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Regulation of Flagellar Morphogenesis by Temperature: Involvement of the Bacterial Cell Surface in the Synthesis of Flagellin and the Flagellum

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When cells of *Proteus vulgaris* were transferred from 37 to 42 C, a temperature at which they continue to grow almost optimally, they ceased to form flagella after approximately one generation time. This failure was due to a lesion in the flagellin-synthesizing process rather than the inability of these cells to assemble the organelle from constituents once formed. After transfer back to 37 C, these cells regained their ability to synthesize flagellin and form flagella, after one generation. When added during this period, chloramphenicol, rifampin, or penicillin prevented the synthesis of flagellin. The regeneration of the organelle at 37 C, then, requires growth for one generation, a period during which not only ribonucleic acid and protein synthesis, but also the presence of an intact cell envelope or concurrent synthesis of cell wall, are required.

The bacterial flagellum consists of at least three morphologically distinct substructures. A helical microtubule represents over 95% of the organelle and is composed of the protein flagellin (14). This filament is a continuation of the hook, which is distinct from the filament in its morphology and fine structure, stability, and antigenicity (14). The hook originates from the basal structure, which is intimately associated with the cellular surface layers (14).

The formation of bacterial flagella appears to be sensitive to environmental conditions. For example, ferrous, ferric, and aluminum ions suppress the formation of flagella (15). In a Trypticase-yeast extract medium, cells of *Proteus vulgaris* have an average of 7 flagella/cell, whereas in a tryptone-yeast extract medium this number varies between 15 and 70 (E. McGroarty, H. Koffler, and R. W. Smith. Bacteriol. Proc. p. 27, 1971). Glucose prevents motility and the formation of flagella in cells of *Escherichia coli* (2). Furthermore, adenyl cyclase mutants cannot make flagella or exhibit chemotaxis. These mutants, however, synthesize flagella when given 3′,5′-cyclic adenosine monophosphate (AMP). Glucose competitively suppresses the activation of chemotaxis by cyclic AMP (5, 18).

Temperature has a dramatic effect on flagellation. For example, when a culture of *Salmonella paratyphi* is transferred to a suboptimal growth temperature (from 37 to 20 C), the synthesis of flagella is inhibited. Growing cells of *S. typhimurium* above their optimum (at 44 C) prevents flagellation (6, 12); after transfer of a growing culture to 44 C, the existing flagella distribute themselves among the daughter cells as the cells continue to divide. Similar effects of elevated temperatures on flagellation have been observed for cells of *P. vulgaris* (3), *E. coli* (8, 9), and *Pasteurella pseudotuberculosis* (11). When cultures grown at a relatively high temperature are shifted back to lower temperatures, the flagella are resynthesized after a short lag. The nature of the lesion caused by growth at higher temperatures is not yet understood. For example, Morrison (8) reported that cells of *E. coli* previously rendered nonflagellated by growth at an elevated temperature were able to regenerate some of their flagella at 20 C, even in the presence of chloramphenicol. One interpretation of this observation could be that the inhibition of flagellation at an elevated temperature occurs somewhere in the polymerization of flagellar proteins; however, this does not seem to be the case. There are indications that growth at a relatively high temperature prevents flagellation through effects exerted on the cell surface structures. Cells of *E. coli* and *S. typhimurium* grown at 44 C are unable to

resynthesize flagella at 37 C in the presence of penicillin (16, 17), which interferes with the synthesis of the peptidoglycan layer of the wall. After growth at 44 C, mutants of *E. coli* incapable of synthesizing diaminopimelic acid, a constituent of this layer, are unable to synthesize flagella at 37 C unless diaminopimelic acid is added (16). Astonishingly, penicillin inhibits the regeneration of flagellin as well. Since the formation of flagellin and flagella can be turned off and on by temperature, this switching represents a useful experimental situation with which the steps in the formation of the flagellum, as well as the regulation of flagellation, can be examined.

MATERIALS AND METHODS

Organism. Cells of *P. vulgaris* strain P used in this study were grown in 2-liter flasks containing 500 or 750 ml of nutrient medium consisting of 1% tryptone (Fisher Scientific Co.) and 0.2% yeast extract (Difco) at *p*H 7.0. Unless specified otherwise the cells were grown at 37 C; aeration was provided by agitation on a New Brunswick gyratory shaker.

Purification of flagellin. Flagellar filaments were isolated according to the procedure of Abram and Koffler (1). The crude pellets of flagellar filaments were resuspended in distilled water to a final concentration of ca. 3 mg/ml. The suspension was adjusted to *p*H 2.0 with 1 N HCl, and was stirred at 26 C for 30 to 60 min; this solubilizes the polymer to give a solution of monomeric flagellin. The "*p*H 2.0 insoluble" material was removed by centrifugation at 12,000 $\times g$ for 30 min at 4 C. The supernatant solution, diluted 4:1 (v/v) with 2.5 M NaH_2PO_4 buffer adjusted to *p*H 7.2 with NaOH, was allowed to sit at 26 C for 6 to 8 hr; this results in the reassembly of flagellin to yield filaments (Mautner and Lowy, *personal communication*). These reconstituted filaments then were sedimented by centrifugation at 78,000 $\times g$ for 60 min at 4 C. When the pellet was resuspended in distilled water and adjusted to *p*H 2.0, the protein was shown to be homogeneous upon electrophoresis in urea-polyacrylamide gels (4, 10).

