The Effect of Temperature on the Formation of Sheathed Flagella by *Pseudomonas stizolobii*

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Production of sheathed flagella by *Pseudomonas stizolobii* strain 0268A was markedly inhibited by growth at 34 °C. Flagella produced during growth at 32 °C, at which some flagellar inhibition was evident, possessed normal sheath and core components, but at 34 °C, irregular ‘tubule’ appendages were sometimes produced in low numbers. Cells rendered aflagellate by growth at 34 °C could regenerate normal sheathed flagella at 28 °C within half a mean generation time after shift-down, and such regeneration was inhibited by chloramphenicol. The use of the sheathed flagellum of *P. stizolobii* as an experimental model for studies of biosynthesis and self-assembly in complex organelles is suggested.

INTRODUCTION

There have been numerous reports describing the effect of temperature on the formation of bacterial flagella, especially with respect to the inhibition of flagellum formation at supra-optimal or near maximum growth temperatures (Quadling & Stocker, 1956; Kerridge, 1960; Lacey, 1961; Vaituzis & Doetsch, 1965; Roberts & Doetsch, 1966; Martinez & Gordee, 1966; McGroarty *et al.*, 1973). However, with the exception of those by Roberts & Doetsch (1966) and Martinez & Gordee (1966), these reports have dealt primarily with the behaviour of peritrichous, sheath-less flagella. Although Roberts & Doetsch (1966) studied the behaviour of polarly distributed monotrichous flagella of the genera *Pseudomonas* and *Xanthomonas* and Martinez & Gordee (1966) that of flagella of *Spirillum serpens*, the effect of temperature on the formation of sheathed, polarly distributed bacterial flagella has not previously been investigated. The type of flagellar sheath referred to here is that membranous continuation of the outer Gram-negative cell wall layer occurring on the flagella of *Pseudomonas stizolobii* (Fuerst & Hayward, 1969), *Vibrio metschnikovii* (Glauert *et al.*, 1963), *Vibrio cholerae* (Das & Chatterjee, 1966), *Bdellovibrio* spp. (Seidler & Starr, 1968) and *Beneckea campbellii* (Allen & Baumann, 1971). Flagella with this type of sheath are composite organelles which might be expected to display characteristic features in the physiology of their formation, possibly reflecting their structural complexity. For instance, synchronous synthesis of flagellar core and membranous flagellar sheath in these organisms might well require some regulatory and coordinating mechanism. It was therefore of interest to examine whether varying the growth temperature of a bacterium known to produce sheathed flagella of this type would separate core synthesis from sheath synthesis, and to compare the effect of temperature on the formation of sheathed flagella of the above type with the known effects of temperature on the formation of naked sheath-less flagella of organisms like *Salmonella typhimurium* and *Proteus vulgaris*.

This communication reports the results of experiments investigating the effect of temperature on the formation of sheathed flagella of *Pseudomonas stizolobii*, a plant pathogenic...
bacterium first described as the cause of a leaf spot of Florida velvet bean (*Stizolobium deerlingianum*). It should be noted that Goto & Starr (1971) and Hayward (1972) consider that *P. stizolobii* is synonymous with *Pseudomonas andropogonis*, the cause of a stripe disease in sorghum; the two species constitute specialized pathovars.

**METHODS**

*Bacteria.* *Pseudomonas stizolobii* strain 0268A, isolated from blackened stems of *Vicia sativa* cv. Golden Tares (Fuerst & Hayward, 1969), was used.

**Media.** For growth of bacteria in temperature experiments, a glucose/Casamino acids/mineral salts (GCMS) broth was used, having the following composition (1 l): glucose, 0.45 g; vitamin-free Casamino acids (Difco), 2.0 g; (NH$_4$)$_2$SO$_4$, 1.0 g; 1 M Na$_2$HPO$_4$/KH$_2$PO$_4$ buffer (pH 6.8), 33 ml; Hutner's vitamin-free mineral base, 20 ml. Hutner's vitamin-free mineral base was identical with the 'concentrated base' of Cohen-Bazire *et al.* (1957) except that vitamins were omitted. Glucose and vitamin-free Casamino acids were autoclaved separately, as more concentrated solutions, at 115 °C for 30 min and added aseptically to a solution of the other components which had been autoclaved in the same way.

