Alcaligenes latus, a New Species of Hydrogen-Utilizing Bacteria

NORBERTO J. PALLERONI¹ AND ALICIA V. PALLERONI[†]

Department of Chemical Research, Hoffmann-La Roche Inc., Nutley, New Jersey 07110,¹ and Department of Zoology, University of California, Berkeley, California 94720

Three strains of hydrogen-utilizing bacteria were found to constitute a new species which we propose to assign to the genus *Alcaligenes* with the name *Alcaligenes latus* sp. nov. The phenotypic properties of this species are described, and a minor modification of the definition of *Alcaligenes* is proposed. The type strain is *A. latus* strain H-4 (=ATCC 29712).

The gram-negative bacteria that are capable of autotrophic growth by the use of molecular hydrogen as an energy source are currently assigned to the genus *Pseudomonas*, *Alcaligenes*, *Paracoccus*, *Corynebacterium*, or *Aquaspirillum*. In this report, the isolation of a hydrogen-utilizing bacterium and its characterization as a new species of *Alcaligenes*, *Alcaligenes latus* sp. nov., are described.

MATERIALS AND METHODS

Bacterial strains. The two strains (H-1 and H-4) of the new organism to be described in detail below were isolated from soil samples collected from two widely separated sites of the Berkeley campus of the University of California. Strains similar to H-1 and H-4 were repeatedly isolated in subsequent experiments from samples collected at the two sites. At a later date, a third strain (H-N) was obtained from a sample collected at Orara Reserve, Manly Vale, Australia. This soil sample was sent to us by J. Baker. A small portion of the soil was heated in an oven at 120°C for 1 h following the procedure of Nonomura and Ohara (4) for the isolation of *Microbispora* and *Streptosporangium* strains. A fraction of the heated sample was used for an enrichment culture for hydrogen bacteria, and from the resulting mixed culture, strain H-N was isolated. This strain of the species is not described below since it was not fully characterized, but it possesses many of the basic properties of the new species.

Culture media. The basal mineral medium described by Palleroni and Doudoroff (5) and used in this study for enrichment and growth under both autotrophic and heterotrophic conditions has the following composition: Na-K phosphate buffer, pH 6.8, 0.033 M; NH_4Cl , 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; ferric ammonium citrate, 0.005%; and CaCl_2 , 0.0005%. For heterotrophic growth, various carbon sources were added to a final concentration of 0.1%. The medium was used as a liquid or was solidified with 1.8% of purified agar (Difco).

Enrichment, isolation, and autotrophic cultivation. For enrichments, the liquid mineral medium

was dispensed in 10-ml portions into 50-ml Erlenmeyer flasks and then sterilized. After inoculation with small portions of soil samples, the flasks were placed in a desiccator in which the air was then replaced with an atmosphere of 50% hydrogen, 8% oxygen, 5% carbon dioxide, and 37% nitrogen. After incubation for 2 or 3 days at 30°C, at which time growth was noticeable in most of the flasks, the mixed culture in each flask was streaked onto plates of solid mineral medium. The plates were incubated under the autotrophic conditions described above at 30°C, and colonies could be picked after 4 or 5 days of incubation. The cultures were purified by restreaking.

Methods for study of phenotypic properties. The basic morphological properties of the strains, Gram staining, temperature relationships, oxidase reaction, catalase production, relationship to oxygen, denitrification, nitrate reduction, and gelatinase production were determined following previously described conventional bacteriological techniques (7-9). For the study of the nutritional spectrum, the solid mineral medium was supplemented with each of 141 organic carbon compounds tested for use by the strains as sole carbon and energy sources. These substrates were added as filter-sterilized solutions to give a final concentration of 0.1%. Exceptions were geraniol and naphthalene, which were not dissolved in the medium but instead were placed in the lid of the petri dish. Phenol and benzoate were tested as substrates at three concentrations: 0.1, 0.05, and 0.025%. The plates with different media were inoculated with drops of washed suspensions of cells grown under autotrophic conditions. A drop of each suspension, properly diluted to give a turbidity barely visible to the naked eye, was deposited on the surface of each plate containing a given carbon source. The plates were inverted after the liquid of the drops had been absorbed by the agar and were incubated at 30°C. Positive results could be recorded in 2 or 3 days, but the plates were kept in the incubator considerably longer and were observed periodically for late growth. The density of growth was judged visually by comparison with that obtained on a plate containing no carbon substrate. Growth on this control plate was almost negligible. For determining the mechanisms of cleavage of dihydroxylated intermediates in the metabolism of aromatic compounds, the method recommended by Stanier et al. (9) was