Preparation of antiflagellin antiserum. Purdue Dutch rabbits were used for the production of antiflagellin antibodies. All injections were made subcutaneously behind the neck. Purified flagellin solutions were adjusted to *p*H 7.0 with NaOH, and were buffered with 0.01 M NaH_2PO_4, adjusted to *p*H 7.0 with NaOH. All rabbits received two injections of 1 mg of flagellin in an incomplete Freund's adjuvant (45% Marcol 52, 5% Arlacel, v/v), 7 to 10 days apart. Two additional injections consisted of 0.5 to 1 mg of flagellin in 0.01 M NaH_2PO_4, *p*H 7.0, 7 to 10 days apart. Ten days after the fourth injection, the rabbits were bled from the marginal vein of the ear. Two weeks after the rabbits were bled, 0.5 to 1 mg of flagellin was again injected; 10 days later the rabbits were bled again. This cycle was repeated four to five times.

The whole blood collected from the rabbits was allowed to sit at 26 C for approximately 1 hr, and then overnight at 4 C. The clots were centrifuged at 5,000 $\times g$ for 30 min. The supernatant solutions were centrifuged in the same manner to remove all blood cells. The serum was then frozen rapidly in liquid nitrogen and then stored in a freezer at −20 C.

Extraction of total flagellin. Cultures of *P. vulgaris* were harvested at the middle of the logarithmic phase of growth and were fixed with potassium cyanide and chloramphenicol at final concentrations of 0.002 M and 20 μg/ml, respectively. After the cells had been washed, they were resuspended in a solution of 0.1% lysozyme, 0.02 M tris(hydroxymethyl)aminomethane buffer (*p*H 8.0), 0.055 M sodium ethylenediaminetetraacetate, and 0.55 M lactose (20%). This suspension was incubated at 37 C for 1 hr and then centrifuged at 15,000 $\times$ *g* for 30 min. The resulting spheroplasts were resuspended in 0.01 M KCl, and lysate was incubated in the presence of 20 μg of deoxyribonuclease/ml for 30 min. The solution was then adjusted to a *p*H of 2.0 with 1 N HCl and centrifuged at 15,000 $\times$ *g* for 25 min. The pellet was washed once with 0.01 M KCl at *p*H 2.0, and the resulting supernatant solutions were combined. This solution was then dialyzed against HCl (*p*H 2.0) for 8 to 10 hr and then against 0.01 M NaH_2PO_4 at *p*H 7.0 for 8 to 10 hr. After dialysis at a neutral *p*H, a precipitate formed, which was removed by centrifugation at 15,000 $\times$ *g* for 30 min. The pellet was suspended in 0.01 M NaH_2PO_4 at *p*H 7.0 and centrifuged as before. The combined supernatant solutions were concentrated 20- to 50-fold by ultrafiltration with an Amicon ultrafiltration device equipped with a UM 10 membrane.

Extraction of acid-soluble flagellin. Cells were grown to the middle of the logarithmic phase of growth. Portions of the culture, 100 ml in size, were removed and fixed with KCN and chloramphenicol. These cells were washed once with 0.15 M NaCl-0.01 M NaH_2PO_4 at *p*H 7.0. The pellets were resuspended in 25 ml of HCl (*p*H 2.0); the resulting suspensions were stirred for 30 to 60 min at room temperature, and were subsequently centrifuged at 12,000 $\times$ *g* for 30 min. The "*p*H 2.0 insoluble" material was washed once with 10 ml of HCl (*p*H 2.0). The supernatant liquids were combined, adjusted to *p*H 7.0 with 0.1 M KOH, and buffered in 0.01 M NaH_2PO_4 (*p*H 7.0). Adjustment of the *p*H to 7.0 resulted in the formation of a precipitate, which was sedimented at 12,000 $\times$ *g* for 30 min and washed once with 10 ml of 0.01 M NaH_2PO_4 buffer at *p*H 7.0. The combined supernatant fluids were then concentrated by ultrafiltration as before.

Mechanical deflagellation. Cultures grown to an A_{560} of approximately 0.25 (i.e., the middle of the logarithmic growth phase) were blended in 700 to 750 ml of the used culture medium for 45 sec in a Waring blendor. Immediately after this treatment, fewer than 1% of the cells were motile, and the cells contained less than 6% of the amount of acid-soluble flagellin present prior to deflagellation. The turbidity of the culture changes only slightly owing to blending,

and after little or no lag the cells resumed growth at the same rate as before deflagellation.

