The Sheathed Flagellum of *Pseudomonas stizolobii*

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SUMMARY

Organisms of two strains of *Pseudomonas stizolobii* possessed one polar flagellum of unusual thickness. Negative-contrast staining and ultrathin sectioning indicated that the flagella are sheathed and are comparable in structure to the sheathed flagella described in *Vibrio* and *Bdellovibrio*. In some instances, flagella displayed sheath and core structure after negative contrast staining. Distal 'tubules' and 'knobs' apparently consisting entirely of sheath material were also seen. The thickness of the sheath, which in section consisted of an outer dense component and an inner lighter component, was similar to that of the outer double track membrane of the cell wall.

INTRODUCTION

During the examination of a collection of species of *Pseudomonas* for the presence of fimbria-like appendages (Fuerst & Hayward, 1969), unusual polar flagella were seen on *Pseudomonas stizolobii* (Wolf) Stapp, 1935. This species is a plant pathogen causing a disease of the leaves and stems of mainly leguminous plants (Burkholder, 1957; Rothwell & Hayward, 1964). Whereas flagella of most bacteria are 120 to 150 Å in diameter and, under the electron microscope at high resolution, preparations of negatively stained material exhibit a helical aggregate of globular subunits, (Joys, 1968; Doetsch & Hageage, 1968), the flagella of *P. stizolobii* were of greater thickness and lacked the substructure characteristic of unsheathed bacterial flagella. Our aim was to determine by electron microscopic techniques the nature of the flagellum of *P. stizolobii* and to compare it with the sheathed flagella which are known to occur in a very few genera of Gram-positive and Gram-negative bacteria.

METHODS

Organisms and media. *Pseudomonas stizolobii* NCPPB 450 and *P. stizolobii* strain 0268 A, as listed by Fuerst & Hayward (1969), were grown at 28° in either motility medium (Difco bacto peptone, 5.0 g.; glycerol, 5.0 g.; MgSO₄·7H₂O, 0.25 g.; K₂HPO₄, 0.5 g.; Difco agar, 3.0 g.; distilled water, 1 l.), peptone yeast extract broth (see Fuerst & Hayward, 1969), or glucose peptone yeast extract broth (glucose, 1 g.; peptone yeast extract broth, 100 ml.).

Organisms not to be treated with reagents were carried through two 72 hr serial subcultures in Craigie tubes of motility medium and 5 or 8 serial subcultures of variable duration in 6 × ½ in., tubes containing 5 ml. peptone yeast extract broth. Organisms to be treated with various reagents (including distilled water controls) were

grown in tubes of peptone yeast extract broth for 70 hr or tubes of glucose peptone yeast extract broth for 20 hr. Organisms for ultrathin sectioning were grown in 100 ml. medical flats containing 10 ml. of glucose peptone yeast extract broth and incubated for 26 hr on their wide sides.

Initial inocula in all cases were from stock agar slopes. Purity checks were performed on all cultures examined by plating out from washed cell suspensions.

Negative contrast staining of untreated flagella. Cells were harvested by centrifugation and washed once in sterile distilled water before resuspension in the wash fluid. A 1 % solution of sodium phosphotungstate (pH 7.0 or 7.3) containing bovine plasma albumin to 0.03 % was employed as a negative contrast stain. Negative contrast preparations were made by applying drops of washed suspension and negative contrast stain to carbon-coated copper grids as described by Fuerst & Hayward (1969).

Treatments of flagella with reagents and negative contrast staining following treatment. Flagellated cells were treated with various reagents according to a modification of the procedure of Follett & Gordon (1963). Unlike Follett & Gordon (1963), cells were not fixed with formalin before treatment and ammonium molybdate was not used as a negative contrast stain. In addition Follett & Gordon in all cases treated cells first with a 1 % potassium iodide (KI.) solution before treatment with other reagents. Although a pretreatment with 1 % KI. was included in some cases, this treatment was often omitted because it was not known what effect the potassium iodide itself might have on flagellar structure.

Carbon-coated grids were floated on top of washed cell suspensions. The grids were then floated on a number of wash solutions and reagents. Treatments with N/100 HCl (pH 2.2), 6 M-urea, 10^{-3} M-sodium ethylenediaminetetra-acetic acid (Na_2EDTA) and 0.05 % phenol were interpolated between two distilled water washes. Negative contrast stain was applied by floating grids from the second distilled water wash on top of a 1 % sodium phosphotungstate solution (pH 7.4) containing 0.03 % bovine plasma albumin, removing excess fluid and placing in the microscope specimen chamber while still wet. The time of exposure to 6 M-urea was much shorter than that used by Follett & Gordon (1963) since, if longer times were used, no cells could be found adhering to the grid after such treatment. This may well have affected results and similar qualifications may apply to treatments with phenol and Na_2EDTA , which were also necessarily short.

