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Micromorphology of Gram-Negative Hydrogen Bacteria

I. Cell Morphology and Flagellation

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Abstract. The cell morphology, the arrangement and fine structure of flagella and the piliation of the following Gram-negative aerobic hydrogen bacteria have been studied: *Alcaligenes eutrophus*, *Alcaligenes paradoxus*, *Alcaligenes ruhlandii*, *Pseudomonas flava*, *Pseudomonas pseudoflava*, *Pseudomonas palleronii*, *Pseudomonas facilis*, *Aquaspirillum autotrophicum*, *Paracoccus denitrificans*, *Corynebacterium autotrophicum*, and strains MA 2 and SA 35. The identity of the bacteria was examined by their substrate spectra and type of flagellation. Three types of flagellar fine structure were differentiated. The presence of pili was noted in strains of *Alcaligenes paradoxus*, *Pseudomonas flava*, *P. pseudoflava*, *P. palleronii*, and *P. facilis*.

Key words: Ultrastructure – Micromorphology – Gram-negative hydrogen bacteria – Flagellation – Flagellar fine structure – Pili.

Aerobic hydrogen bacteria are facultative chemolithoautotrophs. They were until recently considered as a taxonomical unit and grouped in the genus *Hydrogenomonas*, Orla-Jensen, 1909. Mainly on the basis of their flagellation, Davis et al. (1969) proposed to assign the peritrichously flagellated forms to *Alcaligenes* (*A. eutrophus*, *A. paradoxus*) and the polarly flagellated forms to *Pseudomonas* (*P. facilis*, *P. saccharophila*, *P. flava*, *P. palleronii*, *P. ruhlandii*). Later *P. ruhlandii* proved to be peritrichously flagellated and was assigned to *Alcaligenes* (Aragno and Schlegel, 1977a). Strains related to, but differing in many respects from *P. flava* are now regarded as a new species, *P. pseudoflava* (Auling et al., 1977).

There are further distinctive genera and species among the hydrogen bacteria: They include spirilla (*Aquaspirillum autotrophicum*, Aragno and Schlegel, 1977b), Gram-negative cocci (*Paracoccus denitrificans*, Davis et al., 1970), Gram-negative coryneforms ("*Corynebacterium*" *autotrophicum*, Baumgarten et al., 1974) as well as Gram-positive bacteria (not included in the present study) belonging to *Arthrobacter* (Canevascini and Eberhardt, 1975), *Nocardia* (Hirsch, 1961; Aggag and Schlegel, 1973) and *Mycobacterium* (Lukins and Foster, 1963; Park and DeCicco, 1974).

At present, the morphology and cytology of hydrogen bacteria has not been systematically studied. It was not known whether the taxonomic diversity of hydrogen bacteria is paralleled by ultrastructural peculiarities. The present paper is concerned with the cell morphology, the arrangement and fine structure of flagella, and the occurrence of pili. It is supplemented by a study on the fine structure of the cell envelope, of membrane systems and cytoplasmic inclusions, as revealed by electron microscopy of ultrathin sections (Walther-Mauruschat et al., 1977).

MATERIALS AND METHODS

Strains Studied. Table 1 lists the various strains investigated.

Culture. The following basal mineral medium (after Schlegel et al., 1961) was used: $Na_2HPO_4 \cdot 12\,H_2O$: 9 g; KH_2PO_4 1.5 g; NH_4Cl 1.0 g; $MgSO_4 \cdot 7\,H_2O$: 0.2 g; ferric-ammonium citrate 0.005 g; $CaCl_2 \cdot 2\,H_2O$ 0.01 g; trace elements solution SL4 according to Pfennig and Lippert (1966), however, without EDTA and ferrous salts: 0.5 ml. It was supplemented for autotrophic growth by 0.05% (w/v) $NaHCO_3$, and for heterotrophic growth by, unless otherwise stated, 0.2% (w/v) succinic acid; pH was adjusted to 7.1 with NaOH. Heterotrophic cultures in 100 ml Erlenmeyer flasks containing 30 ml medium were shaken under air. Autotrophic growth occurred under a continuously flowing mixture of 5% O_2, 10% CO_2 and 85% H_2, in 100 ml baffled Erlenmeyer flasks containing 30 ml medium and agitated on a rotatory shaker. The cells were harvested after three subcultures at identical conditions during the exponential phase of growth.