Methods for detecting flagellin. The concentration of flagellin was determined by a modification of the method described by Ryan (13). The method involves diffusion of the antigen from wells into an agar medium containing specific antibodies. The agar plates were made by melting a solution of 1.2% purified agar which was 0.01 M with respect to NaH_2PO_4 (*p*H 7.0), and mixing this at a ratio of 25:1 (v/v) with antiflagellin serum. Portions of this antibody-agar solution, 15 ml in size, were poured into standard plastic petri dishes (Falcon Plastics) and allowed to solidify. Seven wells, 3.8 mm in diameter, were punched in the agar, and these wells were filled with 20 or 25 μliters of either standard or unknown solutions of flagellin. Values for the diameter of the precipitin rings measured after 6 to 8 hr of incubation at room temperature gave a straight-line relationship when plotted against the logarithm of the flagellin concentration. When the diameters of the rings were measured 36 to 48 hr after the addition of antigen, the logarithms of the diameter of the precipitin rings were directly proportional to the logarithm of the concentration of flagellin. The limit of detection of flagellin was between 7 and 15 μg/ml, and the accuracy of measurement was 10 to 20%.

Quantitative measurements. Protein determinations were performed by the method of Lowry et al. (7). For flagellin determinations, a standard curve was prepared with *P. vulgaris* flagellin as an authentic sample; this preparation had been purified by two cycles of reconstitution of filaments and acid solubilization. The number of flagella per cell was determined electron microscopically; 200 to 250 cells were examined for each determination. Cell culture turbidity measurements were made with a Beckman DU spectrophotometer having a 0.07 mm slit width at 560 nm wavelength.

Biochemicals. Chloramphenicol (*d*-threo) was obtained from Calbiochem and used at a concentration of 20 μg/ml. Rifampin (Mann Research Laboratories) was used at a concentration of 100 μg/ml. Buffered potassium penicillin G used in this work was obtained from E. R. Squibb & Sons. Spheroplasts were prepared according to a modified procedure used by Weinstein (Ph.D. Thesis, Purdue Univ., Lafayette, Ind., 1959). During the logarithmic phase of growth, penicillin was added at a final concentration of 1,000 units/ml with 20% lactose. The incubation was continued for 45 to 120 min at 37 C, and the spheroplasts were centrifuged at 12,000 × *g* for 20 min.

Egg white lysozyme, three times recrystallized, was obtained from Sigma Chemical Co. Bovine pancreatic deoxyribonuclease, 2,200 units/mg, was obtained from Schwarz BioResearch, Inc.

Electron microscopy. Observations were made with a Philips 300 electron microscope operating at 60 kv with a 20 μm objective aperature. Carbon-coated collodion films supported by 200-mesh copper grids were used for viewing all preparations. The specimens were negatively stained with either filtered 1% potassium phosphotungstate, *p*H 7.0, or 2.5% ammonium molybdate, *p*H 7.0

RESULTS

The rate of synthesis of flagellin in cultures of *P. vulgaris* grown in a tryptone-yeast extract medium increased during the logarithmic phase (Fig. 1), as was determined by assaying for acid-soluble flagellin in portions of cells removed from nutrient broth at various times during the logarithmic phase of growth (insert, Fig. 1). The amount of flagellin present in cells growing at 37 C increased at a rate of 0.3 to 0.6 μg of flagellin per mg of cell dry weight per min. The observation that cells in the late logarithmic growth phase contain more flagellin per cell than those early in the growth phase can be accounted for by a larger number of flagella as determined by counts made with an electron microscope.

When normally flagellated cells of *P. vulgaris* growing in nutrient broth at 37 C were shifted to 42.5 C in the early portion of the logarithmic growth phase, flagellin synthesis ceased after a lag of 50 to 60 min, approximately one generation. Figure 2 shows the results of experiments in which cells grown at 37 C were shifted to 42.5 C. The rate of cell growth changed little after transfer to the higher temperature. Portions of 100 ml of the culture were removed at various times after the shift-up and were assayed for acid-soluble flagellin. The results indicate that for the first 50 to 60 min after transfer to 42.5 C flagellin synthesis increased at a rate of 0.4 μg of flagellin per mg of dry weight per min, close to the rate in cells growing at 37 C. After 60 min, the flagellin concentration in the culture remained essentially constant; this suggests either that no new flagella were being formed or that the rates of synthesis and destruction of flagellin and flagella had become equal. Although the flagellin concentration in the culture remained constant, the number of flagella per cell decreased after 40 to 50 min, a predictable event if the existing flagella are distributed among daughter cells as the cells continue dividing (Fig. 3). At 60 to 70 min after the inhibition of net increase of flagellin, the number of flagella per cell had decreased to half. This is approximately the same time necessary for the number of cells to double. Apparently, after a lag of 50 to 60 min the existing flagella do not break down, but new flagella are not being formed.

