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Formation of Bacterial Flagella

I. Demonstration of a Functional Flagellin Pool in *Spirillum serpens* and *Bacillus subtilis*

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ABSTRACT

MARTINEZ, R. J. (University of California, Los Angeles), AND E. Z. GORDEE. Formation of bacterial flagella. I. Demonstration of a functional flagellin pool in *Spirillum serpens* and *Bacillus subtilis*. J. Bacteriol. **91**:870–875. 1966—Exponentially growing cultures of *Spirillum serpens* and *Bacillus subtilis* regained motility and flagella within one generation after mechanical deflagellation. Regeneration of flagella occurred in both cultures in the presence of chloramphenicol at concentrations shown to inhibit flagellin synthesis. Cells labeled with C^{14}-amino acids regenerated radioactive flagella in the presence of chloramphenicol. A conditional mutant of *S. serpens* (T-45) was isolated. This strain did not produce flagella when grown at 45 C, but formed the organelles upon temperature shift to 30 C, even in the presence of chloramphenicol. A reduction of intracellular antibody-precipitable flagellin counts in labeled *S. serpens* T-45 occurred concomitant with the generation of flagella at 30 C. The data suggest that the flagella of *S. serpens* and *B. subtilis* are formed from a pool of intracellular flagellin proteins.

The bacterial flagellum, because of its relative chemical (16) and structural simplicity (9, 10), ease of isolation and purification (12), and its rapid rate of synthesis (15), offers a convenient test system to investigate the biosynthesis of subcellular structures. Pertinent to the mechanism of biosynthesis of the bacterial flagellum is the possible existence of a functional pool of flagellar precursor proteins, the flagellins (3). Kerridge (7) reported that *Salmonella typhimurium* regenerated flagella under conditions where protein or ribonucleic acid (RNA) synthesis, or both, were inhibited. His results suggest either (i) that flagella are assembled from a pool of flagellin or (ii) that the biosynthesis of the protein subunits is resistant to the conventional inhibitors of protein synthesis. The experiments described in this communication were designed to distinguish between these two possibilities.

MATERIALS AND METHODS

Organisms and growth media. The organisms used in this study were *Spirillum serpens*, a bipolarly flagellated bacterium; a mutant of *S. serpens*, *S. serpens* T-45, which is nonflagellated at 45 C but which forms flagella upon temperature shift to 30 C; and *Bacillus subtilis* 168–15, a peritrichously flagellated organism requiring tryptophan and uracil for growth (provided by W. R. Romig). The spirilla were grown in nutrient broth—0.3% yeast extract (N-Y) in a shaker water bath maintained at 30 C for the parent culture and at 45 C for the mutant. Rapid temperature shifts were made by diluting exponentially growing cultures of *S. serpens* T-45 with two volumes of medium at 25 C. *B. subtilis* was grown in a shaker water bath at 37 C in a medium containing (per liter): glucose, 5.0 g; $(NH_4)_2SO_4$, 2.0 g; K_2HPO_4, 0.14 g; KH_2PO_4, 0.06 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; KCl, 7.5 g; sodium citrate, 1.0 g; and 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-HCl buffer (*p*H 7.2); supplemented with 15 μg/ml of uracil, 20 μg/ml of L-tryptophan, and 0.1% Vitamin Free Casamino Acids. The organisms were used while in the exponential phase of growth.