Substrate utilization tests were run on solid (1.7% agar) mineral medium containing 0.2% of sugar or 0.1% of one of the other compounds.

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Table 1. List of strains of hydrogen bacteria studied

Designation	DSM no.	ATCC no.	Isolated by
Alcaligenes eutrophus[a]	531	17697	C. Bovell
Alcaligenes eutrophus H 16	428	17699	E. Wilde (1962)
Alcaligenes paradoxus[a]	66	17713	D. H. Davis
Alcaligenes paradoxus SA 29			C. Schweizer and M. Aragno (1975)
Alcaligenes ruhlandii[a]	653	15749	W. Vishniac
Pseudomonas flava[a]	619		A. J. Kluyver and A. Manten (1942)
Pseudomonas pseudoflava GA 3[a]	1034		G. Auling (1977)
Pseudomonas pseudoflava SA 27			C. Schweizer and M. Aragno (1975)
Pseudomonas palleronii[a]	63	17724	N. J. Palleroni
Pseudomonas palleronii RH 2			M. Reh
Pseudomonas facilis[a]	550	17695	A. Schatz and C. Bovell (1952)
Pseudomonas facilis	620		A. Manten
Aquaspirillum autotrophicum SA 32[a]	732		C. Schweizer and M. Aragno (1975)
Aquaspirillum autotrophicum SA 33	733		C. Schweizer and M. Aragno (1975)
Paracoccus denitrificans[a]	65	17741	M. W. Beijerinck
Paracoccus denitrificans "Morris"	413		J. G. Morris
"*Corynebacterium*" *autotrophicum* 7C[a]	432		D. Siebert
"*Corynebacterium*" *autotrophicum* 14g	431		V. Rudolph
Unnamed strain MA 2			M. Aragno
Unnamed strain SA 35			M. Aragno

[a] Type strain

Electron Microscopy. 2% potassium phosphotungstate or 2% uranyl-acetate was used for negative staining (Valentine et al., 1968). Electron micrographs were taken on Agfa Scientia 23 D 56 sheets with a Philips EM 301 electron microscope. Magnifications were calibrated using a cross-lined grating replica.

Chemicals. All chemicals used were of analytical grade. Uranyl acetate was purchased from Fluka (Buchs, Switzerland), all others from Merck (Darmstadt, West Germany).

RESULTS

Biochemical Features of the Strains Studied

The most characteristic features are shown in Table 2. The results for our tests agree in general with those of Davis et al. (1970) and confirm the identity of the strains.

Cell Morphology and Motility

Alcaligenes and *Pseudomonas* strains, as well as strains MA 3 and SA 35, were straight motile rods (Figs. 1–9), cells of MA 3 and SA 35 were sometimes slightly club-shaped. *Aquaspirillum autotrophicum* strains (Aragno and Schlegel, 1977b) were motile, curved to helically shaped cells (Fig. 10). The length of cells depended strongly on the growth substrate and on the growth phase. *Paracoccus denitrificans* strains were immotile, coccoid cells, and occurred mostly in pairs (in case of type strain) or as short cell chains ("Morris" strain). "*Corynebacterium*" *autotrophicum* cells were immotile. The cell shape varied considerably with the age of cells and the conditions of growth.