**Temperature experiments.** Cells were grown for about 12 generations at the test growth temperature, that is, for about 19 to 22 h through two subcultures of about 7 to 9 h and 12 to 14 h each, in GCMS broth (25 ml per 250 ml conical flask) shaken at 160 strokes min$^{-1}$ in a water bath. The first 'test' broth culture was inoculated with a standardized washed inoculum derived from a GCMS broth culture grown at 28 °C. This first 'test' culture was grown at the test temperature until a standard absorbance was reached; it was then used as the inoculum for the second test broth culture, which was grown under the same conditions as the first culture until a second standard absorbance was reached (equivalent to a cell concentration of 0.42 to 0.45 mg dry wt ml$^{-1}$), when the culture was in the late-exponential or early-stationary phase of growth. At this time, a hanging drop preparation of the culture was examined for motile cells by phase contrast microscopy and 1 ml culture was mixed with 1 ml 1% (w/v) formaldehyde in 0.33 m-phosphate buffer (pH 6.8) as a sample to be prepared for electron microscopy.

In experiments to test the ability of cells to regenerate flagella at 28 °C after growth at 34 °C, the organism was grown through two test subcultures at 34 °C as above, a 1 ml sample of the second culture was formalin-fixed, and the flask of remaining culture was transferred to a water bath at 28 °C and shaken at 160 strokes min$^{-1}$. At 30 and 60 min after transfer to 28 °C, 1 ml samples were taken and formalin-fixed in preparation for electron microscopy as above, and hanging-drop motility preparations were examined. To test the effect of chloramphenicol on the regeneration of flagella at 28 °C, volumes of cultures grown through two subcultures at 34 °Cwere diluted either with fresh GCMS broth alone or with GCMS broth containing chloramphenicol (Sigma; final concentration 200 µg ml$^{-1}$). Diluted cultures (each 20 ml in a 250 ml conical flask) were then shaken in a waterbath at 28 °C, and at intervals 1 ml samples were taken and formalin-fixed.

**Electron microscopy.** For negative staining, samples of culture in formalin were washed once by centrifuging and resuspending in sterile (filtered and autoclaved) distilled water. A drop of washed suspension of fixed cells was placed on a copper grid coated with carbon and nitrocellulose. A drop of 1% (w/v) uranyl acetate containing sucrose (0.4%, w/v) was mixed with the drop of suspension, the mixture was allowed to remain on the grid for 1 min and excess fluid was then removed with filter paper. Grids were examined in a Philips EM300 transmission electron microscope and the proportion of flagellated cells was estimated either directly from the fluorescent viewing screen ($	imes 4000$) or from micrographs ($	imes 4620$ to $	imes 6600$). About 50 to 200 cells from each sample were examined and scored for the presence or absence of a flagellum. A cell was scored only if it was isolated or in a group of two cells with sides in contact so that the poles were clearly visible. If two cells were connected by one flagellum, the latter was assigned to only one of the cells. In some cases, the proportion of cells with tubule appendages was determined. These were distinguished from normal sheathed flagella by their lack of regular wavelength and irregular width (see Results and Discussion).

Lengths of flagella were measured from micrographs, using a map measurer, from flagella included in estimations of flagellated cell proportions where the distal flagellar tip was visible.

In some of the experiments described above, samples were taken for ultra-thin sectioning. The fixation procedure was a modification of that of Burnham *et al.* (1968, 1970). Volumes of culture were mixed with equal volumes of 2% (w/v) glutaraldehyde in 0.05 m-cacodylate buffer, and the cells were centrifuged and resuspended in 6% (w/v) glutaraldehyde in cacodylate buffer for 24 h at 4 °C. Cells were then suspended in agar, washed in cacodylate buffer, fixed with cacodylate-buffered osmium tetroxide, treated with 0.5% (w/v) uranyl acetate, dehydrated through a graded ethanol series and embedded in Araldite. Sections were stained with uranyl acetate and lead citrate.
Temperature effects on sheathed flagella

