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Flagellar Assembly in *Salmonella typhimurium*: Analysis with Temperature-Sensitive Mutants

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The process of flagellar assembly in *Salmonella typhimurium* was investigated by using temperature-sensitive mutants. The mutants were grown at the restrictive temperature and then at the permissive temperature, with radiolabel supplied in the first phase of the experiment and not the second, or vice versa. Flagellar hook-basal body complexes were then purified and analyzed by gel electrophoresis and autoradiography. The extent to which a given protein was labeled in the two phases of the experiment provided information as to whether it preceded or followed the block caused by the mutant protein. We conclude the following concerning flagellar assembly. The M-ring protein (FliF) is stably incorporated in the earliest stage detected, along with two previously unknown proteins, with apparent molecular masses of 23 and 26 kilodaltons, respectively, and possibly one of the switch components, FliG. Independent of that event and all other events, the P-ring and L-ring proteins (FlgI and FlgH) are synthesized and exported to the periplasm and outer membrane by the primary cellular export pathway. Rod assembly occurs by export (via the flagellum-specific pathway) of subunits of four proteins, FlgB, FlgC, FlgF, and FlgG, and their incorporation, probably in that order, into the rod structure; this stage requires the *flhA* and *fliI* genes, perhaps because they encode part of the export apparatus. Once rod assembly is complete, the FlgI and FlgH proteins assemble around the rod to form the P and L rings. The rod structure, which is only metastable while it is being constructed, becomes stable upon P-ring addition. Export (via the flagellum-specific pathway) and assembly of hook protein, hook-associated proteins, and filament protein then occur successively. A number of flagellar proteins, whose genetic origin and structural role are not yet known, were identified on the basis of their dependence on the flagellar master operon for expression.

Bacterial flagella are complex organelles, whose structure spans the cell envelope and extends far beyond it and whose assembly therefore entails special logistical problems. An indication of the overall complexity of the flagellar system is the fact that in *Salmonella typhimurium*, for example, it is encoded by about 40 genes (22, 35). The known structures of the flagellum (Fig. 1) are the basal body, the hook, the hook-filament junction zone, the filament, and the filament cap (11, 14, 23, 24). Within the basal body, the morphology can be further broken down into the inner (M and S) rings, the rod, and the outer (P and L) rings (11). The genes and gene products responsible for most of these morphological features are known (1, 13, 16–19, 25, 26, 27, 31, 33, 39; M. Homma, K. Kutsukake, M. Hasebe, T. Iino, and R. M. Macnab, J. Mol. Biol., in press). Among structures that have not yet been positively identified by electron microscopy, there is the flagellar switch, which is responsible for determining the direction of flagellar rotation (15, 29, 51, 52), and structure associated with two motility proteins, MotA and MotB (8, 9, 42, 43, 49), which may be located in circlets of studs seen in freeze-fracture images (28). There must also be structure associated with the export of external components from the cytoplasm to their final destination, since most of these external components use a flagellum-specific pathway which is believed to utilize the flagellar apparatus itself (12, 17, 19, 20, 27, 34; Homma et al., in press; M. Homma, D. J. DeRosier, and R. M. Macnab, submitted for publication).

How is the flagellum assembled from its components? Early studies had shown that filament growth proceeds by addition of flagellin monomers at its distal end (12, 20). In 1978 and 1981, two major studies addressing the question of basal-body and hook assembly in *S. typhimurium* and *Escherichia coli* were published by Suzuki and co-workers (45, 46), who used electron microscopy to identify flagellar precursors in mutants. The simplest structure they detected consisted of the M ring, S ring, and rod, and was termed a rivet. This structure was thought to require at least 18 genes; current knowledge indicates the true number is at least 21. Suzuki and co-workers concluded that the rivet was converted to the flagellum by the sequential addition of the P ring, L ring, hook, and filament (Fig. 2).

Several years later, with the discovery by Homma and co-workers (18) of the hook-associated proteins (HAPs), and the subsequent identification (by Homma, Ikeda, and co-workers) of their encoding genes and their localization within the flagellum (14, 17, 23, 24), the morphogenetic pathway was expanded to include the successive addition of HAP1, HAP3, and HAP2 to the hook, with subsequent insertion of filament monomers into the distal end of a growth zone between HAP3 and HAP2 (Fig. 2). Thus, the pathway described by Suzuki, Homma, Ikeda, and co-workers proceeded in an essentially linear fashion from more simple to more complex structures.