Cells grown at 42.5 C for more than 12 generations were nonflagellated and nonmotile. When such cells were then grown to the early logarithmic growth phase, less than 0.001% of

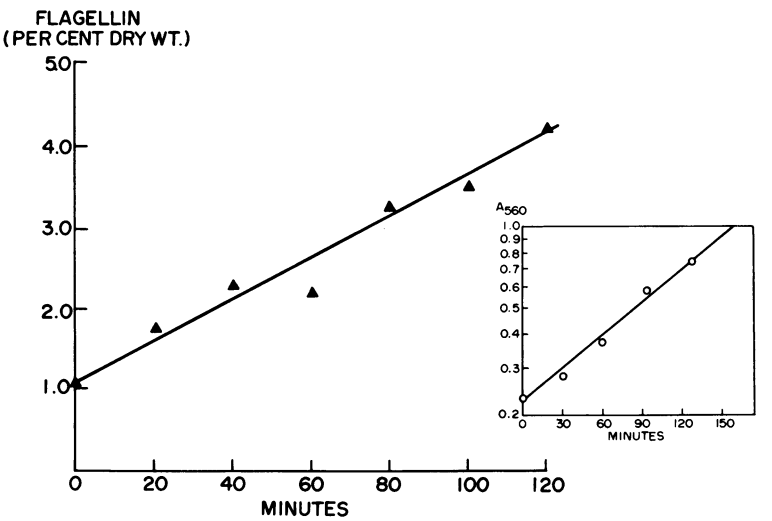

FIG. 1. *Percentage of the dry weight of cells as flagellin in cultures during growth at 37 C. The inset indicates the increase in absorptivity at 560 nm during cell growth.*

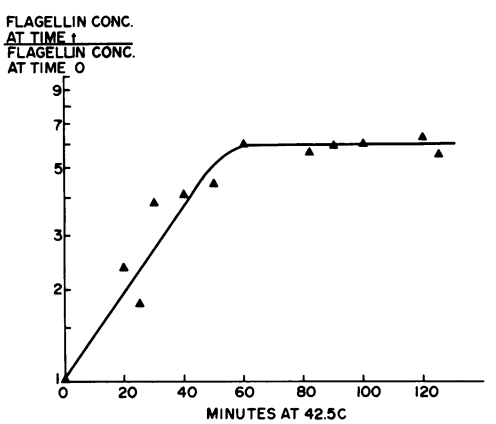

FIG. 2. *Relative amount of acid-soluble flagellin in cells after shifting the culture from 37 to 42.5 C. This relative concentration is the amount of flagellin in the culture at given times after the shift divided by the amount of flagellin assayed at the time of the shift.*

their dry weight was serologically detectable flagellin. Thus, at temperatures slightly above optimum, the major component of the flagellum was not being synthesized, although the cells were still capable of growing and synthesizing several hundred or thousand other proteins. Most likely, the synthesis of other components of the flagellum was also inhibited; for example, hooks and basal components of the flagellum could not be detected electron microscopically in lysates of cells grown for more than 12 generations at 42.5 C. Either the constituent molecules of these structures are not synthesized at 42.5 C or, if synthesized, are incapable of assembling due to other factors, such as the lack of nucleation sites.

When nonflagellated cells grown at 42.5 C were shifted to 37 C, flagellin synthesis began only after a lag of 60 to 70 min. Figure 4 depicts the results from experiments in which cells rendered nonflagellated by growth at 42.5 C were transferred to 37 C and assayed for total flagellin at various times after the transfer. Flagellar filaments also first became visible by ca. 60 min after the shift. The characteristic rate of flagellin synthesis for cells growing at 37 C was reached by 90 to 95 min after shift-down, and full flagellation was reached by approximately 120 min.

The actual assembly of the flagellum from already formed components, however, did not appear to be impaired at the relatively higher temperature (Table 1). Cells rendered nonflagellated by growth at 42.5 C were shifted to 37 C for various periods of time. Portions were then shifted back to 42.5 C, and the cells were observed for motility and flagellation at the end of 120 min after the initial shift. Cells that had been incubated for 45 to 75 min at 37 C and shifted back to 42.5 C were flagellated at the end of 120 min. Normally, however, cells shifted from 42.5 to 37 C did not begin or resynthesize flagella until 60 min after the shift. Thus, cells exposed for 45 to 60 min to 37 C were able to develop flagella even at the higher temperature. The minimal recovery time at 37 C appeared to be 45 min. The lesion caused by high temperature, therefore, does not reside in

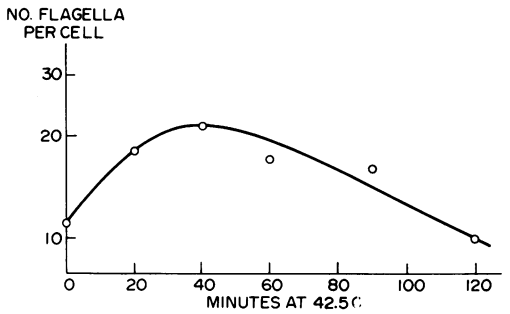

FIG. 3. *Average number of flagella per cell in a culture after shifting from 37 to 42.5 C.*