Incorporation of labeled amino acids into flagellin and flagella. The incorporation of C^{14}- or H^3-labeled amino acids was carried out in N-Y medium diluted 1:5 for the spirilla and in the growth medium for *B. subtilis*. The labeled amino acids were used at 0.25 to 0.5 μc/ml at different specific activities; incubations were in a shaker water bath at temperatures specified for the individual experiments. At timed intervals, samples of the cultures were rapidly chilled to 0 C and deflagellated by mechanical shearing in a Servall Omni-mixer at 24,000 rev/min for 1 min; the cells were separated from the flagella by centrifugation (6,000 × *g* for 10 min), and the flagella-containing supernatant fluid was treated with an excess of

specific antiflagellar antibody. The antigen-antibody complex was harvested by centrifugation, washed, and resuspended in 5% trichloroacetic acid containing 1% Casamino Acids (8). The trichloroacetic acid precipitate was collected on a Gelman type GM-6 membrane filter (2.5-cm diameter) and washed, and its radioactivity was determined with a liquid scintillation spectrometer by use of appropriate channels. Incorporation of labeled amino acids into cellular proteins was determined by a procedure similar to that described by Hartwell and Magasanik (6).

Determination of flagella regeneration. Four methods were used for measuring the formation of flagella: (i) resumption of motility by direct phase-contrast microscopic observation, (ii) detection of flagella by electron microscopy in either phosphotungstic acid- or uranyl acetate-stained preparations, (iii) appearance of specific flagellar antigen with the agglutination-inhibition technique (12), and (iv) appearance of flagellar antibody-precipitable counts from radioactive cells. The latter assay was used by Kerridge and was found to give only a minimal number of flagellar-associated counts (8).

Materials. Uniformly C^{14}-labeled L-leucine, lysine, and valine, and H^3-labeled lysine were obtained from New England Nuclear Corp., Boston, Mass., or Volk Radiochemical Co., Burbank, Calif. Chloramphenicol was obtained from Worthington Biochemical Corp., Freehold, N.J. Antisera were prepared as previously described (12).

RESULTS

Exponentially growing cultures of *S. serpens* and *B. subtilis*, when deflagellated and subcultured into their respective media, regenerated flagella within one doubling time. Similar results were obtained when *S. serpens* T-45 was incubated at 30 C; i.e., within one doubling time (70 min at 30 C) approximately 40 to 50% of the cells showed motility. To determine whether nondividing cells of *S. serpens* regained motility after deflagellation, chloramphenicol was used at concentrations which inhibit cell division (20 to 40 μg/ml). Under these conditions, a portion of the population regained motility, flagellar antigen was detected, and flagellated cells were observed in electron micrographs (Fig. 1). *S. serpens* T-45, grown at 45 C, was found to behave in a similar fashion when incubated at 30 C (Table 1); i.e., in the presence of 40 μg/ml of chloramphenicol, approximately 50% of the viable cells became motile after 2 hr of incubation.

To ascertain whether chloramphenicol inhibits the biosynthesis of flagellin, the following experiments were carried out. Exponentially growing cultures of *S. serpens* were deflagellated, the cells were separated from the flagella by centrifugation