Ultrathin-sectioning. Procedures used for obtaining ultrathin sections were those used by Fuerst & Hayward (1969).

Electron microscopy. A Siemens Elmiskop IA was used at accelerating voltages of 80 or 100 kV.

RESULTS

Negative contrast staining

Both strains of *Pseudomonas stizolobii* possessed one polar flagellum per cell. The cells were usually bullet shaped, being pointed at the flagellate pole and rounded at the aflagellate pole (Pl. 1, fig. 2). The tip of the flagellum as seen in untreated preparations was most commonly even, but occasionally an irregular appendage appeared to extend from the tip (Pl. 1, fig. 2). The typical appearance of intact flagella is seen in Pl. 1, fig. 1. A cell of strain NCPPB 450 displays a flagellum of diameter 250 to 286 Å. The tip of the flagellum is rounded and no substructure of any kind is evident, although

untreated flagella which displayed core and sheath were seen in some cases. An enlarged portion of a flagellum of strain 0268 A is shown in Pl. 2, fig. 3; the diameter (262 to 311 Å) is similar to that of strain NCPPB 450, the tip is rounded and there is a lack of substructure.

Chemical treatments known to enhance the contrast between the core and sheath of vibrio flagella (Follett & Gordon, 1963) were applied to some preparations of *Pseudomonas stizolobii* prior to negative contrast staining. In other cases cells were treated with solutions of the disodium salt of ethylenediaminetetra-acetic acid (Na₂EDTA) or with phenol, also in the hope of delineating sheath and core. These reagents, among others, were shown by Varon & Shilo (1968) to inhibit the motility of *Bdellovibrio bacteriovorus*, an organism with a single polar sheathed flagellum. They postulated that this inhibition might be connected with the sheathed flagellum structure.

After potassium iodide treatment alone prior to negative staining, delineation of sheath and core structure was seen in some cases, and the flagella were in some instances unusually thick. Thin, irregularly folded 'tubules' were often seen at the distal ends of the flagella. In Pl. 2, fig. 4 an enlarged portion of a flagellum of strain NCPPB 450 is shown after potassium iodide treatment and treatment with N/100 HCl solution prior to negative staining. The flagellum is 225 to 295 Å in diameter and displays core and sheath. Two dark lines traverse the interior of the flagellum. If only the outer light portions on both sides of the dark lines are taken to represent sheath material, then the sheath is 46 to 78 Å and the core is 130 to 155 Å in diameter. Plate 4, fig. 7 shows a flagellum after treatment with urea, and serves as a general example of the 'irregular tubule' and 'knob' appearance. The main portion of the flagellum in this micrograph is 280 to 312 Å in diameter. The 'tubule' at its distal end appears to be continuous with the outer parts of the flagellum, and is 100 to 166 Å in diameter. The round 'knob' appears to be continuous with the 'tubule' and is 425 Å in diameter.

Treatments with urea or hydrochloric acid did not result in appearances similar to those of flagella of *Vibrio* species (Follett & Gordon, 1963) and *Bdellovibrio bacteriovorus* (Seidler & Starr, 1968) after similar treatments. This may have been due to the different times of exposure to the reagents used in this study, or to differences in relative stabilities of the sheaths of different species.

Core and sheath delineation and distal 'tubules' and 'knobs' were also seen in phenol- and EDTA-treated preparations (see Pl. 3, fig. 5, 6). In Pl. 3, fig. 6 the more proximal part of the flagellum does not show core and sheath structure, but towards the distal portion near to the 'tubule' and 'knob' such a structure is more clearly seen. The core is 125 to 150 Å wide where there is a well-defined core and sheath while the sheath material on either side is 50 to 75 Å in diameter.

Appearance of sheathed flagella in ultrathin sections

Ultrathin sections of cells of *Pseudomonas stizolobii* in certain instances displayed what appeared to be flagella in longitudinal section (Pl. 4, fig. 8, 9). The sheath appears to be composed of at least an outer dense component and an inner light component, and there are indications of another dense line bounding the inner light component, particularly in Pl. 4, fig. 8. In Pl. 4, fig. 8, the sheath is 42 to 58 Å wide while the core is 100 to 133 Å wide. Although no obvious continuity between the outer layers of the cell wall and the sheath could be discerned in these sections, the outer triple-layered

membrane around the sectioned cells is comparable in thickness to the sheath bounding the flagellar core.