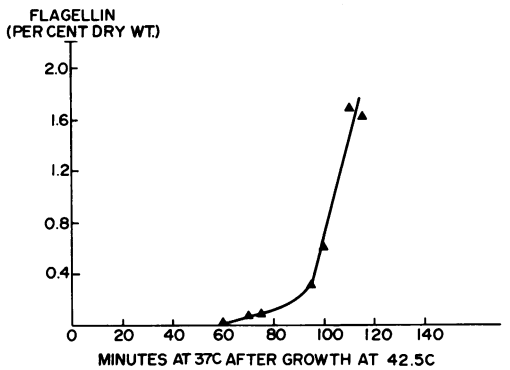

FIG. 4. *Percentage of the dry weight of cells as flagellin in cultures after shifting the cells from 42.5 to 37 C. The flagellin was isolated according to the procedure for total flagellin. Prior to the shift to 37 C, the cultures were grown at 42.5 C for more than 12 generations.*

TABLE 1. *Flagellation of cells grown at 42.5 C and then exposed to 37 C for various time intervals*

Time after growth at 42.5 C (min)		Percent motile cells	Percent flagellated cells	No. of flagella per cell
At 37 C	Then 42.5 C			
0	120	0.8	0	0.0
15	105	0.2	1	0.01
30	90	1.0	0	0.0
45	75	39	27	0.7
60	60	56	65	6.1
75	45	91	86	12
120	0	98	100	26

the polymerization of already formed components.

To test whether inhibition of protein synthesis also inhibits the formation of flagella, chloramphenicol was added to cells after they had been shifted from 42.5 to 37 C. Normally, when chloramphenicol at a concentration of 20 μg/ml is added to growing cultures of *P. vulgaris*, the rates of growth and protein synthesis are reduced to less than 10% within 5 to 10 min. Cultures grown at 42.5 C were shifted to 37 C. After various periods of time, chloramphenicol was added, and incubation at 37 C was continued until the total time at 37 C was 120 min. The cells then were fixed with 0.002 M KCN and assayed for total flagellin. The results of these experiments, given in Table 2, indicate that if chloramphenicol is added at any time during the lag after shift to 37 C prior to the formation of flagella no flagellin can be demonstrated. If chloramphenicol is added after flagellin synthesis has started, further synthesis is inhibited. Chloramphenicol added at 75 min, a time at which both flagellin and flagella had begun to reappear, reduced the amount of flagellin synthesized by 120 min. The final amount of flagellin in a culture which was treated with chloramphenicol at 75 min after shift-down and further incubated for 45 min was approximately the same as that from cells harvested at 75 min after shift-down.

That ribonucleic acid (RNA) synthesis is necessary for the regeneration of flagella was also examined. Rifampin at a concentration of 100 μg/ml added to normally growing cells of *P. vulgaris* reduced total RNA synthesis to less than 10% after 20 to 25 min. Cells rendered nonflagellated by growth at 42.5 C were exposed to rifampin at various times after shifting to 37 C. The cells were incubated for a total of 120 min at 37 C, and then assayed for total flagellin. The results (Table 3) show that when rifampin was added during the early part of the lag after the cells had been shifted to 37 C, they were incapable of synthesizing either flagellin or flagella. If the inhibitor was added at 50 min or later, the cells were capable of forming these only in much reduced amounts.

To detect whether these inhibitors act on flagellin synthesis, the regeneration of flagellar

TABLE 2. *Effect of chloramphenicol on the regeneration of flagellin and flagella*

Time of addition of chloramphenicol (min after shift from 42.5 to 37 C)	Flagellin (percent dry wt after 120 min at 37 C)	No. of flagella per cell (after 120 min at 37 C)	No. of flagella per cell in absence of chloramphenicol
0	<0.03	0	0
25	<0.05	0	0
50	<0.04	0.19	0.7
75	0.06	0.31	12

TABLE 3. *Effect of rifampin on the regeneration of flagellin and flagella*

Time of addition of rifampin (min after shift from 42.5 to 37 C)	Flagellin (percent dry wt after 120 min at 37 C)	No. of flagella per cell (after 120 min at 37 C)	No. of flagella per cell in absence of rifampin
0	<0.05	0	0
25	<0.06	0	0
50	0.09	0.31	0.7
75	0.56	1.6	12