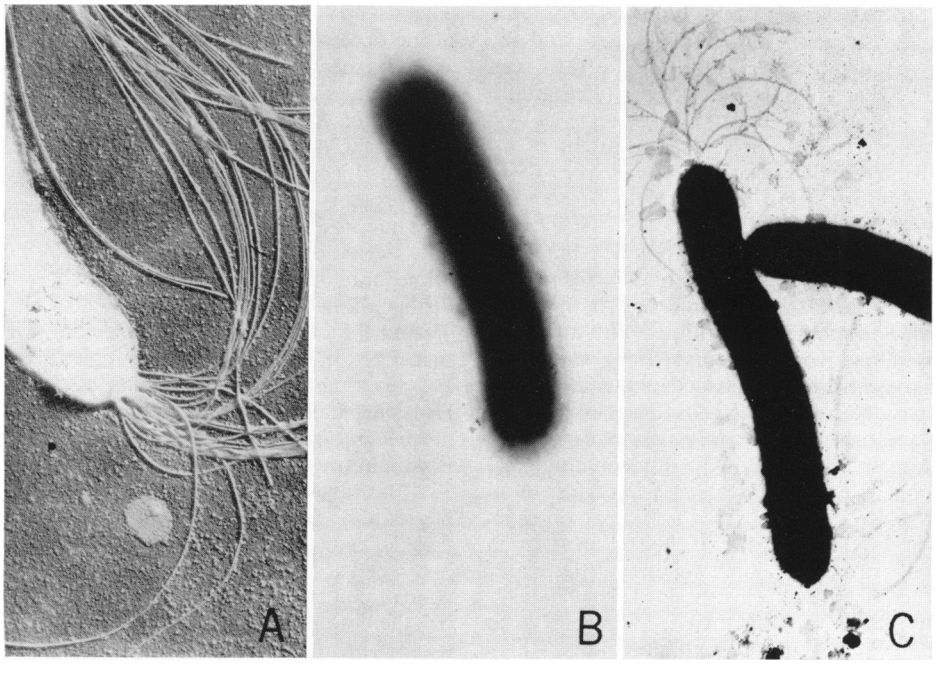

FIG. 1. *Regeneration of flagella by Spirillum serpens in the presence of chloramphenicol. (A) Platinum-palladium shadowed. (B) Mechanically deflagellated, phosphotungstic acid-stained. (C) A sample of the same culture as in B, showing regenerated flagella after 60 min in the presence of chloramphenicol as described in the text.*

TABLE 1. *Effect of chloramphenicol on the growth of Spirillum serpens T-45**

Condition	Viable-cell count/ml		Per cent motile cells after 2 hr at 30 C (based on total count)
	Initial	Final	
Control.....	1.23×10^8	3.2×10^8	30–40
Chloramphenicol treated....	1.33×10^8	8.0×10^7	25–35

* Exponential-phase cultures of *S. serpens* T-45 growing at 45 C were stepped down to 30 C by dilution with two parts of cold medium; viable counts were made immediately and after incubation at 30 C for 2 hr, and the per cent motile cells was determined at the end of the experiment by direct phase-microscopic observation.

and the cell paste was resuspended in diluted N-Y broth. To this culture, chloramphenicol was added to a final concentration of 40 μg/ml, and the culture was incubated at 30 C with shaking for 20 min. Valine-, lysine-, or leucine-C^{14} was then added, and the incubation was continued for another 2 hr. During this latter period, flagella were regenerated, as evidenced by the resumption of motility in 25 to 40% of the population. The newly formed flagella were removed from the cells, and radioactivity of the cell paste and the flagella was determined (Table 2). It can be seen in Table 2 that chloramphenicol inhibited amino acid incorporation into flagellar proteins as well as into other cellular proteins; the extent of inhibition into cellular proteins ranged from 65 to 98% and into flagellin proteins from 76 to 97% in the three experiments shown.

In an attempt to test directly for a flagellin pool, exponentially growing cultures of *S. serpens* were incubated with C^{14}-valine for 2 hr. At this time the labeled amino acid was diluted with an excess of nonradioactive compound (1% Casamino Acids); the culture was deflagellated, and the cells were separated from the flagella. The deflagellated culture was permitted to regenerate flagella by incubation in diluted N-Y broth containing 40 μg/ml of chloramphenicol. At intervals, 5-ml samples ($\cong 5 \times 10^8$ cells per milliliter) were removed and deflagellated, and the radioactivity in the flagella was determined. Assuming that chloramphenicol inhibits the synthesis of flagellin, as is suggested from Table 2, then it is reasonable to assume that antibody-precipitable counts, which appear during the incubation with chloramphenicol, arise from flagellin synthesized in the initial incubation with the labeled amino acids. After 2 hr of incubation with chloramphenicol, the antibody-precipitable counts in flagella were essentially equal to those obtained in control cultures without the drug (Table 3). Similar experiments with *B. subtilis* 168-15 have shown essentially the same results.

