrificans</i> V	18	2.7
<i>Paracoccus</i> sp. N4	36	3.4
<i>Paracoccus</i> sp. N1	9	2.9
<i>Paracoccus</i> sp. N2	9	3.3
<i>Paracoccus</i> sp. N3	18	2.9
<i>Paracoccus</i> sp. N5	18	2.9
<i>Paracoccus</i> sp. N6	18	3.0
<i>Paracoccus</i> sp. N7	18	3.0
<i>Paracoccus</i> sp. N9	18	2.6
<i>Paracoccus</i> sp. N10	9	2.9

^a Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen, Göttingen Federal Republic of Germany.

the competition and direct-binding methods of Palleroni and Doudoroff (15). They found close relatedness of the hydrogen-oxidizing species *P. facilis* and the nonautotrophic species *Pseudomonas delafieldii* as well as no relatedness between the species *Alcaligenes eutrophus* and other autotrophic and nonautotrophic pseudomonads studied. The taxonomic concept of Davis et al. (5, 6) establishing *A. paradoxus* and *P. palleronii* (with only two strains under investigation) as separate species has been confirmed by our DNA-DNA reassociation data (see Table 2). None of the hydrogen-oxidizing strains tested in our renaturation studies was found to be identical or closely related to *P. flava* DSM 619. There does exist moderate DNA homology between *P. flava* and the still expanding species *P. pseudoflava* (see Table 2 of this study and Auling et al., 1978).

Davis et al. (5, 6) have proposed the species *Paracoccus denitrificans* for gram-negative, coccoid, denitrifying, hydrogen-oxidizing bacteria on the basis of the two or three strains examined. Differences between *P. denitrificans* S and *P. denitrificans* M with respect to cell wall composition and nutritional spectrum have

been reported by Walther-Mauruschat et al. (21). Our DNA renaturation studies with 12 autotrophic strains of the genus *Paracoccus*, including nine denitrifying bacteria newly isolated by T. Nokhal from our institute, revealed differences between the hitherto described strains as well as between the new strains, tentatively identified as *P. denitrificans* by T. Nokhal. The data presented in Table 3 and Fig. 1 show that the denitrifying, hydrogen-oxidizing strains are interrelated at various levels of DNA homology. None of the *Paracoccus* strains tested exhibited a close relationship to strain S of *P. denitrificans*, the type species of the genus. At least two clusters of very high genome-DNA relatedness were found; these were grouped around either *P. denitrificans* M or *Paracoccus* sp. N4.

Phenotypic characterization of the newly found denitrifying strains and their comparison with the previously described strains of *P. denitrificans* is currently under investigation. The taxonomic and nomenclatural implications of the results of this investigation on the genus *Paracoccus* will be reported more fully in the near future (Nokhal and Schlegel, manuscript in preparation).

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REPRINT REQUESTS

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