Table 1. Effects of growth temperature on the formation of flagella

<table>
<thead>
<tr>
<th>Growth temp. (°C)</th>
<th>Cells flagellated* (%)</th>
<th>Cells with tubules* (%)</th>
<th>Range of flagellum lengths‡ (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>53.6±13.8 (5)</td>
<td>0 (3)</td>
<td>0.2–4.5 (97)</td>
</tr>
<tr>
<td>(weighted mean 54.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>23.1±9.2 (2)</td>
<td>0 (2)</td>
<td>0.2–3.5 (61)</td>
</tr>
<tr>
<td>33</td>
<td>4.5±6.4 (2)</td>
<td>1.0±1.5 (2)</td>
<td>0.4–1.7 (13)</td>
</tr>
<tr>
<td>34</td>
<td>0.5±1.1 (7)</td>
<td>3.3±5.3 (7)</td>
<td>0–2–3.5 (61)</td>
</tr>
</tbody>
</table>

* Mean values ± standard deviations are given, with the number of replicates in parentheses.
† +, Motile cells detected; ±, most cells not motile, but rare translationally motile cells detected.
‡ Numbers of flagellum lengths measured are given in parentheses.

RESULTS AND DISCUSSION

The proportion of flagellated cells decreased with increasing growth temperature over the range 28 to 34 °C, a decrease which was evident even at 32 °C (Table 1). The lowest proportion of flagellated cells in any experiment at 28 °C was still above the values found at 32 °C. There was pronounced inhibition of flagellum production at 34 °C. However, in some experiments at 34 °C, cell surface appendages differing from normal sheathed flagella were found in low proportions, in addition to some apparently normal sheathed flagella. These ‘tubule’ appendages (Fig. 1) were also found in one experiment at 33 °C. They were highly irregular in width and lacked a regular wavelength, in contrast to the normal sheathed flagella of cells grown at 28 °C, but, like those flagella, they lacked a regular substructure and were in some cases attached to cell poles. These tubules may represent empty sheath material, devoid of core. However, even where tubules were found in 34 °C experiments, the combined proportion of cells possessing tubules and cells with normal sheathed flagella was much less than the lowest proportion of cells with sheathed flagella found in experiments at 28 °C. Thus, even if tubules do represent empty sheath material, there would seem to be relative inhibition of production of all components of the sheathed flagellum after growth at 34 °C. Of special relevance here are the findings of Yokota & Kuwahara (1974) with respect to flagellum formation in adenosine 3',5'-cyclic monophosphate (cyclic AMP)-deficient mutants of Vibrio cholerae (biotype El Tor). Although flagellum formation was inhibited in these mutants in the absence of cyclic AMP, straight flagella were sometimes produced under those conditions. They suggest that since flagella of vibrio species have sheaths, it may be that such straight ‘flagella’ consist only of sheath materials which do not require cyclic AMP. This interpretation seems questionable, as empty sheath material without rigid core would be expected to be irregular in outline rather than straight. Their straight flagella seem likely to contain at least some rigid core material, perhaps composed of modified flagellin.

Flagella detected in cultures grown at 32 °C were similar to the normal sheathed flagella formed at 28 °C both in appearance after negative staining, when they sometimes exhibited delineation of sheath and core (Fig. 2), and in fine structure exhibited in ultra-thin sections (Fig. 3); that is, longitudinally sectioned flagella exhibited a dark central core bounded on either side by at least two layers of sheath, an outer dark layer and an inner light layer. Rare flagella of normal appearance and substructure for negatively stained preparations of sheathed flagella formed at 28 °C were also seen in cultures grown at 33 and 34 °C. The maximum length found for sheathed flagella appeared to decrease as the growth temperature was increased from 28 to 33 °C, suggesting that the amount of flagellum formation per cell may be decreased with temperature increase, in addition to the proportion of cells producing flagella. Cell growth was not inhibited at 34 °C, since doubling times for P. stizolobii strain 0268A in the medium used for temperature experiments were 1.81 and 1.65 h for growth at 28 and 34 °C, respectively.
All micrographs are of *Pseudomonas stizolobii* strain 0268A. Bar markers represent 0.1 µm.

Fig. 1. Cell grown at 34 °C (negatively stained with uranyl acetate/sucrose) showing a 'tubule' appendage of highly irregular width and surface appearance.

Fig. 2. Broken flagella from a culture grown at 32 °C (negatively stained with uranyl acetate/sucrose) displaying extensions of sheath material beyond the break and delineation of sheath and core along their length.

Fig. 3. Section of a cell grown at 32 °C possessing an attached sheathed flagellum with a central dark core bounded on either side by a sheath with a dark outer and light inner layer. Continuity of the two layers of sheath with the corresponding outer cell wall membrane layers may be discerned on one side of the flagellum base.