Figs. 1–9. Flagellation of hydrogen bacteria

Fig. 1. *Alcaligenes eutrophus* H 16

Fig. 2. *Pseudomonas pseudoflava* SA 27

Fig. 3. *P. pseudoflava* SA 27, biflagellated cell

Fig. 4. *P. flava*

Fig. 5. *P. facilis* DSM 620

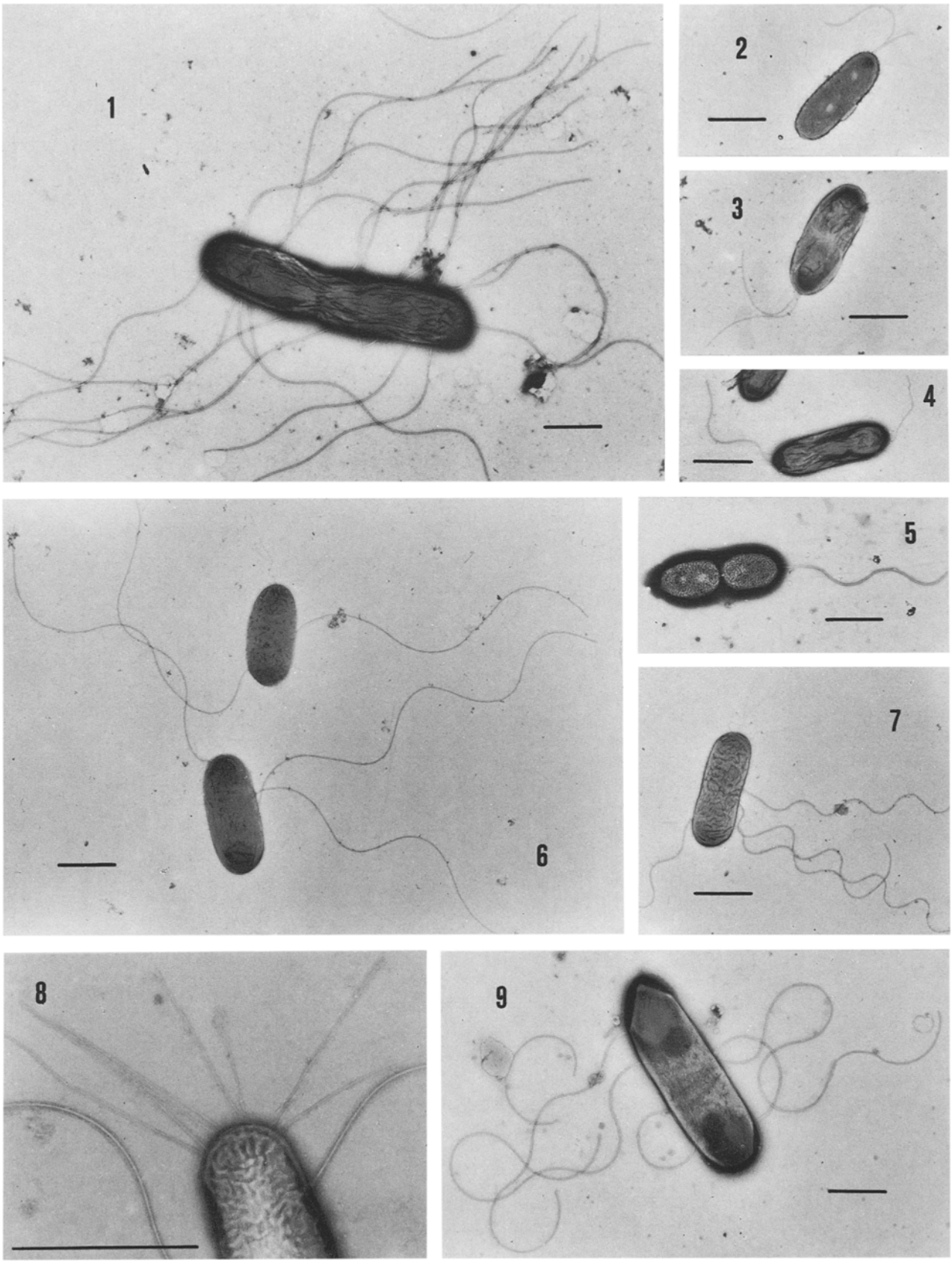
Fig. 6. *Alcaligenes paradoxus* SA 29

Fig. 7. *A. paradoxus* SA 29, with "curly" flagella

Fig. 8. *A. paradoxus*, showing pili on the polar cap

Fig. 9. Strain MA 2

Figs. 1–4, 6, 7, and 9. Negatively stained with 2% uranyl acetate. Figs. 5 and 8. Negatively stained with 2% phosphotungstate. Bar represents 1 μm