[†] Present address: Hoffmann-LaRoche Inc., Nutley, NJ 07110.

followed. Suspensions of cells grown on the appropriate inducing substrates were toluenized and incubated with either catechol or protocatechuate at final concentrations of 1, 2, 5, 10, and 25 mM. The reactions appeared particularly clear at the first three concentrations. Gentisate oxygenase was assayed by the method described by Wheelis et al. (10). Oxygen uptake was followed manometrically in a conventional Warburg apparatus at 37°C.

Electron microscopy. Cultures in liquid media were centrifuged, and the resulting pellet was washed once and then suspended in 0.5% ammonium acetate in 2% sucrose. Twenty-microliter volumes of the suspensions were spotted on carbon-stabilized Formvar-coated grids (200 to 300 mesh size), allowed to settle for about 30 min, and then stained with 1% phosphotungstic acid. Excess liquid was removed with filter paper, and the grids were allowed to air dry. The grids were examined in a Zeiss MS9 electron microscope.

RESULTS

Enrichment-isolation of the strains. In the three enrichment cultures that yielded the new strains, faster-growing hydrogen bacteria were always present, as were various heterotrophs. Therefore, it was found convenient to streak the culture on agar plates as soon as the cells of the new organism, which are very characteristic, were present in the mixed culture and to examine the plates carefully under the dissecting microscope as soon as the colonies were visible. As described below, the colonies of the new organism are also very characteristic, and they can be easily identified among those of other hydrogen bacteria. Transfer of a loopful of the liquid enrichment culture to a second flask of liquid medium occasionally resulted in the disappearance of the new organism and the predominance of faster growing autotrophs.

Phenotypic characterization of the new strains: morphology. The colonies of freshly isolated strains were wrinkled, and various surface patterns were observed on mineral agar after incubation under autotrophic conditions (Fig. 1). The colonies were rather coherent, but they broke readily into pieces when picked with the loop; however, the cells remained aggregated into large clumps and did not suspend easily in liquid. Upon cultivation under heterotrophic conditions for a number of transfers, this "rough" behavior was sometimes lost, and a smooth variant of circular, creamy colonies predominated in the population. Liquid cultures in mineral medium with glucose were frequently clumpy whereas those grown on other carbon sources (for instance, *m*-hydroxybenzoate) were uniformly turbid. The color of the colonies was grayish pink, but that of the Australian isolate H-N was yellowish. Methanol extracts of pellets of cells of strain H-N did not have the absorption

spectrum characteristic of carotenoids in the visible region.

The cells were short, almost coccoid rods (Fig. 2) with average dimensions of 1.1 by 1.6 to 2.4 μ m for strain H-1 and of 1.3 to 1.4 by 1.9 to 2.4 μ m for strain H-4. The cells were gram negative, nonsporulating, peritrichous (Fig. 3), and rather sluggishly motile. They accumulated the carbon reserve material poly-beta-hydroxybutyrate under both autotrophic and heterotrophic conditions, particularly in nitrogen-deficient media. When the cells were suspended in salt solutions of certain concentrations, plasmolysis was practically instantaneous and was easily observed under the microscope (Fig. 4). Plasmolysis with KCl was apparent at concentrations of 0.07 M or higher. The proportion of plasmolyzed cells increased with the salt concentration and was close to 100% with 0.1 M KCl. When the suspension in 0.1 M KCl was diluted 1:1 with water, the cells immediately took on their normal appearance. These changes did not reduce the viability of the cells.