These studies resulted in a thorough characterization of the later stages of flagellar assembly. However, the approach of Suzuki and co-workers would have failed to detect precursors that were unstable, did not fractionate properly, or had too small or simple a morphology; the fact that no precursor structures at all were detected for mutants defective in at least 18 different genes suggests many early steps were left uncharacterized. Also, because the partial structures were not obtained in purified form, their biochemical

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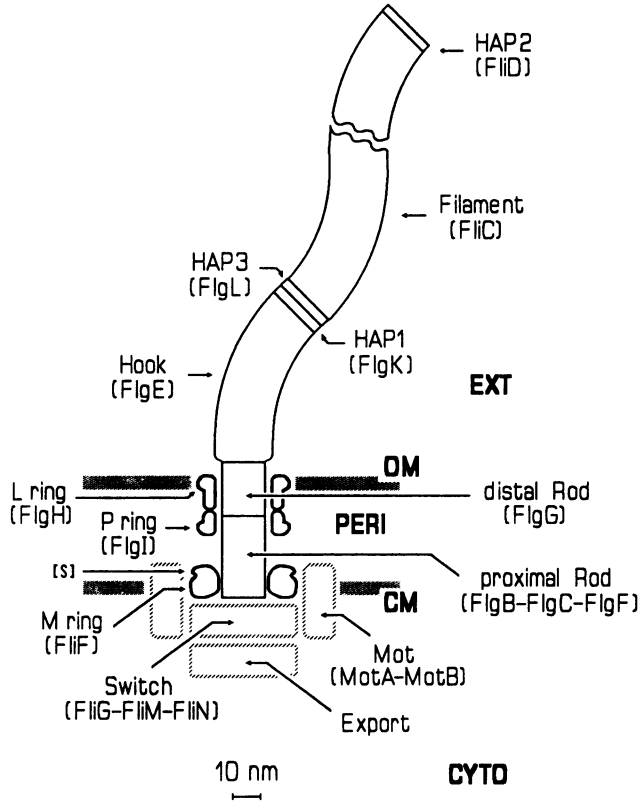

FIG. 1. Schematic illustration of the bacterial flagellum of *S. typhimurium*. Morphological features such as rings, rod, etc., are indicated, together with the gene products from which they are constructed. The origin of the S ring is unknown. The order of the three proximal rod proteins is tentatively assigned from this study as being (from most proximal to most distal) FlgB-FlgC-FlgF. The locations shown for the MotA and MotB proteins, the three switch proteins, and the (unknown) proteins that comprise the flagellum-specific export apparatus are based on a variety of lines of indirect evidence; these structures are indicated in dashed outline. CYTO, Cytoplasm; CM, cytoplasmic membrane; PERI, periplasmic space; OM, outer membrane; EXT, external medium; HAP, hook-associated protein.

composition could not be determined; nor in general was the genetic origin of a given morphological feature in the basal body known.

We have taken a different approach to the question of flagellar morphogenesis. Flagella of temperature-sensitive (Fla^{ts}) mutants were allowed to assemble in two phases. In the first phase, during growth at the restrictive temperature, flagellar assembly proceeded up to the block caused by the mutation. In the second phase, at the permissive temperature, the block was relieved and assembly proceeded to completion. Use of radiolabel in one phase but not the other then permitted a distinction to be made between components assembled before and after the block.

This approach was applied to the substructure termed the hook-basal body (HBB) complex, which can be obtained in purified form (1, 10) and consists of the basal body, the hook, and the first of the two proteins at the hook-filament junction zone (HAP1). It has allowed us to dissect portions of the assembly pathway preceding the rivet structure and to expand on some aspects of the subsequent process. It has also provided information regarding some of the dynamic aspects of the assembly process and has resulted in the identification of several previously unknown flagellar components.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. Fla^{ts} mutants and one Mot^{ts} (paralyzed) mutant were derivatives of *S. typhimurium* wild-type strain ST1 (2) and are listed in Table 1. They were isolated and their mutations were mapped essentially as described previously (1, 53).

Chemicals and enzymes. Acrylamide and *N,N'*-methylenebisacrylamide (electrophoresis grade) were obtained from Bio-Rad Laboratories, Richmond, Calif.; silver nitrate (reagent grade) was obtained from Aldrich Chemical Co., Milwaukee, Wis.; lysozyme (grade I) was obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals used were of at least reagent grade and were obtained from standard commercial sources.

Growth and in vivo radiolabeling of Fla^{ts} mutants. Vogel-Bonner citrate glycerol minimal medium (VBCG) (48) was made with magnesium supplied as $MgCl_2$ instead of $MgSO_4$, sulfur supplied as Na_2SO_4, and glycerol (1%, vol/vol) supplied as the main carbon source.