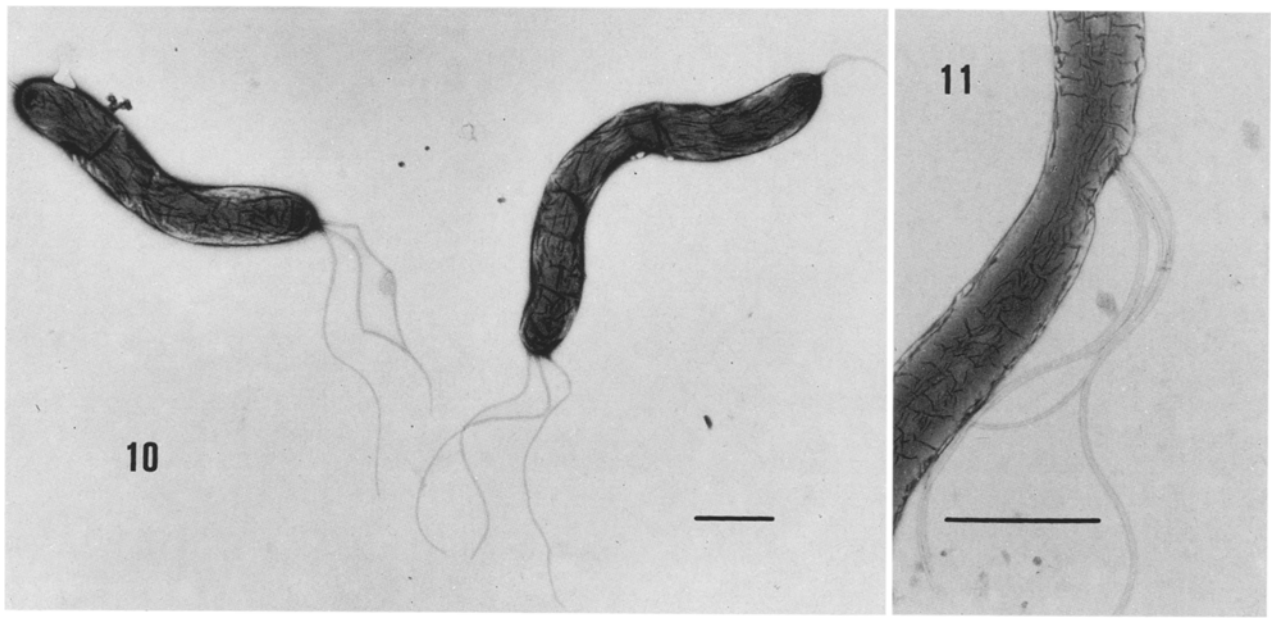


Figs. 1–9

Table 2. Utilization of substrates as single C-source by the strains of hydrogen bacteria studied

	D-Glucose	D-Fructose	L-Arabinose	D-Fucose	L-Rhamnose	D-Galactose	Sucrose	Trehalose	Citrate	Lactate	Malonate	L-Tartrate	Meso-tartrate	Mesaconate	Gluconate	Benzoate	p-OH-Benzoate	Urate	Allantoin	n-Butanol	Mannitol	Ethanol	PHB
Alcaligenes eutrophus type	M	+	−	−	−	−	−	−	+	+	M	−	+	+	+	+	+	+	+	−	−	−	−
Alcaligenes eutrophus H 16	M	+	−	−	−	−	−	−	+	+	−		+	M	+	+	+	+	+	−	−	−	−
Alcaligenes paradoxus type	+	+	+	+	−	+	−	−	+	+	+	+	+	−	+	−	+	+	+	+	+	−	+
Alcaligenes paradoxus SA 29	+	+	+	+	−	+	−	−	+	+	+	+	+	−	+	−	+	+	+	+	+	−	+
Alcaligenes ruhlandii	+	−	−	−	−	+	−	−	+	+	−	−	+	+	+	−	−	−	−	+	−	−	−
Pseudomonas flava	+	+	+	−	+	+	+	+	+	+	−	−	+	−	+	−	−	−	−	−	+	−	−
Pseudomonas pseudoflava GA 3	+	+	+	−	−	M	+	+	+	+	−	−	−	−	+	−	+	+	−	+	+	sl	−
Pseudomonas pseudoflava SA 27	+	+	+	−	−	+	+	+	+	+	−	+	−	−	+	−	+	+	−	M	+	+	−
Pseudomonas palleronii type	sl	−	−	−	−	−	−	−	+	+	−	−	+	−	+	−	+	+	−	−	−	−	−
Pseudomonas palleronii RH 2	M	−	−	−	−	−	−	−	+	+	−	−	+	−	+	−	+	+	−	−	−	−	−
Pseudomonas facilis type	+	+	+	−	−	+	−	−	d	+	−	−	+	−	+	−	+	−	−	−	+	−	+
Pseudomonas facilis DSM 620	+	+	+	−	−	−	−	−	−	+	−	−	+	−	+	−	+	−	−	−	+	−	+
Aquaspirillum autotrophicum type	−	−	−	−	−	−	−	−	+	+	+	−	−	+	+	−	+	+	−	−	−	−	−
Aquaspirillum autotrophicum SA 33	−	−	−	−	−	−	−	−	+	+	+	−	−	+	+	−	+	+	−	−	−	−	−
Paracoccus denitrificans type	+	+	−	−	−	−	+	+	+	+	+	+	−	−	+	−	+	+	−	+	+		−
Paracoccus denitrificans "Morris"	+	+	+	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	−	+	+		−
"*Corynebacterium*" *autotrophicum* type	−	+	−	−	−	−	+	−	+	+	+	+	+	−	+	−	+	+	−	+	−	+	−
"*Corynebacterium*" *autotrophicum* 14 g	−	−	−	−	−	−	−	−	+	+					+	−	−					+	−
Strain MA 2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−
Strain SA 35	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−