Cells grown for 24 h at 30°C in mineral medium to which 0.5% of yeast extract had been added appeared large and heavily granulated (Fig. 5). Large cells similar to the so-called "involution forms" of other species sometimes appeared in old cultures grown under heterotrophic conditions at the expense of a single organic carbon source.

The deoxyribonucleic acid of strain H-4 was analyzed by M. Mandel, and the guanine-plus-cytosine content was found to be 69.4 mol%, as estimated from measurements of the buoyant density in CsCl gradient.

Physiological properties. The salient physiological properties of the new strains are the following. The strains are absolute aerobes since they were incapable of growth under anaerobic conditions. Growth in anaerobiosis in the presence of nitrate was also negative. The oxidase and catalase reactions were positive. No gas was produced from nitrate, but there was reduction of nitrate to nitrite. Gelatin was liquefied. Starch was slowly hydrolyzed. The optimal temperature for growth was around 35°C. Growth occurred at 40°C but not at 43°C. The doubling time of strain H-1 in mineral medium supplemented with 0.2% glucose in a 37°C bath with rotatory shaking at 280 rpm was about 1.2 h (μ : 0.57 h⁻¹). There was good growth under autotrophic conditions in the mineral medium in contact with an atmosphere containing 8% oxygen. Growth was severely inhibited when an atmosphere with 20% oxygen was used instead, but spontaneous mutants insensitive to high oxygen concentrations were readily obtained.

FIG. 1. Colonies of various subcultures of strain H-4. Solid mineral medium, incubation at 30°C under autotrophic conditions for 1 week, except for lower right, which have been incubated for 2 weeks. Some of the rough colonies at lower left have smooth outgrowths at the edges. Magnification, $\times 10$.

Cultures of the strains were preserved by vacuum drying cell suspensions in water without freezing prior to the evacuation of the vials. Dried cells of strains H-1 and H-4, prepared

from cultures which had grown under autotrophic conditions, or with glucose or *m*-hydroxybenzoate as carbon sources, were assayed for viability after 1 year of storage at room temper-

FIG. 2. Cells of strain H-4. Phase contrast. Magnification, $\times 3,000$.

ature. For reactivation of the cells, the same growth conditions as those used prior to drying were used. The autotrophically grown cells revived normally under autotrophic conditions, but both of the dry samples from the glucose and the *m*-hydroxybenzoate media appeared to be dead. Further experiments showed, however, that this was probably not the case, since cells from other vials of these batches could be reactivated by inoculation into mineral medium and incubation under autotrophic conditions. In other words, reactivation of the dried cells was ineffective under heterotrophic conditions but was perfectly normal under autotrophic conditions irrespective of the carbon source used for the growth of the cells before drying. This observation is puzzling in view of the fact that the hydrogenase system is inducible and seems to be almost totally inactive in glucose-grown cells.

Cells of the three strains grown heterotrophically with glucose, and suspended either in skim milk or in sucrose solution, were subjected to freeze-drying at the American Type Culture Collection. Samples of these batches sent to us by the American Type Culture Collection could be reactivated in glucose medium after 1 month of storage. Reactivation in mineral medium with

an atmosphere of hydrogen, carbon dioxide, and oxygen was also normal, but in this case the conditions of reactivation were not truly autotrophic due to the presence of organic carbon substrates (sucrose or skim milk solutes) in the fluid in which the cells had been suspended prior to freeze-drying.

Nutritional characters. Either ammonium or nitrate, but not molecular nitrogen, was used by the strains as a nitrogen source. A total of 141 organic compounds were tested as carbon and energy sources for the growth of strains H-1 and H-4. The results can be summarized as follows.