Fig. 4. Section of cells grown at 34 °C and then incubated at 28 °C for 60 min before fixation. One of the cells has an attached sheathed flagellum with a dark central core bounded on either side by a sheath with an outer dark and inner light layer.
Table 2. Effect on the formation of flagella of transfer of cells grown at 34 °C to 28 °C

<table>
<thead>
<tr>
<th>Time after transfer</th>
<th>Cells flagellated* (%)</th>
<th>Cells with tubules* (%)</th>
<th>Range of flagellum lengths† (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before transfer</td>
<td>0 (3)</td>
<td>4·1±7·2 (3)</td>
<td>±</td>
</tr>
<tr>
<td>30 min after transfer</td>
<td>15·6±12·6 (3)</td>
<td>1·2±1·7 (2)</td>
<td>0·2-2·3 (57)</td>
</tr>
<tr>
<td>60 min after transfer</td>
<td>35·9±20·4 (3)</td>
<td>0 (2)</td>
<td>0·4-3·7 (71)</td>
</tr>
</tbody>
</table>

* Mean values ± standard deviations are given, with the number of replicates in parentheses.
† +, Motile cells detected; ±, most cells not motile, but rare translationally motile cells detected.
‡ Numbers of flagellum lengths measured are given in parentheses.

Table 3. Effect of chloramphenicol (200 μg ml⁻¹) on the formation of flagella after transfer of cells grown at 34 °C to 28 °C

<table>
<thead>
<tr>
<th>Time after transfer</th>
<th>Cells flagellated* (%)</th>
<th>Cells with tubules* (%)</th>
<th>Motility†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before transfer</td>
<td>1·9±1·7 (2)</td>
<td>5·2±6·0 (2)</td>
<td>±</td>
</tr>
<tr>
<td>Control, 60 min after transfer</td>
<td>42·0±4·4 (2)</td>
<td>4·2 (1)</td>
<td>+</td>
</tr>
<tr>
<td>Chloramphenicol-treated, 60 min after transfer</td>
<td>0·4±0·6 (2)</td>
<td>0 (1)</td>
<td>±</td>
</tr>
</tbody>
</table>

* Mean values ± standard deviations are given, with the number of replicates in parentheses.
† +, Motile cells detected; ±, most cells not motile, but rare translationally motile cells detected.

If cells rendered aflagellate by growth at 34 °C were transferred to 28 °C, flagella were regenerated (Tables 2 and 3). In four out of the five experiments performed, the proportion of flagellated cells increased within 60 min to proportions approximately equal to or greater than the minimum proportion of flagellated cells found after growth at 28 °C for about 12 generations (37·5%). Flagella appeared within 30 min of transfer (Table 2), well within the doubling time at this temperature (109 min). The apparently gradual increase in the proportion of flagellated cells upon transfer indicates that cells began to produce new flagella at different times rather than synchronously. Ranges of flagellar lengths found in these experiments (Table 2) are consistent with this interpretation. Variation in length would be expected solely on the basis of delays in initiation of flagellum outgrowth, as noted for flagellar regeneration after mechanical deflagellation in *Escherichia coli* by Novotny et al. (1969). Regenerated flagella appeared to be identical to normal sheathed flagella formed on cells grown at 28 °C, as examined in both negatively stained preparations and ultra-thin sections (Fig. 4). A core was present in sheathed flagella found in samples taken 30 min and 60 min after transfer to 28 °C. Where tubules were present in a culture grown at 34 °C before transfer, the proportion of cells possessing them appeared to decrease on incubation at the lower temperature (Tables 2 and 3), suggesting possible conversion of tubules to sheathed flagella by growth of core.