M = growth of mutant; sl = slight growth; d = delayed growth



Figs. 10 and 11. *Aquaspirillum autotrophicum*

Fig. 10. Whole cells, negatively stained with 2% uranyl acetate

Fig. 11. Laterally inserted bundle of flagella, near an area of incomplete fission (negatively stained with 2% phosphotungstate). Bar represents 1 μm

Flagellation

Three types of flagellation were recognized: peritrichous, monopolar monotrichous, and bipolar polytrichous (Table 3). The peritrichous type was represented by the strains of the genus *Alcaligenes* (Figs. 1, 6, and 7) and by the strains MA 2 (Fig. 9) and SA 35. The *Alcaligenes paradoxus* strains (Figs. 6 and 7) and strains MA 2 and SA 35 may be considered as oligotrichous [so-called "degenerated peritrichous", Davis et al. (1969)]; they contained 2–3 laterally inserted flagella. Among the strains of *Alcaligenes eutrophus* and in *A. ruhlandii* the number of flagella varied considerably, and depended on the growth substrate and on the growth phase (Figs. 12 and 13).

The polar monotrichous type was represented by the strains ranged within *Pseudomonas: P. flava* (Fig. 2), *P. palleronii*, *P. facilis* (Fig. 5) and *P. pseudoflava* (Fig. 4). Few cells of *P. flava* were observed with two flagella at one pole (Fig. 3).

The bipolar polytrichous type was represented by the *Aquaspirillum autotrophicum* strains (Fig. 10). The number of flagella varied considerably with the growth substrate and the growth phase. In long cells laterally inserted tufts of flagella were seen, close to a delayed division area (Fig. 11).

The flagellar wave length is apparently not a species specific property (Table 3). In one culture the flagella of *Alcaligenes paradoxus* had two distinctive wavelengths, one being about half as long as the other (Figs. 6 and 7). Such phenomena had already been observed in a strain related to *Alcaligenes* (Leifson and Hugh, 1953) and in *Proteus* strains (Leifson et al., 1955).

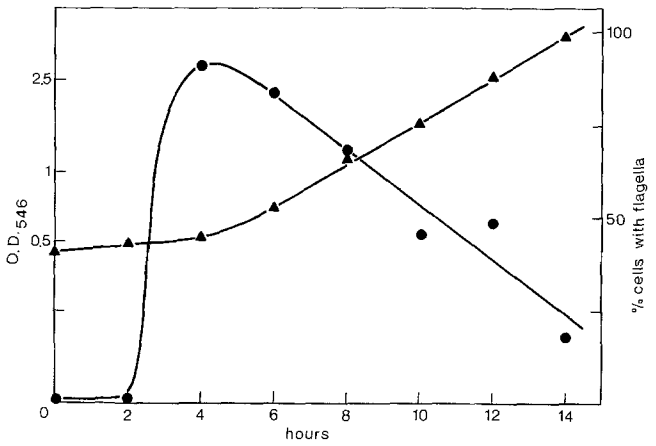


Fig. 12. Variation of the percentage of flagellated cells of *Alcaligenes eutrophus* H 16 during growth on fructose. Each point represents the evaluation of 50–100 cells on negatively stained preparations. The percentages in this and in Figure 13 are mean values of two separate experiments (●——●) % cells with flagella; ▲——▲ OD_{546}