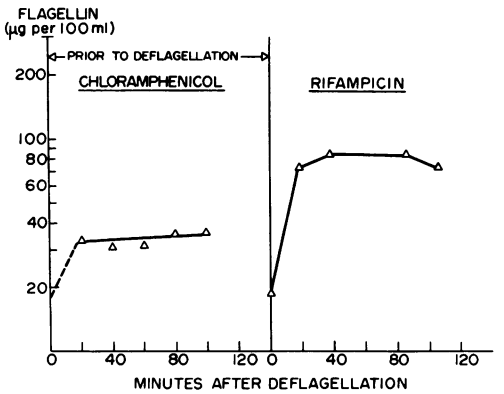

FIG. 5. *Amount of acid-soluble flagellin from cells in cultures grown at 37 C, mechanically deflagellated, and then incubated in the presence of either 20 μg of chloramphenicol/ml or 100 μg of rifampin/ml at 37 C.*

filaments by mechanically deflagellated cells was examined. Unlike cells in which the formation of the complete flagellum is prevented at the higher temperature, mechanically deflagellated cells retain their basal structure and often the hooked portion of the flagellum. Thus, it is possible to distinguish between the effect of inhibitors on the formation of the whole flagellum or just on the filament. Flagellated cells sheared in a Waring blendor to remove their flagellar filaments regenerated the full complement of filaments within 60 to 80 min. Mechanically deflagellated cells treated with chloramphenicol were incubated further at 37 C. At various intervals of time after addition of the inhibitor, samples of the culture were assayed for acid-soluble flagellin (Fig. 5). The results indicate that the resynthesis of flagellin in the presence of chloramphenicol was reduced to 5 to 10% of the level prior to deflagellation. Similar experiments performed with mechanically deflagellated cells inhibited by rifampin indicated that this antibiotic also inhibited the synthesis of flagellin (Fig. 5). Rifampin reduced flagellin synthesis to levels lower than the amount prior to deflagellation even after 100 min. That rifampin-inhibited cells contain more flagellin than chloramphenicol-inhibited cells probably is due to the fact that the synthesis of messenger RNA for flagellin initiated before the addition of rifampin would be completed, and therefore such messenger RNA could participate in the synthesis of flagellin, whereas chloramphenicol stops the synthesis of flagellin more directly.

To test the hypothesis that flagellin synthesis may require the synthesis of other proteins during the lag after shift-down, cells were grown at 42.5 C and then shifted to 37 C in the presence of either chloramphenicol (Fig. 6) or rifampin (Fig. 7). The cultures were incubated at 37 C for 90 min in the presence of one of the inhibitors and then washed free from the inhibitor; 90 min was required for flagellin synthesis to reach a constant rate at 37 C after growth of cells at 42.5 C in the absence of inhibitor. Half of the cells were incubated further at 37 C and the other half at 42.5 C, both without antibiotic. After the removal of the antibiotic, the cells were observed for regeneration of flagella and the resumption of motility; they started to divide after a lag of 60 to 70 min. As Fig. 8 and 9 indicate, motility and flagella were detectable 180 to 200 min after the removal of the inhibitor and incubation at 37 C. This represents a lag of 110 to 140 min after the resumption of growth. Regeneration occurred only if the cells were incubated at 37 C; therefore, there appears to be a lag at 37 C during which both protein and RNA synthesis must occur before flagella can form. The synthesis of whatever is being made

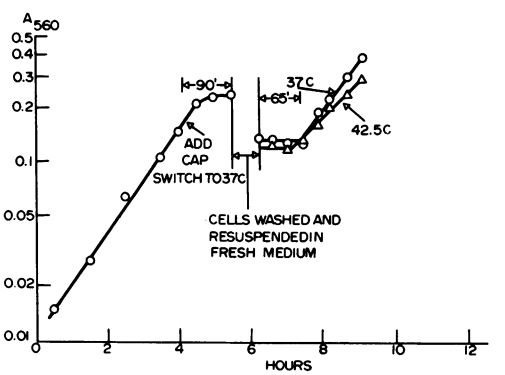

FIG. 6. *Absorptivity at 560 nm of a culture grown at 42.5 C, shifted to 37 C in the presence of chloramphenicol (CAP) for 90 min, washed with fresh medium, and then incubated further at either 37 or 42.5 C. Prior to the initial shift to 37 C, the culture was grown at 42.5 C for more than 12 generations.*

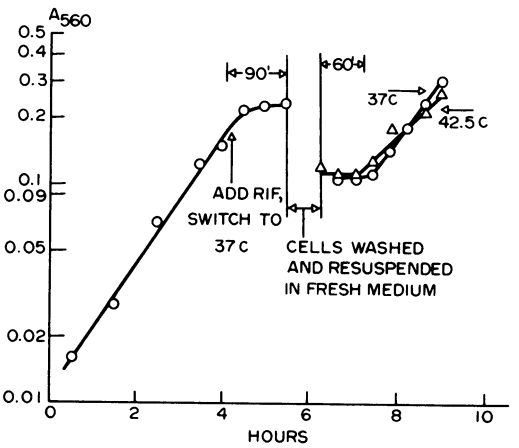

FIG. 7. *Absorptivity at 560 nm of a culture grown at 42.5 C, shifted to 37 C in the presence of rifampin (RIF) for 90 min, washed with fresh medium, and then incubated further at either 37 or 42.5 C. Prior to the initial shift to 37 C, the culture was grown at 42.5 C for more than 12 generations.*