TABLE 2. *Inhibition of amino acid incorporation into cellular and flagellar protein of Spirillum serpens by chloramphenicol*

Labeled precursor	Condition	Count/min in cells*	Per cent inhibition	Count/min in flagella*	Per cent inhibition
L-Leucine	Control	2,040	—	700	—
	Chloramphenicol	639	69	138	80
L-Lysine	Control	993	—	33	—
	Chloramphenicol	20	98	8	76
L-Valine	Control	1,330	—	56	—
	Chloramphenicol	461	65	2	97

* Determined in 5 ml of culture.

The data suggest that cells of *S. serpens* and *B. subtilis* possess a pool of preformed flagellin proteins which is functional and serves as a precursor of the ordered flagellar structure.

To further test this hypothesis, a double-label experiment was carried out as follows. Log-phase cultures of *B. subtilis* were incubated with 0.25 μc/ml of C^{14}-valine and 12.5 μc/ml of H^3-lysine for 2 hr. Incorporation of the labeled amino acids into proteins was stopped by adding an excess of Casamino Acids and centrifuging the cells. The cells were resuspended in phosphate-buffered saline and deflagellated, and the isotopes were further diluted with Casamino Acids. After separating the cells from the flagella, the cells were suspended in growth medium supplemented with 1% Casamino Acids containing 12.5 μc/ml of H^3-lysine. Chloramphenicol was added to one half of the culture (40 μg/ml, final concentration). Both cultures were incubated with shaking at 37 C; samples were removed at intervals and deflagellated, the cells were separated from the flagella, and the C^{14} and H^3 in the antigen-antibody pellet were determined (Table 4). In the control culture incubated without chloramphenicol, there was a decrease in the C^{14}–H^3 ratio during the 1.5-hr incubation with H^3-lysine, implying that flagellin was constantly being synthesized, and that the newly synthesized protein mixed freely with the pre-existing pool of flagellin. In the chloramphenicol-treated culture, the C^{14}–H^3 ratio

TABLE 3. *Regeneration of radioactive flagella from prelabeled flagellin by Spirillum serpens in the presence of chloramphenicol*

Time of incubation in the presence of chloramphenicol	Count/min in flagella*
min	
30	2,097
60	3,121
90	2,824
120	4,748
150	4,473
150†	4,462

* Determined in 5 ml of culture. The zero-time counts (264) have been subtracted.

† Control (no chloramphenicol).

TABLE 4. *Regeneration of C^{14} and H^3 flagella from prelabeled flagellin by Bacillus subtilis 168-15 in the presence of chloramphenicol*

Condition	Regeneration time	C^{14}-H^3 ratio of flagella
	hr	
Control	0	0.258
	0.5	0.164
	1.0	0.125
	1.5	0.057
Chloramphenicol-treated	0	0.258
	0.5	0.167
	1.0	0.167
	1.5	0.187

remained relatively constant after the first 30 min. The incorporation of H^3-lysine during the first 30 min in the chloramphenicol-treated culture may be ascribed to the lack of preincubation with the drug prior to the addition of the H^3 amino acid. These experimental findings support the hypothesis that a flagellin pool exists in *B. subtilis* 168-15 and *S. serpens* cells.

S. serpens T-45, which is nonflagellated when grown at 45 C, was also found to possess a pool of flagellin proteins at the elevated temperature; this pool, upon temperature shift to 30 C, served as a precursor to the ordered flagellar structure (Table 5). The number of antibody-precipitable counts obtained after 3 hr of incubation with chloramphenicol at 30 C was similar to that obtained in the control culture incubated without chloramphenicol.

Since we are proposing that flagella are formed from a pool of flagellin in the cells, it should be possible to demonstrate a reduction in the internal flagellin pool of *S. serpens* T-45 as a result of

TABLE 5. *Formation of radioactive flagella at 30 C from prelabeled flagellin by Spirillum serpens T-45 in the presence of chloramphenicol**

Time of incubation at 30 C with chloramphenicol	Count/min in flagella†
hr	
1	2,221
2	1,279
2.5	2,562
3	3,586
3‡	3,039

* Log-phase cultures of *S. serpens* T-45 were incubated in 1:5 N-Y broth containing 0.5 μc/ml of C^{14}-valine for 2 hr at 45 C. After isotope dilution, the cultures were incubated at 30 C in N-Y broth containing 40 μg/ml of chloramphenicol. Samples were removed at intervals and deflagellated, and the radioactivity in the flagella was determined.