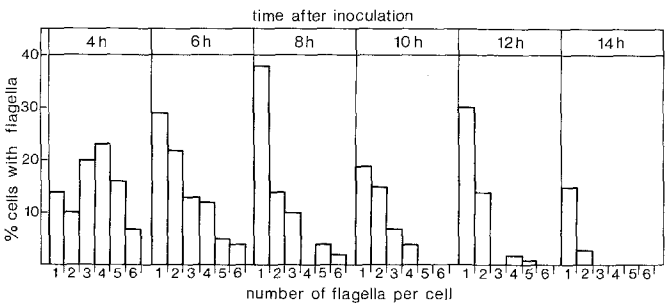


Fig. 13. Variation of the number of flagella per cell during growth of *A. eutrophus* H 16. Experimental conditions are identical with those described in Figure 12

Table 3. Flagellation and flagellar fine structure patterns of motile hydrogen bacteria

	Flagellation	Number of flagella	Wave length (μm)	Diameter of flagella (nm)	Type of fine structure	Angles of rows[c]: α	Angles of rows[c]: λ	Angles of rows[c]: δ	Centre-to-centre	Distance of subunits (nm)
Alcaligenes eutrophus	per[a]	3–14	1.9–2.4	12 –13	II	–5°	24°	–33°	7.2[d]	4.4[e]
Alcaligenes paradoxus	per	1–3	1.2–1.3 2.2–2.5	15 –17	II	4°	31°	–23°	8.7[d]	9.8[f] 4.4[e]
Alcaligenes ruhlandii	per	4–12	1.9–2.3	17.5–18.5	III	nd	nd	nd	nd	
Pseudomonas flava	mpl	1 (–2)	1.2–1.3	13.5–14	I	nd	63°	nd	nd	
Pseudomonas pseudoflava	mpl	1	1.4–1.9	13.5–14	[b]	nd	nd	nd	nd	
Pseudomonas palleronii	mpl	1	1.2–2.0	14 –16	I	–2°	60°	–62°	4.4[d]	43[e]
Pseudomonas facilis	mpl[a]	1	1.4–1.7	19 –20	I	5°	70°	–59°	4.2[d]	
Aquaspirillum autotrophicum	bpl[a]	2×(1–6)	2 –2.3	15	I	nd	60°	nd	nd	
MA 2/SA 35	per	2–5	1.3–1.7	14.5–16	I	nd	63°	nd	4.4[d]	

[a] per = peritrichous
mpl = monopolar
bpl = bipolar

[b] Indistinct

nd = not determined

[c] See Figure 14a

[d] Along the A axis, see Figure 14a

[e] Along the L axis, see Figure 14a

[f] Along the D axis, see Figure 14a

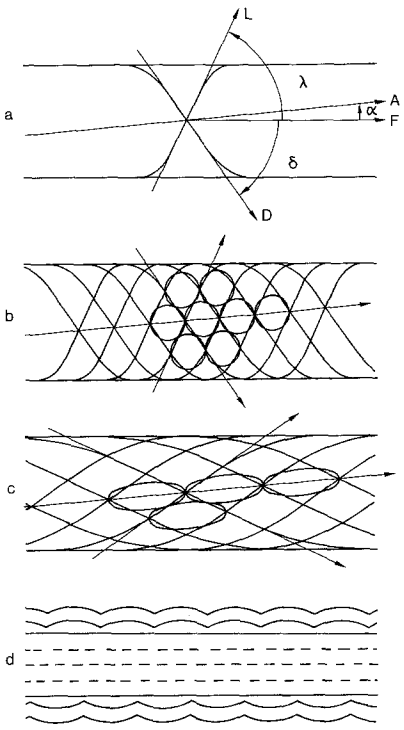


Fig. 14a–d Diagrammatic representation of flagellar fine structure. (a) Symbols used (see text). (b) Type I structure. (c) Type II structure. (d) Type III structure

Flagellar Fine Structure

In our model of the fine structure of flagella, the following symbols are used (Fig. 14a): A is the axis of the longitudinal rows of subunits, α is the angle which this axis forms with the filament axis; L and D are the axes tangential to the left and right oblique rows of subunits, and λ and δ are the corresponding angles with the axis of the flagellum. The centre-to-centre distance between subunits is referred to the axis along which it has been measured. Angles and distances given are the mean values of at least 10 measurements.