Cells were grown with shaking throughout. A single colony of a Fla^{ts} mutant was grown up in nutrient broth at 37°C; once growth was apparent, 50 μl of culture was used to inoculate 7.5 ml of VBCG supplemented with Na_2SO_4 to 200 μM. This culture was grown overnight at 42°C. A 250-μl sample of this overnight culture was used to inoculate 7.5 ml of fresh VBCG with no sulfate. This culture was grown at 42°C until, ca. 4 h later, the rate of growth (determined turbidimetrically) began to slow as a result of sulfur depletion.

Two 500-ml flasks, each containing 250 ml of VBCG supplemented with Na_2SO_4 to 25 μM, were inoculated with the sulfate-depleted cells to a density of ca. 1.5×10^6 cells ml^{-1}. One flask was further supplemented with 1 mCi of $^{35}SO_4$ (as H_2SO_4 in H_2O, carrier-free [ICN Radiochemicals, Irvine, Calif.]). These parallel cultures were grown at 42°C until the growth rate again began to slow; this took approximately 24 h but varied depending on the strain used. At this point, the prelabeled culture was supplemented with Na_2SO_4 to 1 mM (thereby diluting the label to negligible activity), and the other culture was supplemented with 1 mCi of $^{35}SO_4$ and Na_2SO_4 to 28 μM (in addition to the original, but now mostly depleted, 25 μM). Both cultures were grown at 30°C until the growth rate of the postlabeled culture again began to slow, at which point the cell density had approximately doubled. The cells were then harvested and their HBB complexes were purified essentially as described previously (1).

Gel electrophoresis, densitometry, and autoradiography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously (1) except that HBB complexes were suspended directly in sodium dodecyl sulfate sample buffer containing 1% 2-mercaptoethanol. Gels were silver stained according to the protocol of Morrissey (37) modified slightly as described elsewhere (25) and dried between sheets of dialysis membrane. The silver stain patterns were used to verify that the quantity and composition of the HBB complexes were essentially the same in the gel lanes of the pre- and postlabeled cultures. The gels were then exposed to Kodak X-Omat AR film at room temperature for times ranging from 5 days to several weeks.

RESULTS

Isolation of Fla^{ts} mutants. To be useful, a temperature-sensitive mutant had to (i) have normal flagellation when

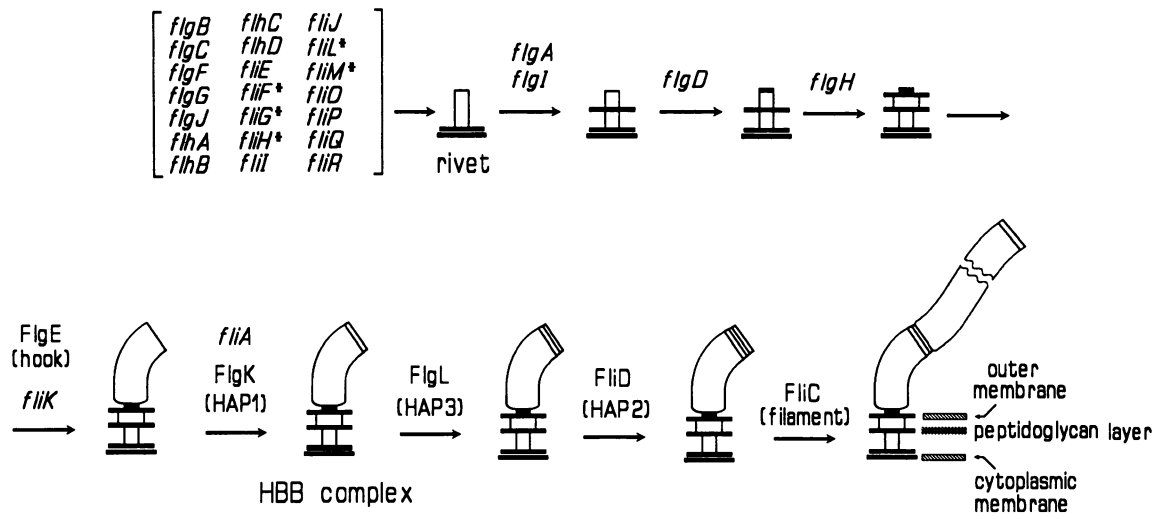

FIG. 2. Morphogenetic pathway for the flagellum of *S. typhimurium* as originally deduced by Suzuki and co-workers (45) and amplified by Homma, Ikeda, and co-workers to include the steps involving the HAPs (14, 17, 18, 23, 24). (The pathway has been simplified here by exclusion of aberrant structures branching from the main pathway.) *fliF*, *fliG*, and *fliH* (32, 52) were at the time believed to be a single gene, as were *fliL* and *fliM* (15) (*). The requirement for *fliJ*, *fliL* or *fliM*, *fliO*, and *fliR* was deduced from a parallel study in *E. coli* (46); *fliN* is not included in the pathway, as only *mot* alleles of it had been recognized in *E. coli*, and it had not yet been recognized in *S. typhimurium*. Where a gene product had been identified biochemically, and its encoding gene had been established, the product is indicated (as, e.g., FlgE); otherwise, only the gene is indicated (as, e.g., *flgB*). Note the large number of genes necessary for the simplest structure detected, the M ring–S ring–rod complex, or rivet. The partial structure seen with *flgL* mutants is called the HBB complex.