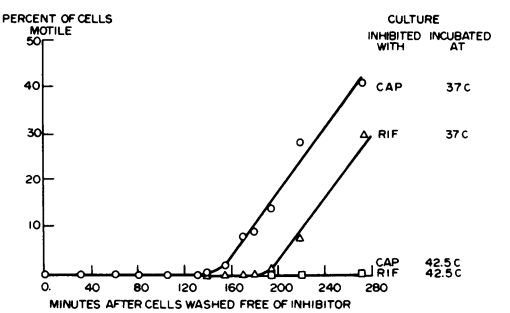

FIG. 8. *Percentage of cells that were motile in cultures which were grown at 42.5 C, shifted to 37 C in the presence of either chloramphenicol (CAP) or rifampin (RIF) for 90 min, washed with fresh medium, and then incubated further at either 37 or 42.5 C. Prior to the initial shift to 37 C, the cultures were grown at 42.5 C for more than 12 generations.*

during this lag is inhibited during growth at 42.5 C.

To test whether the inhibition of cell wall synthesis has any effect on the regeneration of flagella, the following experiments were performed. Cells of *P. vulgaris* rendered nonflagellated by growth at 42.5 C were shifted to 37 C, and penicillin was then added at various times; 20 min after the addition of penicillin, cells began to show small protrusions indicative of the fact that portions of their wall were no longer functional. The cells were incubated for a total of 120 min at 37 C, fixed with KCN and chloramphenicol, and then prepared for electron microscopy. Table 4 indicates the degree of flagellation of pencillin-treated cells and the

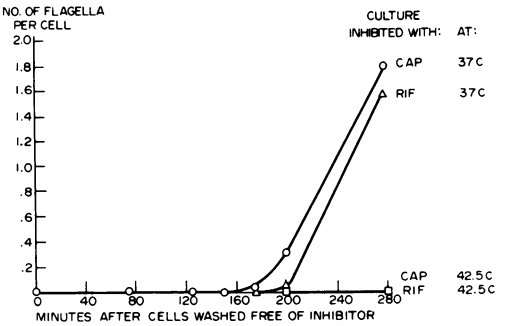

FIG. 9. *Average number of flagella per cell in cultures that were grown at 42.5 C, shifted to 37 C in the presence of either chloramphenicol (CAP) or rifampin (RIF) for 90 min, washed with fresh medium, and then incubated further at either 37 or 42.5 C. Prior to the initial shift to 37 C, the cultures were grown at 42.5 C for more than 12 generations.*

TABLE 4. *Effect of penicillin on flagellar regeneration after shift from 42.5 to 37 C*

Addition of penicillin (min at 37 C)	No. of flagella per cell after 120 min at 37 C	Percent motile cells after 120 min at 37 C	No. of flagella per cell in absence of penicillin
0	0	0	0
20	—	0	0
30	0	0	0
40	0.09	0	0.1
50	0.5	6.1	0.7
60	0.8	17	3
70	1.4	31	6
80	2.8	44	14

relative number of motile cells at the end of 120 min. If penicillin was added early during the lag after shift to 37 C, the number of flagella produced at the end of 120 min was greatly reduced. To test the effect of penicillin specifically on flagellin synthesis, cells were grown at 42.5 C and shifted to 37 C; portions of the culture were inhibited with penicillin at various times before and after the shift to 37 C. These cells were incubated for a total of 120 min at 37 C and then assayed for total flagellin. The results (Fig. 10) indicate that when penicillin was added within 30 min after the shift to 37 C the cells contained only 0.04% of their dry weight as flagellin, as compared to 1.8% in untreated cells, by 120 min. Increasing amounts of flagellin were synthesized as the addition of penicillin after shift-down was postponed. Another antibiotic capable of interfering with cell wall synthesis, cycloserine, added within 40 min after shift to 37 C at a concentration of 100 μg/ml to cultures rendered nonflagellated by

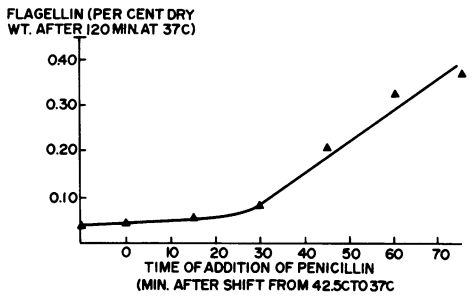

FIG. 10. *Percentage of the dry weight of cells as flagellin in cultures grown at 42.5 C, shifted to 37 C, inhibited with penicillin at various times before and after the shift, incubated for 120 min at 37 C, and then fixed with 0.002 M KCN and 20 μg of chloramphenicol/ml. The flagellin was isolated according to the procedure for total flagellin. All cultures were grown for more than 12 generations prior to the shift to 37 C.*

growth at 42.5 C, completely inhibited motility as determined by examination at the end of 120 min of incubation at 37 C.