When evaluating electron micrographs artifacts due to shrinkage and flattening of the flagella have to be considered. The measured dimensions of flagella and subunits may deviate from those of native flagella. Therefore, only specimens treated in an identical manner were compared. The angles between the rows of alignment of subunits were considered not to have been changed by the preparation procedures. The results of our measurements and calculations are summarized in Table 3.

Three types of flagellar fine structure were recognized: Type I (Fig. 14b): The angles between the axes A, L, D approach 60°. This type is represented by the strains of *Pseudomonas facilis* (Fig. 15), *P. flava*, *P. palleronii* (Fig. 16) and by the unidentified strains MA 2 (Fig. 1) and SA 35. The flagella of both strains of *Aquaspirillum autotrophicum* probably belong to this type (Fig. 18). Type II (Fig. 14c): The angles between the axes A, L, D are less than 60°. This type is represented by the strains of *Alcaligenes eutrophus* (Fig. 19) and of *A. paradoxus* (Fig. 20). Type III (Fig. 14d): The flagellum is sheathed. The sheath appears as a succession of incurved projections of units, about 9 nm in length, with the convex side inwards. The fine structure of the core is more indistinct. Only longitudinal rows can be seen. This type is represented by the *Alcaligenes ruhlandii* strain (Fig. 21). The interpretation of the fine structure of the flagella of *Pseudomonas pseudoflava* remained uncertain (Fig. 22).