grown in minimal medium at the permissive temperature, (ii) be nonflagellate when grown at the restrictive temperature, and (iii) acquire flagellation within one generation after a shift to the permissive temperature.

Wild-type strain ST1 (2) grew well in minimal medium and was motile at both growth temperatures. We proceeded to isolate spontaneous Fla^{ts} derivatives, selecting for the ability to survive infection by the flagellotropic phage χ (36) at the restrictive temperature (42°C) and the ability to swarm at the permissive temperature (30°C). Seventy-one independent temperature-sensitive mutants, with defects in 17 different flagellar genes, were isolated.

Mutations were very unevenly distributed among the flagellar genes, with four genes (*flgE*, *flgG*, *flgI*, and *fliF*) accounting for 61% of the mutants, and *flgG* alone accounting for 24%. Many genes failed to yield any Fla^{ts} mutations; these included *flgB* (which encodes one of the four rod proteins), *flgH* (L-ring protein), *flgL* (HAP3), *fliC* (flagellin), *fliD* (HAP2), and *fliM* (a switch protein). Similar results have been obtained with another *S. typhimurium* strain (1; S.

TABLE 1. Temperature-sensitive mutants of *S. typhimurium* used in this study[a]

Strain no.	Mutant gene	Product function and location	Reference(s)[b]
MY645	*flhC*	Master operon; control of expression of all other flagellar genes	3, 21, 30
MY652	*flhD*	Master operon; control of expression of all other flagellar genes	3, 21, 30
MY669	*fliN* (Fla^{ts})[c]	Flagellar switch; peripheral to cytoplasmic membrane?	29, 51, 52
MY630, MY650	*fliG* (Fla^{ts})[c]	Flagellar switch; peripheral to cytoplasmic membrane?	29, 41, 51, 52
MY617, MY646	*fliF*[d]	Basal-body M ring; cytoplasmic membrane	1, 13
MY606, MY632, MY634	*flgI*[d]	Basal-body P ring; periplasmic space	1, 16, 25, 26, 38, 45
MY638, MY654	*flhA*	Unknown function and location	53
MY644	*fliI*	Unknown function and location	15, 53
MY629	*flgC*[d]	Basal-body rod; periplasmic space	Homma et al., in press
MY616, MY658	*flgF*[d]	Basal-body rod; periplasmic space	1, 19; Homma et al., in press
MY614, MY635, MY641	*flgG*[d]	Basal-body rod (distal portion); periplasmic space	1, 19, 39; Homma et al., in press
MY651	*flgD*	Rod modification; unknown location	45
MY643	*flgE*[d]	Flagellar hook; external to cell	1, 19, 33
MY648	*flgK*[d]	HAP1; hook-filament junction; external to cell	14, 17, 18, 24
MY636	*fliG* (Mot^{ts})[c]	Flagellar switch; peripheral to cytoplasmic membrane?	29, 41, 51, 52

[a] All mutants are derived from wild-type strain ST1 (2) and were obtained in this study. They are listed roughly in order of their effect on the labeling patterns of HBB proteins, from those causing late patterns to those causing balanced patterns (see text).
[b] References are to the identification of genes and their product functions and locations.
[c] Different mutant alleles in these genes can give rise to a nonflagellate (Fla$^-$), paralyzed (Mot$^-$), or nonchemotactic (Che$^-$) phenotype.
[d] Proteins present in the HBB complex.

Yamaguchi, personal communication). It is not known why some flagellar genes give rise to so many temperature-sensitive mutants and others so few.

Most of the mutants we isolated were completely immotile at the restrictive temperature, but a few showed some (ca. 1%) motile cells. Not all were able to produce, during one generation of growth at the permissive temperature, sufficient quantities of flagella for analysis. Twenty-two Fla^{ts} mutants, with defects in 14 genes, were judged suitable for use. We also included one *fliG* switch mutant with a temperature-sensitive paralyzed (Mot^{ts}) phenotype.

Table 1 lists each mutant used, the defective gene, and the function of the gene product, where known. Seven of the genes represented encode HBB components.