† Determined in 5 ml of culture. The zero-time counts (182) have been subtracted.

‡ Control (no chloramphenicol).

temperature shift and flagella production in the presence of chloramphenicol. The following experiment was designed to test this possibility. *S. serpens* was incubated in diluted N-Y broth containing 0.5 μc/ml of C^{14}-lysine for 3 hr at 45 C. After diluting the isotope, a sample of the culture was lysed with lysozyme-ethylenediaminetetraacetate (12), and the lysate was centrifuged at 100,000 × *g* for 1.5 hr. The supernatant fluid was treated with antiflagellin antiserum, and the antibody precipitable counts were determined. An equal portion of the culture was permitted to form flagella at 30 C in the presence of chloramphenicol; the flagella were then removed from the cells, precipitated with antibody, and counted for radioactivity. The deflagellated cells were washed and lysed as above, and the 100,000 × *g* supernatant fluid was similarly treated with antiflagellin antiserum and was counted. It is apparent (Table 6) that there was a drastic reduction (88%) in the intracellular antibody-precipitable counts concomitant with the generation of flagella at 30 C. We interpret this to mean that *S. serpens* T-45 is capable of producing a pool of flagellin at 45 C, but is incapable of organizing the protein subunits to the ordered structure at the elevated temperature.

DISCUSSION

The data presented support the view that the flagella of *S. serpens* and *B. subtilis* 168-15 are aggregated from a pool of intracellular flagellin proteins. This conclusion is based on the following

TABLE 6. *Reduction of flagellin antibody-precipitable counts from prelabeled Spirillum serpens T-45 after temperature shift and flagella formation*

Sample	Count/min in antigen-antibody pellet*
45 C cell lysate	522
45 C → 30 C cell lysate	63
45 C → 30 C flagella	839

* Determined in 5 ml of culture.

observations. (i) In the presence of chloramphenicol at concentrations which inhibit growth, there is a resumption of motility, appearance of specific flagellar antigen, and regeneration of flagella as seen in electron micrographs. (ii) Cells prelabeled with C^{14}-amino acids produce radioactive flagella in the presence of chloramphenicol. (iii) A reduction of intracellular antibody-precipitable flagellin counts in labeled *S. serpens* T-45 occurs concomitant with the generation of flagella at 30 C in the presence of chloramphenicol.

To explain the formation of flagella under conditions which inhibit flagellin synthesis, two processes must be involved which can be schematically represented as follows:

$$\text{Amino acids} \xrightarrow{(I)} \text{flagellin} \xrightarrow{(II)} \text{flagella}$$

Chloramphenicol presumably inhibits only the first process; thus, flagella can be synthesized in the presence of the drug provided that a pool of flagellin is present in the cell.

Weinstein, Koffler, and Maskowitz (Bacteriol. Proc., p. 63, 1960) and Kerridge (8) examined by serological means *Proteus vulgaris* and *Salmonella typhimurium*, respectively, for the presence of flagellin. Both of these groups were successful in demonstrating the existence of an antigen capable of reacting with antisera (prepared against flagella or flagellin) in the soluble fraction of cells. Kerridge (8), however, has cast some doubt on the validity of interpreting these data as evidence for the existence of a flagellin pool in *Salmonella*. Further, from measurement of the kinetics of isotope incorporation into the flagella of *Salmonella*, Kerridge (8) has concluded that a functional pool of flagellin proteins is probably not present in these organisms. The divergence between our results and those reported by Kerridge may be due to significant differences in experimental approach and to the different organisms employed. By using procedures similar to ours, however, Brinton (*personal communication*) was able to demonstrate that the pili of *Escherichia coli* may be removed by mechanical means and regenerate in the presence of chloramphenicol. Further, it appears that the pool of pili precursor proteins is considerable, since depilation and subsequent regeneration take place several times in the presence of chloramphenicol. It is possible that other bacterial organelles, besides pili and flagella, may be assembled from pools of precursor proteins.