DISCUSSION

Sheathed flagella are known to occur in few species of bacteria, including Gram-negative and Gram-positive organisms and organisms with peritrichous as well as those with polar flagella. Present information is summarized in Table 1. The sheathed flagella of species of *Vibrio* and *Bdellovibrio bacteriovorus* have been most fully studied. The sheathed flagellum of *Pseudomonas stizolobii* resembles those of *Vibrio* and *Bdellovibrio* in diameter and structure. It is of interest to determine whether *P. stizolobii* has other characters in common with *Vibrio*. However, there is little evidence to support such a relationship. A detailed description of the plant disease caused by *P. stizolobii*, and of the characteristics of this species will be reported else-

Table 1. *Reports of sheathed flagella in bacteria*

Bacterium	Flagellum diameter	Reference
<i>Bacillus brevis</i>	?	De Robertis & Franchi (1951)
<i>Vibrio metchnikovii</i>	270 ± 20 Å	van Iterson (1953)
		Follett & Gordon (1963)
		Glauert, Kerridge & Horne (1963)
<i>Vibrio cholerae</i> (sic)	300 Å	Das & Chatterjee (1966)
<i>Vibrio eltor</i>	270 ± 20 Å	Follett & Gordon (1963)
<i>Vibrio</i> sp.	?	Tweedy, Park & Hodgkiss (1968)
<i>Bdellovibrio bacteriovorus</i>	280–290 Å	Abram & Shilo (1967)
		Seidler & Starr (1968)
		Burger, Drews & Ladwig (1968)
<i>Pseudomonas rhodos</i>	177 Å	Lowy & Hanson (1965)
		Lowy (1965)
<i>Proteus vulgaris</i>	?	Lowy & Hanson (1965)
<i>Spirillum</i> spp.	?	Lockard & Boler (1968)

where. (Allen, Hayward, Halliday & Fulcher, to be published). *P. stizolobii* differs from the human and animal pathogenic vibrios in which sheathed flagella have been described in having an oxidative metabolism of glucose, a negative oxidase reaction, and in failing to hydrolyse starch or gelatin. *P. stizolobii* is a short straight rod, containing refractile sudanophilic inclusions, which never exhibits the marked degree of cell curvature and of pleomorphism in old cultures which is characteristic of *Vibrio* (Park & Holding, 1966). *P. stizolobii* is thus the first member of the genus *Pseudomonas* in which a sheathed flagellum of the kind observed in *Vibrio* and *Bdellovibrio* has been identified. In *P. rhodos* sheathed flagella have been reported but they are clearly different in size and organization, being more slender (*cf.* Table 1) and having a sheath consisting of two helically wound ribbons which were easily visible by negative contrast (Lowy & Hanson, 1965); furthermore the fine structure of the core could be seen through the sheath when negative contrast methods were employed. There is therefore good reason to think that the sheath of this type is different in structure and size from those occurring in *Vibrio*, *Bdellovibrio* and *P. stizolobii*.

The value of the occurrence of sheathed flagella as a taxonomic criterion should be stressed. With the advent of improved negative contrast staining and sectioning techniques, and the more general use of electron microscopy, this is a feature of the fine structure of the bacterial cell which, like the occurrence of fimbria-like appendages,

will be of use in the characterization and identification of bacteria. Sheathing appears to be a stable feature of *Pseudomonas stizolobii* since one of the cultures used, NCPPB 450, was isolated in 1954 and had been maintained since then in various culture collections. In a survey of some *Pseudomonas* species for the presence of fimbria-like appendages, only the two strains of *P. stizolobii* examined in that survey possessed sheathed flagella. The remaining 20 strains representing 14 species all possessed flagella of a diameter and substructure which were normal for the naked flagella of bacteria (Fuerst & Hayward, 1969). However, a much more extensive survey for the presence of sheathed flagella in *Pseudomonas* is required before general conclusions can be made.

At present nothing is known about the functional significance of the sheath in motile bacteria. A comparison of the motility of *Bdellovibrio*, *Vibrio* and *Pseudomonas stizolobii* with the motility of polarly flagellate Gram-negative bacteria with naked flagella, or with sheathless mutants of normally sheathed bacteria, may provide information about function. The sheath may be 'protective' or be an integral part of the flagellum (Doetsch & Hageage, 1968).

'Tubules' and 'knobs' at the distal ends of sheathed flagella were frequently seen in this study. It is not clear whether these structures are an artifact resulting from autolytic effects or the effects of chemical treatment, or whether they occur naturally as a result of a lack of synchrony between synthesis of core and sheath. The 'tubules' were always irregularly folded structures. Follett & Gordon (1963) considered similar structures to be empty sheaths. Abram & Shilo (1967) and Seidler & Starr (1968) have also noted that sheath material often extends beyond the distal end of the flagellar filament. The evidence from the present study seems to confirm the general opinion that these 'tubules' represent sheath material.