Table 2 lists the proteins that were known to be components of the HBB complex or are now believed to be components. Also listed are their encoding genes, molecular masses, and the corresponding substructures, where known. Flagellin is included because, being present in large amounts (ca. 20,000 subunits) in the intact flagellum, it can usually be detected in HBB preparations.

Rationale for the temperature shift protocol. Sulfur-depleted cells of a given Fla^{ts} mutant were added to two flasks containing medium with enough sulfate to support growth to mid-exponential phase, and [^{35}S]sulfate was added to one culture. The cultures were then grown at the restrictive temperature until growth began to slow. The label in the first culture was diluted with excess unlabeled sulfate, the second culture was supplemented with [^{35}S]sulfate plus limiting additional sulfate, and growth was continued at the permissive temperature for approximately one further generation. HBB complexes were then purified and analyzed.

Cultures labeled before and after the downshift in temperature are termed prelabeled and postlabeled, respectively. Since no label was present in the postlabeled culture before the shift, and the activity of the label in the prelabeled culture was negligible after the shift, the labeling pattern of a protein should represent its relative composition in terms of preshift versus postshift synthesis, and the two cultures should therefore contain complementary information.

A protein more heavily labeled in the prelabeled versus the postlabeled culture we term early, one equally labeled in both cultures we term balanced, and one more heavily labeled in the postlabeled culture we term late. These are operational definitions and do not necessarily indicate whether the protein is actually assembled early or late.

We postulated the following sequence of events during such an experiment (Fig. 3). At the restrictive temperature, flagellar assembly would proceed up to the point where the mutant protein was required and stop there. The partial structures would accumulate, and flagellar proteins not incorporated into structure would be degraded. Upon shifting to the permissive temperature, assembly of partial structures would proceed to completion, using components synthesized after the temperature shift. Also, an approximately equal number of flagella would be constructed de novo during the one generation of growth at the permissive temperature. For proteins assembling before the block, the radiolabeling pattern would derive half from growth at the restrictive temperature and half from growth at the permissive temperature and would therefore appear balanced (not early). For proteins assembling after the block, the pattern would appear late, essentially all deriving from growth at the permissive temperature.

Two key assumptions were made in the above description. The first was that partial structures would be stable and able to further assemble once the block had been removed. If this assumption was not valid, all flagellar proteins would have a late labeling pattern, and proteins that in fact comprised the unstable partial structure could be mistakenly viewed as assembling after the protein causing the block. The second assumption was that unincorporated monomers would be degraded. If this assumption was not valid for a given protein, the protein would have a balanced labeling pattern and be mistakenly viewed as being assembled before the block; an even more extreme case would be one in which a protein could assemble before the block but additionally exist as a pool of stable monomer, in which case an early pattern would be seen.

As we shall see, these two assumptions proved to be valid in many but not all cases. The significance of exceptions will be evaluated in Discussion.

Labeling patterns in wild-type cells. We needed to establish whether the use of different temperatures or an abrupt temperature shift had any general effect on the assembly process.

The labeling patterns of all known HBB components were indistinguishable when wild-type cells were grown at constant temperatures of 30 or 42°C; furthermore, the pre and postlabeled patterns appeared balanced at either temperature (data not shown). These proteins also appeared balanced when wild-type cells were grown with a temperature shift (Fig. 4A and B). Thus, the process of flagellar assembly at the two temperatures appears to be essentially the same, and the temperature shift does not significantly perturb the process.

The balanced patterns of the known HBB proteins also indicated that they were not present as large unincorporated pools. This did not appear to be true of two previously unidentified proteins, with molecular mass values of 23 and 26 kilodaltons (kDa), which had strikingly early patterns.

We now proceed to a description of the labeling patterns in various mutants. Illustrative examples of autoradiograms are given in Fig. 4, and a complete summary of the data is given in Table 2.

Labeling patterns in master regulatory mutants. Flagellar genes constitute a regulon whose expression is absolutely dependent on expression of a master operon, the *flhD-flhC* operon (21, 30). A Fla^{ts} mutant with a defect in *flhD* or *flhC* should therefore act as a negative control, with no flagellar structure being assembled at the restrictive temperature. As expected, the labeling patterns of all known HBB components appeared extremely late in these mutants (Fig. 4C and D).

We had noted in the past a number of proteins appearing in HBB preparations but did not know whether they were authentic flagellar components. We now conclude they are, since they all appeared late in *flhD* or *flhC* mutants. Proteins with molecular mass values of 34, 28, 26, 24, 23, and 18 kDa (Table 2) appeared most consistently. Others, with molecular mass values of 50, 43, 39, 33, and 9 kDa, and two with very high molecular mass values, appeared inconsistently and will not be discussed further.