These results indicate that some of the processes occurring during the regeneration of both flagellin and flagella after the shift from 42.5 to 37 C may be associated with changes or development of the cell envelope. The steps in regeneration that occur during the first 30 min after the shift to 37 C appear to be especially sensitive to the inhibition of cell wall formation.

DISCUSSION

The synthesis of the flagellum appears to be sensitive to the environment and under control of one or more specific regulatory mechanisms. There appears to be some regulation of flagellation throughout the growth cycle, at least in the closed system that nonsynchronized flask cultures represent. Cultures of *P. vulgaris* growing in nutrient media synthesized flagellin at an increasing rate throughout the logarithmic phase of growth.

Above 42 C, cells are no longer capable of forming flagella, in spite of the fact that they are able to multiply rapidly; this inhibition appears to act at some step prior to flagellin synthesis. Cells grown at 42.5 C contain no serologically detectable flagellin. Neither hooks nor basal structures could be detected in preparations of lysates when viewed electron microscopically. It is possible, but unlikely, that the individual proteins essential to the formation of these structures are being synthesized, but cannot be assembled under the prevailing circumstances. Synthesis of flagellin after growth at the higher temperature requires approximately one generation of growth at the lower temperature. A similar lag of 40 to 60 min is required before flagellin synthesis is turned off upon shift-up to 42.5 C. This temperature regulation may be due to the activation and inactivation of some specific regulatory system, since at the high temperature the cells seem to grow normally in every other aspect. The higher temperature does not interfere with the assembly of the organelle from previously formed constituents because flagella are formed at 42.5 C after a short exposure to 37 C. Therefore, a temperature above 42 C must inhibit the formation of flagella at some point other than at the polymerization of subunits.

It appears that active messenger RNA for flagellin is not being synthesized at a temperature above the optimal growth temperature, since rifampin, which acts at the level of transcription, inhibits the synthesis of flagellin after shift-down of nonflagellated cells to 37 C. Probably the messenger is not being made until 50 min or later after the shift to 37 C.

The lag in the synthesis of flagellin after cells have been shifted from 42.5 to 37 C may be caused by the necessity to synthesize RNA and proteins essential to the formation of flagellin. This requirement is implied by the recurrence of an additional lag in the formation of flagella after the removal of chloramphenicol or rifampin (Fig. 8 and 9).

The formation of cell wall is associated with the regeneration of flagella by cells grown at high temperatures. Since the basal structure of the flagellum appears to be closely associated with the cell surface, this is not surprising. However, penicillin, when added to nonflagellated cells during the first 30 min after a shift from 42.5 to 37 C, also inhibits by 98% the regeneration of flagellin. This result implies that the processes occurring during the early part of the lag before the regeneration of flagella also require either the presence or the concurrent synthesis of intact surface structures.

How the absence of cell wall or cell wall synthesis inhibits the formation of both flagellin and flagella is not obvious. The lack of flagellin synthesis in the presence of penicillin may be due to some indirect result of cell wall inhibition; that is, it may be caused by changes in metabolism due to the formation of spheroplasts. On the other hand, the lack of wall may directly influence the membrane sites at which flagellin is being synthesized so that synthesis can no longer take place.

A system regulating flagellar synthesis has recently been discovered. Yokota and Gots (18) and Dobrogosz and Hamilton (5) have shown that adenyl cyclase mutants of *E. coli* cannot synthesize flagella. Motility and chemotaxis

occur in cultures of these mutants only in the presence of 3',5'-cyclic AMP, an effect which can be antagonized by the addition of glucose. Previously, Adler and Templeton (2) had observed that glucose repressed the production of flagella, suggesting that the synthesis of the flagellum may be under control of catabolite repression. The fact that flagellation increases markedly as cultures become older is in agreement with the notion that some metabolite capable of repressing flagellation decreases in amount or becomes exhausted as the culture ages. It is also possible that a metabolic product capable of acting as an inducer accumulates as the culture ages.

The processes responsible for turning flagellin synthesis off and on during temperature shifts may involve a more complicated mechanism than that of catabolite repression. This is suggested by the fact that RNA and protein synthesis and an intact cell envelope or concurrent cell wall formation are required before flagellin synthesis can take place. It is possible that unique flagellin-synthesizing sites exist, the components of which are specifically inactivated at 42.5 C. This would explain the necessity for RNA and protein synthesis preceding the regeneration of flagellin and flagella after cells had been grown at 42.5 C and returned to 37 C. Furthermore, if these sites also were associated with the cell envelope, interference with the normal characteristics of the cell surface or concurrent cell wall synthesis might also disrupt the formation or functioning of these sites.

In summary, growth at a temperature slightly above the optimum inhibits the formation of the flagella in cells of *P. vulgaris*. The inhibition appears to occur at some step prior to the synthesis of flagellin, the main component of the organelle. The regeneration of the organelle requires growth at 37 C for one generation time, a period during which not only RNA and protein synthesis, but also the presence of an intact cell envelope or concurrent synthesis of cell wall, are required.

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