Recently, Hartwell and Magasanik (6) demonstrated that *B. subtilis* produces the inducible enzyme histidase as an inactive protein which can be converted to the active enzyme by reactions insensitive to chloramphenicol inhibition. The conversion of the histidase precursor to the active enzyme parallels the conversion of pili and flagella precursor proteins to their respective structures in that the processes apparently do not involve peptide bond formation.

Assuming that the conversion of amino acids to flagellin occurs via a set of reactions normally associated with protein synthesis, we must still account for the second reaction which is necessary for the synthesis of the ordered structure, i.e., the aggregation of flagellin molecules to form the flagellum. The experiments of Abram and Koffler (1), Asakura, Eguchi, and Iino (2), Lowy and McDonough (11), and Nossal, Ada, and Austin (14) show that flagellin molecules may undergo self-assembly to form a structure which is almost indistinguishable from the native flagellum (11). Both Abram and Koffler (1) and Asakura et al. (2) have suggested that the in vivo aggregation of the subunits to form the flagellum is a spontaneous phenomenon. In the experiments described by Abram and Koffler (1), aggregation of the subunits appears to be controlled by the *p*H of the solution, as is the final product of the reaction, i.e., straight structures or flagellalike filaments. Asakura et al. (2), on the other hand, reported that "seeds" (flagella fragments) are required as sites of aggregation and growth of the flagellum, and they compared the process of flagellar growth with that of crystallization. The fact that subunits of supermolecular structures can spontaneously aggregate in vitro to form native structures does not apply only to the flagellins; it has long been known that tobacco mosaic virus protein and RNA aggregate abiologically to form infectious particles (5). This ability to aggregate must be inherent in the structure of the subunits (4).

If, indeed, the in vivo biosynthesis of structures occurs by self-assembly independent of biological regulation, then several observations must be explained. Cellular division of a bipolarly flagellated cell such as *S. serpens* results in two daughter cells, each of which has inherited one maternal tuft of flagella. Shortly thereafter, a new tuft of

flagella appears at the nonflagellated pole. The inherited maternal tuft, however, increases neither in length nor in numbers; nor is there any disaggregation at the ends of these flagella permitting uniform growth at both poles. This is verified by the fact that growth media do not contain flagellar or flagellin antigen. Further, if cultures of spirilla are deflagellated and then allowed to regenerate flagella, both poles of the cells appear to be equally competent in regenerating the organelles. These, and other observations, suggest that a biologically controlled mechanism is involved in aggregating the flagellins to an ordered structure. Such a control mechanism is difficult to envision if the final step in the production of the flagellum is a spontaneous phenomenon, especially since a pool of flagellin has been shown to be present intracellularly in spirilla and *B. subtilis*.

Finally, the nonflagellated *S. serpens* T-45, which we have shown to possess a pool of flagellin proteins, but which is incapable of organizing these proteins to the organelle at 45 C, must be explained. We have made several observations which bear on this problem. It is possible that at 45 C flagellin is aggregated to a structure which is unstable at the elevated temperature and disaggregates upon extrusion. If this were the case, we would anticipate finding flagellin antigen in growth media. This has not been found, nor are nascent flagella formed upon temperature shift to 30 C dissociated upon elevating the temperature of the culture to 45 C. Rather, we found a gradual loss of motility of the cells concomitant with an increase in cell numbers, i.e., a dilution of the pre-existing organelles.

In conclusion, we suggest that the in vivo aggregation of flagellins (and possibly other subunit proteins) to the ordered structure is not a spontaneous occurrence, but rather that it is mediated by a biological organizing principle.

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