Labeling patterns of individual proteins. We next describe (roughly in order from earlier to later) the patterns observed for known HBB components. Then we describe the patterns observed for the other proteins that we now believe to be HBB components. Descriptions refer to patterns in all mutants other than master regulatory mutants (which were presented above).

(i) M-ring protein (FliF). The M-ring protein appeared late only in Fla^{ts} mutants defective in one of the switch genes

TABLE 2. Proteins in the HBB complex of *S. typhimurium*: radiolabeling patterns in temperature shift experiments with temperature-sensitive mutants

Protein detected in HBB complex			Radiolabeling patterns[a] of given mutant gene and function of its product														
			Master operon		Switch (Fla^{ts})		M ring, *fliF*	P ring, *flgI*	Unknown		Rod			Rod modification, *flgD*	Hook, *flgE*	HAP1, *flgK*	Switch (Mot^{ts}), *fliG*	Wild type
Molecular mass (kDa)	Gene product[b]	Substructure	*flhC*	*flhD*	*fliN*	*fliG*			*flhA*	*fliI*	*flgC*	*flgF*	*flgG*					
23	?	?	L	L	E	E	L	E	(E)	E	E	E	E	E	E	E	E	E
26	?	?	L	L	(E)	E	(=)	E	E	E	(E)	E	E	E	(E)	E	E	E
65	FliF	M ring	L	L	=	L	[L]	=	=	=	=	=	=	=	=	=	=	=
38	FlgI	P ring	L	L	(L)	=	=	[L]	=	=	=	=/L	=/L	=	=	=	=	=
27	FlgH	L ring	L	L	=	=	=	=	=	=	=	=	=	=	=	=	=	=
16	FlgB	Proximal rod	L	L	L	L	L	L	L	L	(E)	(E)	(E)		=	=	=	=
14	FlgC	Proximal rod	L	L	L	L	L	L	L	L	[L]	L	=/L		=	=	=	=
32	FlgF	Proximal rod	L	L	L	L	L	L	L	L	L	[L]	L	=	=	=	=	=
30	FlgG	Distal rod	L	L	L	L	L	L	L	L	L	L	[L]	=	=	=	=	=
42	FlgE	Hook	L	L	L	L	L	L	L	L	L	L	L	L	[L]	=	=	=
60	FlgK	HAP1	L	L	L	L	L	L	L	L	L	L	L			[(=)]	=	=
56	FliC	Filament	L	L	(L)	L	L	L	L	L	L	L	L	(L)		L	=	=
28	?	?	L	L	(L)	L	L	L	L	L	(L)	L	L	(L)	L	(L)	=	=
24	?	?	L	L	(L)	L	L	L	L	(L)		L	L		(L)	(L)	=	=
18	?	?	L	L	(L)	(L)	(L)	L	L	(L)		(L)	L		(L)	(L)	=	=
34	?	?	(L)	L	L	L	L	L	(L)	L	(L)	L	L	L	(L)	L	L	=

[a] E, Early; =, balanced; L, late; =/L, balanced in one (*flgF*) or two (*flgG*) mutants and late in another. For explanations of these terms, see text. (), Tentative result; boxed entry, pattern of mutant protein itself; blank entry, pattern could not be described for technical reasons.

[b] References to gene products are given in Table 1, except for FlgH (1, 19, 25, 26, 38, 45), FlgB (1; Homma et al., in press), and FliC (27).