It should again be emphasized that at this stage it is not possible to attribute any of the effects on the sheathed flagella seen after various treatments to particular reagents. Distal 'tubules' and delineation of core and sheath were sometimes seen in preparations which had not been treated with potassium iodide or any other reagent. The treatments were undertaken either because other workers had found similar procedures successful or because it seemed possible that they might enhance delineation of sheath and core. One might note that these qualifications may also apply to the results of treatments of sheathed flagella performed by Follett & Gordon (1963) and Seidler & Starr (1968).

Continuity of the outermost envelope of the cell wall with the sheath covering the flagellum has been determined in ultrathin sections of *Bdellovibrio* by Abram & Shilo (1967) and by Seidler & Starr (1968), and in *Vibrio metchnikovii* by Glauert, Kerridge & Horne (1963). Sections showing a continuity with the cell wall were not obtained in this study, although it was established that the thickness of the sheath was comparable to that of the outer membrane of the cell wall. There is no reason to doubt that more favourable sections would show a continuity between these layers.

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EXPLANATION OF PLATES

Abbreviations used in figures: T, distal 'tubule'; K, distal 'knob'; C, core; S, sheath.

PLATE 1

Fig. 1. *Pseudomonas stizolobii* strain NCPPB 450 after two 72 hr serial subcultures in motility medium + 5 serial subcultures in peptone yeast extract broth incubated at 28°. A single polar flagellum without visible substructure arises from the cell. Negatively stained with sodium phosphotungstate, pH 7.0. $\times 56,000$.

Fig. 2. *P. stizolobii* strain NCPPB 450 after two 72 hr serial subcultures in motility medium + 8 serial subcultures in peptone yeast extract broth incubated at 28°. Several cells with single polar flagella are visible. Negatively stained with sodium phosphotungstate, pH 7.3. $\times 20,000$.

PLATE 2

Fig. 3. *P. stizolobii* strain 0268 A after two 72 hr serial subcultures in motility medium + 8 serial subcultures in peptone yeast extract broth incubated at 28°. An enlarged portion of a sheathed flagellum (note the rounded tip). Negatively stained with sodium phosphotungstate, pH 7.3. $\times 160,000$.

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Fig. 4. *P. stizolobii* strain NCPPB 450 after growth for 70 hr in peptone yeast extract broth incubated at 28° followed by treatment for 10 sec. with 1 % KI. + 10 sec. distilled water + 20 sec. N/100 HCl (pH 2.2) + 10 sec. distilled water. Delineation of core and sheath structure is visible. Negatively stained with sodium phosphotungstate, pH 7.4. $\times 130,000$.

PLATE 3

Fig. 5. *P. stizolobii* strain NCPPB 450 after growth for 20 hr in glucose peptone yeast extract broth incubated at 28°, followed by treatment for 5 sec. with distilled water + 45 sec. 10^{-3} M- Na_2 EDTA + 5 sec. distilled water. An irregularly folded distal 'tubule' extends from an apparently intact flagellum. Negatively stained with sodium phosphotungstate, pH 7.4. $\times 160,000$.

Fig. 6. *P. stizolobii* strain NCPPB 450 after growth for 20 hr in glucose peptone yeast extract broth incubated at 28°, followed by treatment for 5 sec. with distilled water + 12 sec. 0.05 % phenol + 5 sec. distilled water. Core and sheath delineation and distal 'tubule' and 'knob' are visible. Negatively stained with sodium phosphotungstate, pH 7.4. $\times 160,000$.

PLATE 4

Fig. 7. *P. stizolobii* strain NCPPB 450 after growth for 20 hr in glucose peptone yeast extract broth incubated at 28°, followed by treatment for 5 sec. with distilled water + 15 sec. 6 M-urea + 5 sec. distilled water. A distal 'tubule' and 'knob' extend from an apparently intact flagellum. Negatively stained with sodium phosphotungstate, pH 7.4. $\times 160,000$.

Fig. 8. *P. stizolobii* strain NCPPB 450 after growth for 26 hr in glucose peptone yeast extract broth incubated at 28°. Sheath and core structure is visible in the sectioned flagellum. Glutaraldehyde-osmium tetroxide fixation. $\times 120,000$.

Fig. 9. *P. stizolobii* strain NCPPB 450 after growth for 26 hr in glucose peptone yeast extract broth incubated at 28°. Sheath and core structure is visible in the sectioned flagellum. Glutaraldehyde-osmium tetroxide fixation. $\times 120,000$.