Aquaspirillum autotrophicum, a New Species of Hydrogen-Oxidizing, Facultatively Autotrophic Bacteria

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A facultatively autotrophic, hydrogen-oxidizing spirillum was isolated from a small eutrophic lake in Switzerland. It is medium-sized (0.6 to 0.8 μm) and possesses bipolar tufts of flagella. The guanine-plus-cytosine content of its deoxyribonucleic acid is 60 to 62 mol%. Based upon its source, morphology, and biochemical features, the organism belongs to the genus *Aquaspirillum* Hylemon et al. 1973. Because this organism does not closely resemble any of the previously described medium-sized aquaspirilla, it is proposed here as belonging to a new species, *Aquaspirillum autotrophicum*. The specific epithet refers to the outstanding property of this organism to grow on hydrogen and carbon dioxide as energy and carbon sources, respectively. The type strain of *A. autotrophicum*, strain SA 32, was deposited with the Deutsche Sammlung von Mikroorganismen under the number DSM 732.

The facultatively chemolithoautotrophic, hydrogen-oxidizing bacteria were, until recently, placed in the genus *Hydrogenomonas* Orla-Jensen 1909. The discovery of "hydrogen" bacteria that clearly belonged to other genera, such as *Paracoccus* (4), *Nocardia* (1, 6), *Mycobacterium* (10, 12), or *Corynebacterium* (2), emphasized the taxonomic heterogeneity of this physiological group of organisms (14). Moreover, Davis et al. (3) showed that there were differences in the flagellation of the species previously placed in the genus *Hydrogenomonas*. They proposed, therefore, to reject the genus *Hydrogenomonas* and to place the peritrichous organisms in the genus *Alcaligenes* and the polarly flagellated species in the genus *Pseudomonas*.

From the water of a eutrophic lake, we recently isolated two strains of a spirillum able to grow autotrophically in a mineral medium under an atmosphere of $\text{H}_2\text{-O}_2\text{-CO}_2$. This finding strongly confirmed the observation that the hydrogen bacteria are taxonomically diverse. The present report is concerned with the characterization and taxonomy of this spirillum.

MATERIALS AND METHODS

Bacterial strains. The two spirillar strains of hydrogen bacteria studied, SA 32 and SA 33, were isolated at the Institute of Botany, University of Neuchâtel, Switzerland, by C. Schweizer and M. Aragno from water samples of a small eutrophic lake, Le Loclat, near St. Blaise (canton of Neuchâtel). The isolation of these bacteria was as previously described (16): water samples were filtered on membrane filters, and the filters were deposited on mineral agar; incu-

bation was under an atmosphere of 60% H_2 -30% air-10% CO_2 at 30°C. Pure cultures were obtained by repeated subculturing on mineral agar plates incubated under a hydrogen-air-carbon dioxide mixture. Purity of the cultures was determined by plating them on different organic media and by observing them microscopically.

Media. The basal mineral medium used contained: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 9.0 g; KH_2PO_4 , 1.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; NH_4Cl , 1.0 g; ferric-ammonium citrate, 0.005 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g; trace-elements solution SL 6 (solution SL 4 of Pfennig und Lippert [13] minus ethylenediaminetetraacetate and iron salt), 3.0 ml; double-distilled water, 1,000 ml (15). The final pH was about 7.1. For autotrophic growth, 0.05% (wt/vol) NaHCO_3 was added, and, unless otherwise stated, cultures were incubated under 5% O_2 -10% CO_2 -85% H_2 at 30°C. If required, 1.7% (wt/vol) agar (Difco) was added. Stock cultures were maintained autotrophically on mineral agar slants and were transferred to fresh medium every 2 months.

Methods. The utilization of compounds as sole carbon sources was tested on agar plates as described by Stanier et al. (17); the medium used was the basal mineral medium plus 0.2% carbohydrate, 0.01% phenol, or 0.1% of one of a number of other carbon compounds. The control lacked any carbon source. The utilization of compounds as sole nitrogen sources was tested in the same manner as with the carbon sources; however, the medium used was the basal mineral medium without NH_4Cl but with 0.1% (wt/vol) succinate plus 0.1% (wt/vol) of the nitrogen compound examined. Cleavage of *p*-hydroxybenzoate was tested by the method of Holding and Collee (7). Other characteristics—cell morphology (with phase microscopy); nitrate reduction; phosphatase and sulfatase production; sulfide from cysteine; catalase, oxidase, and urease production; gelatin, casein, starch, and esculin hydrolysis; indole production; acid formation from carbohydrates; selenite reduction; and

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growth with 3% NaCl, 1% bile, or 1% glycine—were studied by the methods of Hylemon et al. (8). Intracellular poly- β -hydroxybutyrate was recognized by its typical appearance in electron micrographs (A. Walther-Mauruschat et al., in preparation) and confirmed by its solubility in chloroform and insolubility in ether.