Both strains grew on D-glucose, D-fructose, sucrose, maltose, starch, gluconate, 2-ketogluconate, mucate, formate, butyrate, isobutyrate, malonate, succinate, fumarate, suberate, lactate, DL-glycerate, beta-hydroxybutyrate, L-malate, D(-)-tartrate, aconitate, itaconate, *n*-propanol, isopropanol, 2,3-butylene glycol, propylene glycol, glycerol, quinate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, gentisate, protocatechuate, phenylacetate, L-mandelate, beta-alanine, L-alanine, D-alanine, L-serine, L-threonine, L-leucine, L-citrulline, L-ornithine, gamma-aminobutyrate, L-aspartate, L-asparagine, L-glutamate, L-pro-

FIG. 3. Cell of strain H-4. Electron micrograph; phosphotungstic staining. Magnification, $\times 30,000$.

line, butylamine, hippurate, betaine, sarcosine, and creatine. Growth of these strains on starch, formate, isopropanol, beta-alanine, and L-citrulline was extremely poor. Resting cells of both strains were capable of respiring formate rather actively, and good growth on this substrate could probably be obtained under carefully controlled conditions. Although both strains used the following substrates, growth of H-1 on isobutyrate, malonate, *n*-propanol, 2,3-butylene glycol, and propylene glycol and of H-4 on L-malate and

itaconate was poor. The following substrates did not support growth of either strain: D-ribose, L-arabinose, D-arabinose, D-xylose, L-rhamnose, L-fucose, D-fucose, D-galactose, D-mannose, L-sorbose, cellobiose, lactose, salicin, raffinose, acetate valerate, isovalerate, caproate, heptanoate, caprylate, pelargonate, caprate, *m*-tartrate, oxalate, maleate, glutarate, adipate, pimelate, D-malate, pyruvate, alpha-ketoglutarate, mesaconate, citraconate, levulinate, methanol, geraniol, erythritol, adonitol, inositol, mannitol,

FIG. 4. Cells of strain H-4 plasmolyzed by suspension in 0.1 M KCl solution. Phase contrast. Magnification, $\times 4,600$.

FIG. 5. Cells of strain H-4 after growth overnight in mineral medium with the addition of 0.5% of yeast extract. Magnification, $\times 3,000$.

dulcitol, sorbitol, *o*-hydroxybenzoate, D-mandelate, benzoate, phenol, naphthalene, phthalate, glycine, DL- α -aminobutyrate, L-norvaline, delta-aminovalerate, L-norleucine, L-arginine, L-lysine, L-histidine, L-phenylalanine, L-tryptophane, D-tryptophane, kynurenate, putrescine, histamine, methylamine, alpha-amylamine, acetamide, nicotinate, trigonelline, testosterone, methane, heptane, nonane, decane, and dodecane.

Growth on hippurate but not on its components, benzoate and glycine, may be due either to permeability problems or to toxicity at the concentration used in the medium. Cells of strains H-4 were grown on hippurate and were found to respire this compound at a concentration of 0.009 M with an oxygen quotient [$Q(O_2)$] of 246. When used at the same concentration, benzoate was respired by the same cells with a $Q(O_2)$ of 195, and the rate of respiration was faster [$Q(O_2)$ of 288] with half this benzoate concentration. Glucose-grown cells were unable to respire benzoate above the level of the endogenous respiration. Thus, very likely, benzoate is an intermediate in the pathway of hippurate degradation. A case similar to that of hippurate is posed by L-mandelate utilization. As in the case of hippurate, L-mandelate-grown cells are induced to the respiration of benzoate which, likewise, may be an intermediate in the respiration of the substrate. Benzoate was tested as a substrate in solid medium at concentrations of 0.1, 0.05, and 0.025%, with negative results. Since these concentrations are close to those used in the manometric experiments, where active respiration was observed, exogenous benzoate somehow appears to be inhibitory to cellular biosynthetic processes.

Substrates used by only one strain are listed in Table 1.

Mechanisms of cleavage of dihydroxylated intermediates. Manometric experiments performed with cells grown on various aromatic substrates were carried out in the presence of chloramphenicol (50 μ g/ml of cell suspension) to prevent further induction. The results of these experiments can be summarized as follows.

(i) Cells grown on hippurate or L-mandelate are induced to the respiration of benzoate.

(ii) Cells grown on *p*-hydroxybenzoate respire protocatechuate at a faster rate than glucose-grown cells.