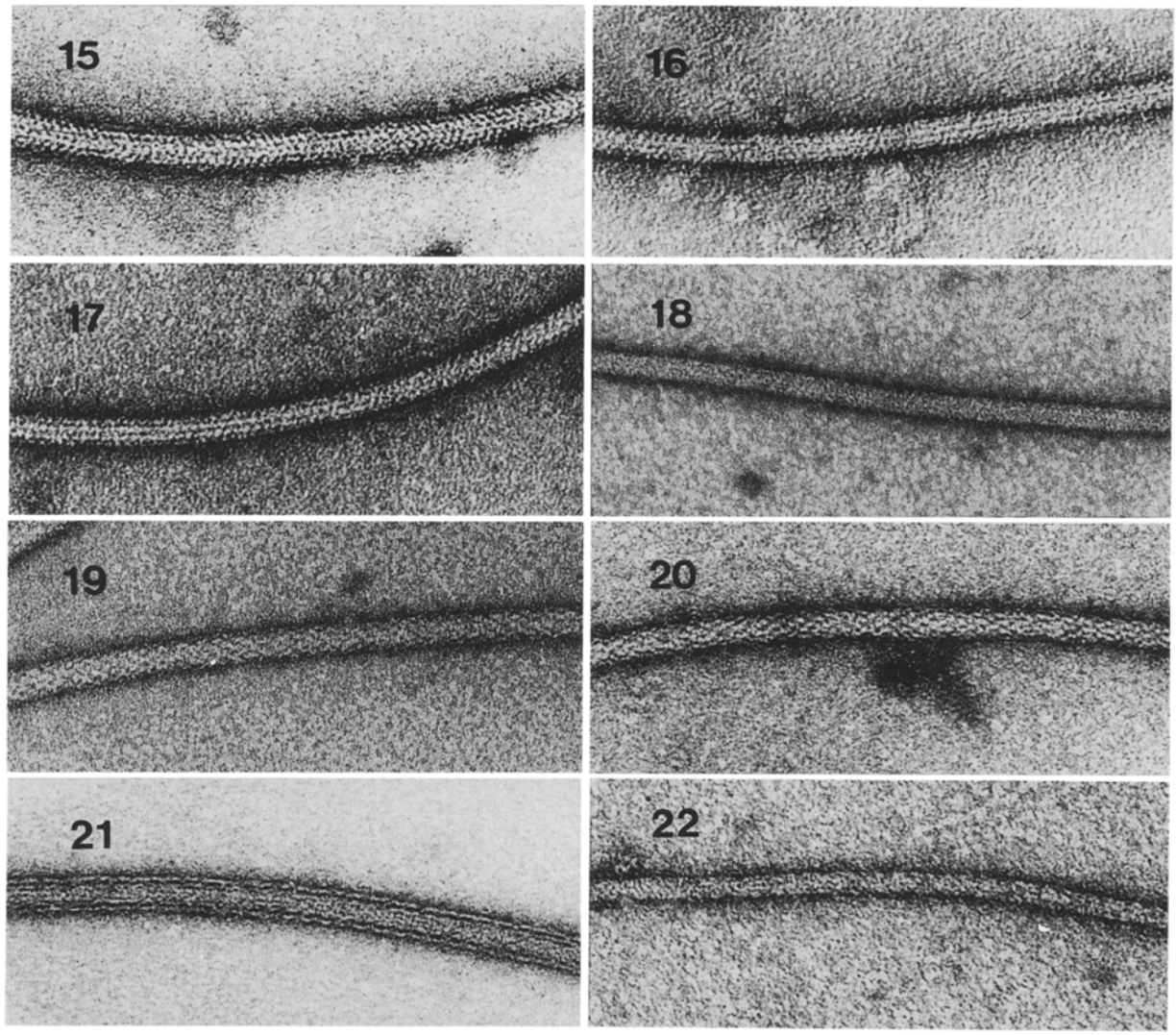
Pili

The occurrence of pili was noted in strains of *Alcaligenes paradoxus*, *Pseudomonas flava*, *P. pseudoflava*, *P. palleronii* and *P. facilis*. In *A. paradoxus* (Fig. 8) and in *P. palleronii* the pili were inserted only at the polar caps; in *P. flava*, *P. pseudoflava* and *P. facilis* they arose from anywhere on the cell surface. They were rarely observed in *P. facilis*, whereas in the other species up to 6 or 8 pili per cell were present in most cases.

DISCUSSION

The present knowledge about flagellar fine structure is far from complete. It is therefore admitted that, in most cases, the flagellar subunits, spherical or lengthened, are arranged in a hexagonal network, as in the models proposed by Lowy and Hanson (1965). Such an arrangement implies that three main rows of alignment of subunits have to be recognized (Fig. 14a).

The structure of type I flagella agrees well with the type A as defined by Lowy and Hanson (1965). It may be interpretated as a hexagonal arrangement of roughly isodiametrical subunits (Fig. 14b). The longitudinal rows are not exactly parallel to the axis of the flagellum (Lowy and Spencer, 1968). The structure of type II flagella may also be interpreted as a hexagonal arrangement of subunits; in this case, however, the smaller λ and δ angles imply that the subunits are lengthened in the direction of the A axis (Fig. 14c). The structure of type III flagella (Fig. 14c) resembles closely that described for *Proteus vulgaris* (Lowy and Hanson, 1965), with periodic undulations of the "sheath". As in *Proteus*, and contrary to *Pseudomonas rhodos* (Lowy and Hanson, 1965; Schmitt et al., 1974), no helical structure of the sheath could be detected. It cannot be excluded, however, that its periodicities are due to a helical arrangement of subunits. The sheath does not envelop the hook (Aragno and Schlegel, 1977a); it is, therefore, quite different from the structure observed in *Bdellovibrio* flagella (Seidler and Starr, 1968). The core structure, with longitudinal



Figs. 15–22. Fine structure of flagella. Magnification: 285000fold. Negatively stained with 2% uranyl acetate

Fig. 15. *Pseudomonas facilis*, type strain

Fig. 16. *P. palleronii*, type strain

Fig. 17. Strain MA 2

Fig. 18. *Aquaspirillum autotrophicum* SA 32

Fig. 19. *Alcaligenes eutrophus* H 16

Fig. 20. *A. paradoxus* SA 29

Fig. 21. *Alcaligenes ruhlandii*

Fig. 22. *Pseudomonas pseudoflava*

rows of subunits, is in accordance with type B as described by Lowy and Hanson (1965).

Taxonomic implications of the above results will be discussed in the next paper (Walther-Mauruschat et al., 1977) along with ultrastructure of cell envelope, membrane systems and cytoplasmic inclusions.

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