The guanine-plus-cytosine (G+C) content of the deoxyribonucleic acid (DNA) was determined by thermal denaturation (T_m) in 0.5 M sodium phosphate buffer plus 25% (vol/vol) formamide, a modification of the method described by Mandel and Marmur (11). T_m values were determined at 270 nm with a Gilford spectrophotometer 240. DNA hybridization was determined by optically following renaturation (T_r) at $T_r = T_m - 25^\circ\text{C}$. The degree of binding was calculated according to the formula of DeLey et al. (5).

Electron microscopy. Negative staining was performed with log-phase cells grown on succinate and with either 2% uranylacetate or 2% potassium phosphotungstate as the negative stain. Observations were made with a Phillips EM 301 electron microscope.

Growth. Growth, either autotrophic or heterotrophic, was followed turbidimetrically by measuring the optical density of a suspension at 436 nm, in 1-cm cuvettes, with a Zeiss PL 4 photometer.

RESULTS

From each of two water samples taken during February 1974 from Lake Le Loclat, one at a 3-m depth and the other at a 7-m depth, a spirillum was isolated that was able to grow aerobically under a hydrogen-oxygen-carbon dioxide mixture. A description of the two strains, which were nearly identical in their morphological and biochemical features, is presented below.

The cells are curved to helical with a clockwise orientation of the spirals. The dimensions, determined by phase microscopy in 18-h-old PSS broth cultures (8) at 30°C , are as follows: cell diameter, 0.6 to 0.8 μm ; cell length, 2.0 to 5.0 μm ; wave length, 3.0 to 4.0 μm . Longer cells (up to 50 μm) were obtained with late-log-phase cultures in gluconate (0.2%, wt/vol)-mineral medium. At pH 6.5 and above, the cells were motile

by means of one to five flagella at each pole (Fig. 1). The Gram reaction was negative. Coccioid bodies were not observed in 3-week-old PSS broth cultures. Intracellular poly- β -hydroxybutyrate granules were present. Colonies on succinate-mineral agar were cream-colored, translucent, up to 3 mm in diameter, circular, and convex.

The growth rate was optimal in succinate-mineral medium at 28°C . There was detectable growth at 10 and 35°C but no growth at 37°C . The pH for optimum growth was between 5 and 8; however, motility was strongly diminished below pH 6.5.

The biochemical properties of the two strains are summarized in Table 1.

The results of the utilization by the two strains

TABLE 1. Biochemical characteristics and penicillin susceptibility of the "hydrogen spirillum"

Test	Result
Nitrate reduction	—
Anaerobic growth with nitrate	—
Phosphatase	+
Sulfatase	—
Oxidase	+
Catalase	+
Urease	+
Protocatechuate cleavage in <i>p</i> -hydroxybenzoate metabolism	ortho
Hydrolysis of gelatin	—
Hydrolysis of casein	—
Hydrolysis of starch	—
Hydrolysis of esculin	—
Production of water-soluble fluorescent pigment	—
Indole formation	—
H ₂ S from cysteine	—
Acids from carbohydrates	—
Growth in the presence of 1% bile salts	+
Growth in the presence of 1% glycine	—
Growth in the presence of 3% NaCl	—
Selenite reduction	—
Minimal inhibitory concn of penicillin G	60 IU/ml

FIG. 1. Electron micrograph of a cell of strain SA 32, negatively stained with uranylacetate. Bar represents 1 μm .

TABLE 2. *Results of sole carbon source utilization tests with strains SA 32 and SA33, using NH₄Cl as the nitrogen source*