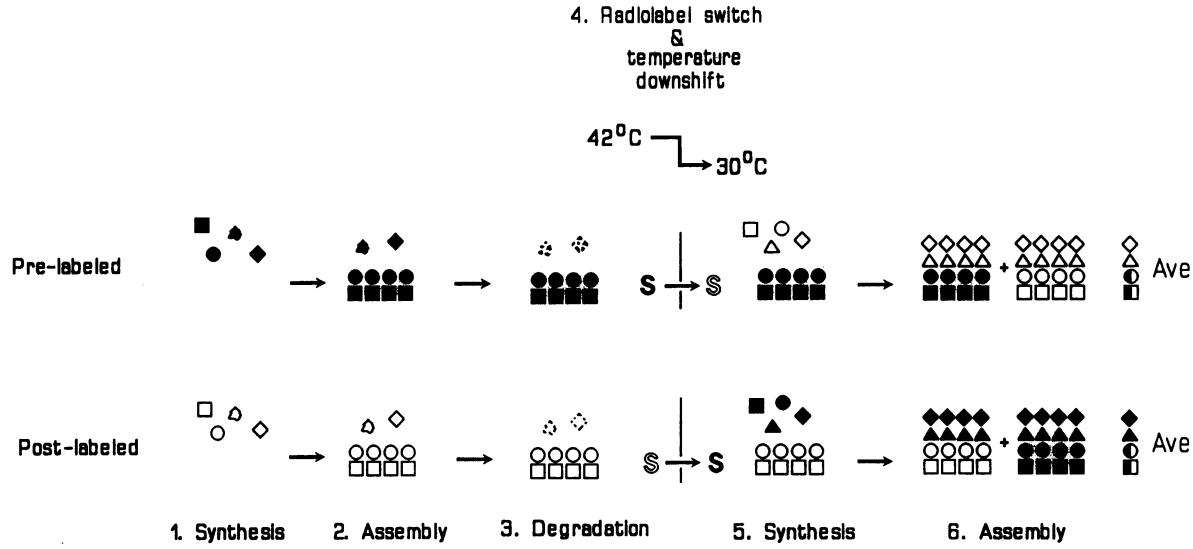

FIG. 3. Postulated events during a temperature shift growth experiment using a Fla^{ts} mutant, with radiolabeling of protein either before (prelabeled) or after (postlabeled) the temperature shift from the restrictive value (42°C) to the permissive value (30°C). Label conditions are indicated by a black S (^{35}S-labeled sulfate) or a white S (unlabeled sulfate); similarly, labeled proteins are indicated in black, and unlabeled proteins are indicated in white. Different proteins in the flagellar structure are indicated by different shapes; the mutant protein in this instance is indicated by a triangle (distorted at the restrictive temperature). Unincorporated subunits are presumed to be degraded. Ave, Average label composition at end of experiment. See text for further explanation.

(*fliG*) and in both *fliF* mutants. It appeared balanced in all other mutants tested.

(ii) S-ring protein. No protein corresponding to the S ring has yet been identified.

(iii) Rod proteins (FlgB, FlgC, FlgF, and FlgG). All four rod proteins appeared late in Fla^{ts} switch mutants, M-ring mutants, mutants defective in two genes of unknown function (*flhA* and *fliI*), and P-ring mutants. They appeared balanced in hook and HAP1 mutants and in *flgD* (rod modification) mutants in the two cases (FlgF and FlgG) where we were able to determine it. FlgC, FlgF, and FlgG appeared late in mutants defective in the corresponding genes.

The patterns of rod proteins in mutants defective in another rod gene were complex: FlgB appeared early to balanced in *flgC*, *flgF*, and *flgG* mutants; FlgC appeared late in *flgF* mutants and balanced or late in *flgG* mutants; FlgF appeared late in *flgC* and *flgG* mutants; and FlgG appeared late in *flgC* and *flgF* mutants. We did not have a suitable *flgB* mutant to test.

(iv) P-ring protein (FlgI). The P-ring protein appeared balanced in Fla^{ts} switch (*fliG*), M-ring, *flhA*, *fliI*, rod modification, hook, and HAP1 mutants and balanced or late in rod mutants. It appeared late when the *flgI* gene itself was defective. It was tentatively scored as late in the *fliN* switch mutant.

(v) L-ring protein (FlgH). The L-ring protein appeared balanced in all mutants, even those in which the P-ring protein appeared late. The fact that the P- and L-ring proteins appeared balanced rather than late in so many mutants was unexpected, on the basis of the morphogenetic pathway shown in Fig. 2 and the assumptions of Fig. 3.

(vi) Hook protein (FlgE). The hook protein appeared late in all Fla^{ts} mutants except the HAP1 mutant, where it appeared balanced.

(vii) HAP1 protein (FlgK). Wild-type HAP1 appeared late in all Fla^{ts} mutants where it could be scored. We were unable to describe its pattern in the rod modification and hook mutants because it was present in insufficient amounts, but we assume on regulatory grounds (21, 30), as well as on the basis of the data of Homma, Ikeda and co-workers, that it would appear late. Mutant HAP1 was tentatively scored as balanced.

(viii) Flagellin (FliC). Flagellin, present as a contaminant in most HBB preparations, appeared late in all Fla^{ts} mutants in which it could be scored.

(ix) Proteins of unknown genetic origin. As mentioned above, a number of proteins copurified with the HBB complex and showed themselves to be under flagellar regulon control. Among these, the 23-kDa protein showed a striking pattern of early labeling, with prelabeling-to-postlabeling ratios of as high as 25:1 (data not shown). Aside from master regulatory mutants, the only mutants in which this protein appeared late were the M-ring mutants. A similar, perhaps less pronounced, pattern applied to the 26-kDa protein. The other consistently observed proteins (molecular mass values of 34, 28, 23, and 18 kDa) appeared late in all Fla^{ts} mutants in which they could be scored.