(iii) Of four possible dihydroxylated intermediates (gentisate, protocatechuate, 2,3-dihydroxybenzoate, and 3,5-dihydroxybenzoate), only gentisate is respired at a rate significantly above the rate of endogenous respiration by *m*-hydroxybenzoate-grown cells. However, this rate is at

TABLE 1. *Organic substrates utilized by one strain or the other of Alcaligenes latus but not by both*

Organic substrate	Utilization by strain:	
	H-1	H-4
Trehalose	—	+
Saccharate	+	—
Propionate	+	—
Sebacate	—	+
Glycolate	+	—
L-(+)-Tartrate	+	—
Citrate	—	+
Ethanol	—	+
<i>n</i> -Butanol	—	+
Isobutanol	—	+
Ethylene glycol	+	—
Xylitol	—	±
D-Arabitol	—	+
L-Isoleucine	+	—
L-Tyrosine	+	—

most only one-third of the rate at which these cells respire *m*-hydroxybenzoate [$Q(O_2)$: 300 to 400 for *m*-hydroxybenzoate]. Gentisate-grown cells respire *m*-hydroxybenzoate at approximately the same rate as gentisate. These results suggest the operation of the gentisate pathway for *m*-hydroxybenzoate degradation.

(iv) Protocatechuate-grown cells appear to be uninduced for *p*-hydroxybenzoate respiration.

The above-mentioned observations received confirmation through the application of the colorimetric test for cleavage mechanisms described by Stanier et al. (9) and of enzymatic assays performed on cell-free extracts. The results of these tests were as follows.

(i) Cells grown on hippurate or L-mandelate carry out an *ortho* cleavage of catechol. No action on protocatechuate is apparent.

(ii) Cells grown on *p*-hydroxybenzoate or on quinate catalyze a *meta* cleavage of protocatechuate and do not give a color reaction with catechol.

(iii) Cells grown on *m*-hydroxybenzoate do not give any color reaction with protocatechuate or catechol. These negative color reactions, taken together with the results of the manometric experiments, supported the hypothesis that *m*-hydroxybenzoate is metabolized through the gentisate pathway. This idea received further confirmation from the assay of gentisate oxygenase by the method of Wheelis et al. (10). Cell-free extracts of strains H-1 or H-4 readily convert gentisate to maleylpyruvate, which can be subsequently isomerized to fumarylpyruvate by the addition of reduced glutathione to the reaction mixture.

In summary, the results strongly suggest that endogenously generated benzoate is degraded by these strains through the beta-ketoadipate path-

way. The metabolism of *p*-hydroxybenzoate does not follow this pathway in view of the demonstrated *meta* cleavage of protocatechuate. *m*-Hydroxybenzoate is degraded following the gentisate pathway. The evidence presented in favor of the operation of these various pathways may however be considered as preliminary and should be confirmed by more detailed enzymatic analyses.

Observations on hydrogen oxidation. No efforts were made to obtain cells in the log phase of growth under autotrophic conditions, and therefore the data given below may not represent the maximum capacity for hydrogen oxidation. Cells grown in flasks of liquid mineral medium in a desiccator, under an atmosphere of H₂, O₂ (8%), CO₂, and N₂, without agitation, were harvested in the stationary phase and washed, and the oxidation of hydrogen was followed in Warburg flasks at 37°C. In contact with an atmosphere of 90% hydrogen and 10% oxygen, the total uptake was 690 μ l of gas per h per mg (dry weight). However, when the gas mixture was composed of 80% hydrogen, 10% oxygen, and 10% carbon dioxide, the uptake increased to 975 μ l per h per mg (dry weight). Since the increment was larger than the autotrophic uptake of carbon dioxide, it was concluded that this new organism gives a positive "Bartha effect" (2, 6), although the effect is very moderate when compared with that of other species of the genus *Alcaligenes* (e.g., *A. eutrophus* and *A. ruhlandii*). Autotrophically grown cells oxidize glucose very poorly [Q(O₂): 37] at a concentration of 0.009 M. On the other hand, glucose-grown cells oxidize glucose more actively [Q(O₂): 132], and the oxidation of hydrogen either in the presence or absence of carbon dioxide is negligible.