Sole carbon source	Utilization ^a	Sole carbon source	Utilization ^a
Organic acids		Amino acids—continued	
Acetate	+	L-Lysine	—
<i>cis</i> -Aconitate	+	L-Methionine	—
Anthranilate	—	L-Ornithine	—
Benzoate	—	L-Phenylalanine	+
Butyrate	+	L-Proline	+
Caproate	—	L-Serine	—
Citrate	+	L-Tryptophan	+
Fumarate	+	L-Tyrosine	+ (slowly)
Glycolate	+	L-Valine	—
<i>p</i> -Hydroxybenzoate	+	Sugar acids	
DL- β -Hydroxybutyrate	+	D-Gluconate	+
Poly- β -hydroxybutyrate (exogenous)	—	α -Ketogluconate	+
DL-Isocitrate	+	Sugars	
α -Ketoglutarate	+	L-Arabinose	—
DL-Lactate	+	Cellobiose	—
L-Malate	+	D-Fructose	—
Malonate	+	D-Fucose	—
Mesaconate	+	D-Galactose	—
Oxaloacetate	+	D-Glucose	—
Propionate	+	Maltose	—
Pyruvate	+	D-Mannose	—
Succinate	+	L-Rhamnose	—
D-(-)-Tartrate	+	Saccharose	—
L-(+)-Tartrate	—	Trehalose	—
<i>meso</i> -Tartrate	—	D-Xylose	—
Amino acids		Alcohols	
L-Alanine	+	<i>n</i> -Butanol	—
L-Arginine	—	<i>t</i> -Butanol	—
L-Asparagine	+	Ethanol	—
L-Aspartate	+	Glycerine	—
L-Citrulline	—	Mannitol	—
L-Cysteine	—	Methanol	—
L-Glutamate	+	Phenol	—
L-Glutamine	+	<i>n</i> -Propanol	—
Glycine	+	Other substances	
L-Histidine	—	Allantoin	—
L-Isoleucine	+	Ethanolamine	+
L-Leucine	—		(slowly)

^a +, Utilized; —, not utilized.

of various compounds as sole carbon sources are summarized in Table 2; 72 compounds were tested, and NH₄Cl was the nitrogen source. Among the compounds utilized were two sugar acids, 20 of 26 organic acids, and 11 of 21 amino acids. No growth occurred with sugars or alcohols. The best growth rates were obtained with succinate (generation time, 1.9 h), L-malate, and D-gluconate (generation time, 2.0 h). Ammonium salts, nitrates, asparagine, and urea were used as sole nitrogen sources, either for autotrophic growth or for growth with 0.1% succinate.

Autotrophic growth was microaerophilic. At low cell concentrations, the optimal oxygen concentration in the gas mixture was about 3%,

corresponding to about 1 mg of dissolved oxygen per liter. A generation time of 4 h was obtained under autotrophic conditions in a 10-liter Biostat fermentor (Braun, Melsungen), with adaptation of the oxygen supply to increasing cell concentration. CO₂ was fixed through the Calvin cycle; hydrogenase and ribulose diphosphate carboxylase were entirely inducible under autotrophic conditions; they were not detectable in heterotrophic cells grown in air (M. Aragno and H. G. Schlegel, in preparation). The hydrogenase was membrane bound only, and it failed to reduce either nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate.

The G+C content of the DNA was 61.7 mol% for strain SA 32 and 60.2 mol% for strain SA

33. The results of the DNA hybridization tests between these two strains and *Aquaspirillum dispar* strains are given in Table 3.

DISCUSSION

Apart from H₂ autotrophy, the other characteristics of strains SA 32 and SA 33, as well as their habitat, fit with the description of the genus *Aquaspirillum* as defined by Hylemon et al. (8). Since H₂ autotrophy is widespread among various bacteria, it is not a useful criterion for separation of taxa at the generic level.

However, there are distinct differences between strains SA 32 and SA 33 and other medium-sized spirilla (Table 4). These differences lie in a combination of features rather than in single unique features, although a number of

unique features such as H₂ autotrophy and utilization of D-tartrate, *p*-hydroxybenzoate, tryptophan, and glycine do exist in these organisms. A dendrogrammatic plot of the relationships between the hydrogen spirilla and the other medium-sized spirilla, drawn from a similarity matrix (Jaccard coefficient) of data from Hylemon et al. (8), Kropinski (9), and the present work, is shown in Fig. 2. The separation between autotrophic spirilla and the other spirilla occurs at a level similar to that of other species between themselves. Furthermore, DNA-DNA hybridization assays between the hydrogen spirilla and *Aquaspirillum dispar* (previously thought to resemble the former) show only few, if any, similarities between the two organisms. Thus, we believe that the hydrogen spirilla represent a new species, for which we propose the name

TABLE 3. DNA-DNA hybridization between the "hydrogen spirillum" and *Aquaspirillum dispar* strains

Source of DNA	Degree of DNA binding ^a with strain:	
	SA 32	ATCC 27510
SA 32	100	28
SA 33	78	ND ^b
<i>Aquaspirillum dispar</i> ATCC 27510	28	100
<i>A. dispar</i> ATCC 27650	8	106

^a Reference 5.