A notable feature of the regeneration of sheathed flagella of *P. stizolobii* after temperature shift-down was the apparent absence of a significant lag period before regeneration, in contrast to the considerable lag periods found for the regeneration of sheath-less flagella of *Salmonella typhimurium* and *Proteus vulgaris* after similar shift-downs (Kerridge, 1960; McGroarty et al., 1973). Kerridge (1960) found with *Salmonella typhimurium* that the lag period before recovery of motility at 37 °C after growth at 44 °C was equal to the mean generation time of the organism, and postulated that the lag might be due to the time taken for synthesis of the system responsible for formation and functioning of the flagellum. McGroarty et al. (1973) found with *Proteus vulgaris* that after shift-down of non-flagellated cells grown at 42·5 °C to 37 °C, regeneration of flagella occurred only after a lag, requiring growth at 37 °C for one generation time; they postulated that the lag may be necessary for
synthesis of RNA and proteins essential to the formation of flagellin, and that unique flagellin-synthesizing sites may exist, the components of which would be specifically inactivated at the elevated temperature. Thus, it appears that in *Salmonella typhimurium* and *Proteus vulgaris*, at least, there is a lag period of about a generation before flagella formation upon shift-down from a non-permissive temperature. In the present study, however, sheathed flagella appeared within half a mean generation time after shift-down from a non-permissive temperature. This absence of a comparable lag is an interesting difference, since the sheathed flagellum is a complex multi-component organelle compared with flagella of the organisms in which regeneration lags are known, in which only flagellin synthesis and polymerization need be considered. The difference may be significant with respect to the type of flagellum structure, but it must be noted that *P. stizolobii* is a monotrichous polar flagellate while the two organisms with regeneration lag are normally peritrichously flagellated. The difference could thus be correlated with flagellum position (Roberts & Doetsch, 1966) or reflect taxonomic differences, in addition to the possibility that it reflects flagellar structure and corresponding modes of synthesis.

Regeneration of flagella at 28 °C by cells of *P. stizolobii* grown at 34 °C was completely inhibited by a growth-inhibiting concentration of chloramphenicol (Table 3). This suggests that protein synthesis is necessary for regeneration of sheathed flagella after temperature shift-down from a non-permissive temperature. One explanation for the absence of a lag before regeneration of flagella, as found above, could be that an intracellular pool of flagellum precursors is present in cells grown at 34 °C which cannot be used for flagellum assembly at 34 °C, but which, when transferred to 28 °C, is able to be used immediately for flagellum assembly. Assuming protein synthesis was not necessary for self-assembly of such precursors into flagella, one would expect that chloramphenicol would have no inhibitory effect on regeneration of flagella at the lower temperature. Conceivably, however, pools of sheath and core precursors may be present at 34 °C but protein synthesis is required for initiation and coordination of their assembly into sheathed flagella. Martinez & Gordee (1966), working with a mutant strain of the polar flagellate *Spirillum serpens*, found that regeneration of flagella after shift-down to a permissive temperature was not inhibited by a growth-inhibiting concentration of chloramphenicol, and they concluded that the strain was capable of flagellum pool production at a non-permissive temperature, but could not assemble an ordered structure from the pool at that temperature.

In summary, the physiology of the effects of temperature on formation of sheathed flagella in *P. stizolobii* strain 0268A is similar in at least some respects to that found previously for flagella of other bacteria. There are, however, certain differences which may reflect fundamental differences in flagellar structure and synthesis. The effects of temperature on the formation of sheathed flagella suggest that there may be a considerable degree of coordination between the synthesis of core and sheath elements of the flagellum, perhaps reflecting temperature-sensitive steps common to both. If the core of the sheathed flagellum grows by addition of flagellin subunits to the distal tip of the core, as found for peritrichous sheathless flagellar filaments of *Salmonella* (Iino, 1969), and the growth rate of the core depends on the distance to be traversed by flagellin subunits from the flagellar basal body through a tubular hole in the core to the tip, as suggested for sheathless flagella by Iino (1969), then coordination between synthesis of core and sheath poses some intriguing problems in organelle growth regulation. Sheath growth would be expected to occur at the base of the flagellum, where plasma membrane-associated synthesis of outer membrane could occur. Coordination of core and sheath synthesis might be effected by a requirement for some common temperature-sensitive component or step in a synthetic pathway, perhaps a step critically dependent on plasma membrane properties and flagellar basal body components. Dependence of coordination on a particular state of the plasma membrane could account for the absence of regeneration lag in sheathed flagella noted above. If the plasma membrane were crucial to coordination, experiments with fatty acid auxotrophs of
various types might be revealing, especially with respect to temperature characteristics of flagellum formation inhibition in organisms grown under conditions producing different membrane lipid composition and thus different temperatures for phase transition in the physical state of membrane lipids. It is proposed that the sheathed flagellum of *P. stizolobii* could serve as a fruitful experimental model for investigating the regulation of biosynthesis and self-assembly in a bacterial organelle with multiple structural components.

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REFERENCES