Experiments performed in H. G. Schlegel's laboratory have shown that *A. latus* grows with a doubling time of about 5 h at 30°C under autotrophic conditions, with a gas atmosphere of 80% hydrogen, 10% oxygen, and 10% carbon dioxide. In addition, it was found that the three strains contain an active membrane-bound hydrogenase which reduces methylene blue. No nicotinamide adenine dinucleotide-reducing soluble hydrogenase has been detected, an important difference with *A. eutrophus* and *A. ruhlandii*, where both soluble and particulate hydrogenase activities can be demonstrated (H. G. Schlegel, personal communication).

DISCUSSION

None of the known gram-negative hydrogen-utilizing bacteria so far described in the literature resemble the strains whose properties are presented in detail in this communication. Based on the basic phenotypic characters of the new

strains, we suggest the assignment of these strains to the genus *Alcaligenes* because their characters fit almost perfectly the generic definition presented by Holding and Shewan (3) in the 8th edition of *Bergey's Manual of Determinative Bacteriology*. The only strain characteristics in disagreement with the generic description are the thickness of the cells, which in the new bacterium appears to reach up to 1.4 μ m, and the number of flagella, which is normally higher than 8. Therefore, we suggest that the definition of *Alcaligenes* be modified to accommodate these strains, which we regard as belonging to a new species for which we propose the name *Alcaligenes latus* (la'tus. L. adj. *latus* broad) because of the plump appearance of the cells under the microscope. A formal description of the species follows.

Alcaligenes latus sp. nov. The cells are short to coccoid, gram-negative rods which measure 1.1 to 1.4 by 1.6 to 2.4 μ m and which occur singly, in pairs, or in short chains. The cells are motile by means of 5 to 10 flagella arranged in peritrichous fashion. No resting stages are known. Colonies are round, grayish, and opaque; they are wrinkled in fresh isolates but become smooth upon subcultivation.

Metabolism is respiratory, and molecular oxygen is the final electron acceptor. Strictly aerobic. Oxidase positive. Nitrates are reduced to nitrites, but denitrification does not occur. Gelatin is liquefied; starch is hydrolyzed, although poorly. Temperature for optimum growth: around 35°C. At least 67 different organic compounds are utilized as sole carbon and energy sources for growth; these include sugars, acids, alcohols, aromatic compounds, and amino acids. Facultatively chemolithotrophic in an atmosphere containing hydrogen, oxygen, and carbon

TABLE 2. Characters useful for the differentiation of the facultatively autotrophic species of *Alcaligenes*

Character	Reaction ^a of:			
	<i>A. eutrophus</i>	<i>A. paradoxus</i>	<i>A. ruhlandii</i>	<i>A. latus</i>
Yellow cellular pigments	—	+	—	—
Gelatin liquefaction	—	—	—	+
Denitrification	+	—	—	—
Utilization of:				
L-Arabinose	—	+	—	—
Maltose	—	(—)	—	+
Glycerol	—	+	—	+
Phenylalanine	+	+	+	—
Betaine	—	—	—	+

^a Symbols: +, positive; —, negative; (—), most strains are negative.

dioxide gases. Under autotrophic conditions, growth is inhibited by high (20% or higher) oxygen concentrations, but mutants resistant to these conditions are readily obtainable.

The guanine-plus-cytosine content of the deoxyribonucleic acid of strain H-4 is 69.4 mol%.

Type strain: H-4. A culture of this strain has been deposited in the American Type Culture Collection under the number 29712.

With the transfer of *Pseudomonas ruhlandii* to the genus *Alcaligenes*, as recently proposed (1), and the assignment of this new species to the same genus, the number of extensively described species of *Alcaligenes* will be increased to six. Of these, four are hydrogen bacteria. *A. latus* can be easily differentiated from the other three facultatively autotrophic species on the basis of the characters given in Table 2.

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REPRINT REQUESTS

Address reprint requests to: Dr. Norberto J. Palleroni, Department of Chemical Research, Hoffman-La Roche, Inc., Nutley, NJ 07110.

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