^b ND, Not determined.

FIG. 2. Dendrogram showing the arrangement of medium-sized strains of *Aquaspirillum* after single-linkage clustering. Similarities were determined using the Jaccard coefficient S_j .

TABLE 4. Characters useful in differentiating between strains SA 32 and SA 33 and other medium-sized aquaspirilla

Character	SA 32 and SA 33	<i>A. dispar</i>	<i>A. aquaticum</i>	<i>A. itersonii</i>	<i>A. peregrinum</i>
Coccoid bodies in 3-week-old cultures	—	—	—	+	+
Nitrate reduction	—	+ (N ₂) ^a	+ (NO ₂ ⁻)	+ (N ₂)	—
Anaerobic growth with nitrate as acceptor	—	+ ^a	—	+	—
Water-soluble pigment	—	+	+	+	+
Sulfide from cysteine	—	+	+	+	+
Urease	+	—	—	—	+
Esculin hydrolysis	—	—	—	+	+
Acid from carbohydrates	—	—	—	+	+
Selenite reduction	—	+	+	—	+
Growth with 1% glycine	—	+	+	—	—
Auxotrophy	—	—	+	—	—
Sole-carbon-source utilization					
Gluconate	+	—	+	—	—
Citrate	+	+	+	—	—
D-(-)Tartrate	+	—	—	—	—
Caproate	—	+	+	—	—
<i>p</i> -Hydroxybenzoate	+	—	—	—	—
Glycine	+	—	—	—	—
Tryptophan	+	—	—	—	—
Ethanol	—	+	—	+	—
NO ₃ ⁻ as sole N source	+	—	+	+	—
Mol% G+C of DNA	60–62	63–65	65	62	62

^a These features differ from those given by Hylemon et al. (8), but we have verified that *A. dispar* is a denitrifier and grows vigorously anaerobically with nitrate as the electron acceptor, producing gaseous nitrogen.

Aquaspirillum autotrophicum. A description of this species follows.

Aquaspirillum autotrophicum sp. nov. (au.to.tro'phi.cum. Gr. pronoun *autos* self; Gr. adj. *trophikos* nursing, tending, or feeding; M.L. adj. *autotrophicus* self-nursing or self-feeding, i.e., able to grow with carbon dioxide as a sole carbon source).

Curved to spiral cells, 0.6 to 0.8 μm wide, 2 to 5 μm long (older cells may be up to 50); the wavelength is 3 to 4 μm ; the helix is oriented in a clockwise fashion. Gram-negative. Motile by means of bipolar fascicles of one to five flagella. Intracytoplasmic granules of poly- β -hydroxybutyrate are present. No coccoid bodies occur in older cultures. Temperature for optimal growth: 28°C; slight growth occurs at 10 and at 35°C. Either chemolithoautotrophic, using H_2 as the electron donor and CO_2 as the carbon source, or chemoorganoheterotrophic; strictly respiratory metabolism, with oxygen as the terminal electron acceptor. Cannot grow anaerobically with nitrate. Hydrogenase is induced by molecular hydrogen, is membrane bound, and is not nicotinamide adenine dinucleotide specific. CO_2 is fixed autotrophically through the Calvin cycle. Catalase, phosphatase, and urease are positive. Sulfatase is not produced. *p*-Hydroxybutyrate is metabolized via the *ortho* cleavage of protocatechuate. Does not hydrolyze starch, casein, gelatin, esculin, or extracellular poly- β -hydroxybutyrate. No acid is produced from sugars. Selenite is not reduced. H_2S is not produced from cysteine. Grows in the presence of 1% bile salts. No growth occurs in the presence of either 1% glycine or 3% NaCl. Water-soluble fluorescent pigment is not produced. Nitrate is reduced but only for assimilation. A wide range of organic acids, amino acids, and sugar acids can serve as sole carbon sources with ammonium ions as a nitrogen source. Sugars and alcohols are not metabolized. Ammonium ions, nitrates, urea, and asparagine can be utilized as sole nitrogen sources. The G+C content of the DNA is 60 to 62 mol% (T_m).

Source: Isolated from the water of a small eutrophic lake near Neuchâtel, Switzerland.

Type strain: Strain SA 32 is designated as the type strain for the species. It has been deposited with the Deutsche Sammlung von Mikroorganismen under the number DSM 732. The description of the type strain is the same as that given above for the species, with the following addition: the G+C content of the DNA is 61.7 mol% (T_m).

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