Labeling patterns in terms of mutant class. We next summarize the labeling patterns from the point of view of the mutant background in which they were observed.

(i) Master regulatory mutants (*flhC* and *flhD*). As described above, all HBB components appeared late in these mutants.

(ii) Switch mutants with Fla^{ts} phenotype (*fliG*, *fliM*, and *fliN*). No *fliM* mutant was available. Most proteins appeared late in the *fliG* and *fliN* mutants. The exceptions were the 23- and 26-kDa proteins (which appeared early), the M-ring protein (which appeared balanced in the *fliN* mutant), and the L- and P-ring proteins (which appeared balanced in most cases).

(iii) Switch mutant with Mot^{ts} phenotype (*fliG*). Since even at the restrictive temperature this mutant was capable of assembling a complete flagellum, most flagellar components ought to appear balanced in this mutant. This was found to be the case for all known flagellar components, including the last known component in the assembly process, flagellin. The 23- and 26-kDa proteins appeared early, and the 34-kDa protein appeared late.

(iv) M-ring mutants (*fliF*). The P- and L-ring proteins, and

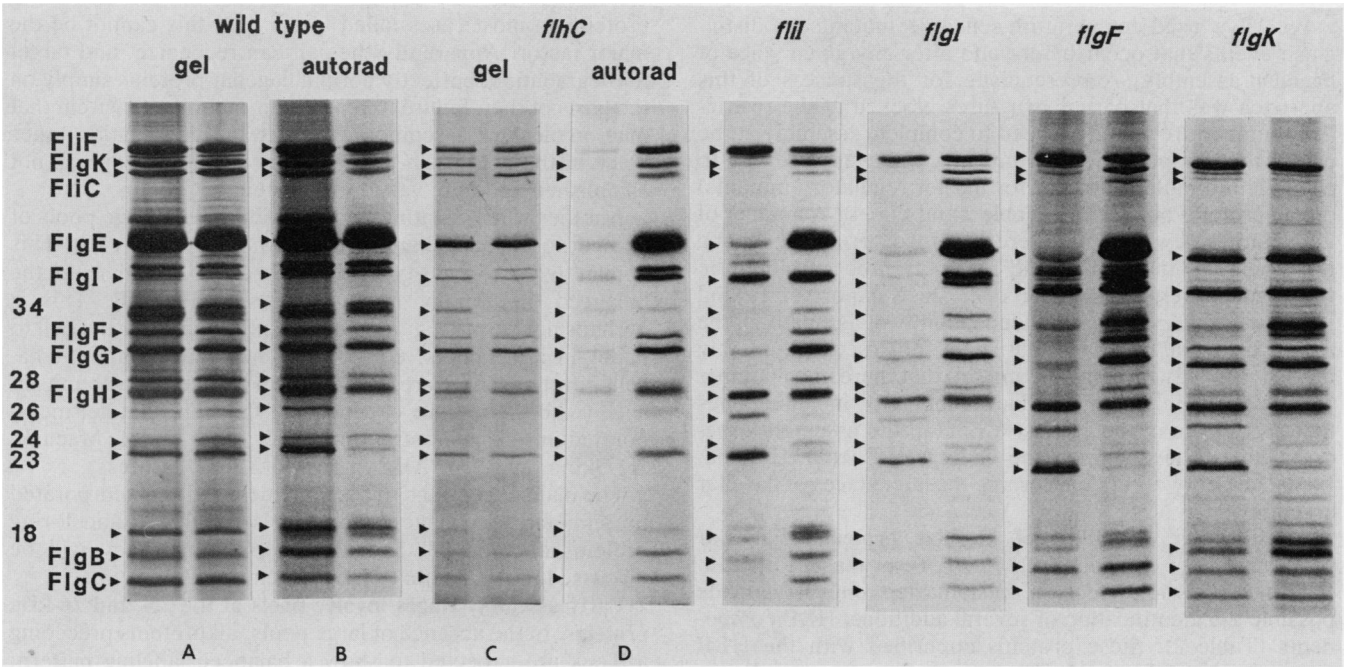

FIG. 4. Radiolabeling patterns in HBB complexes assembled during a temperature shift experiment of the sort shown in Fig. 3, with labeling either before the shift (prelabeling; left lane of each pair) or after the shift (postlabeling; right lane of each pair). Shown are results for a wild-type strain and for temperature-sensitive mutants defective in *flhC* (one of the master regulatory genes), *fliI* (a flagellar gene of unknown function), *flgI* (encoding the P-ring protein), *flgF* (encoding one of the rod proteins), or *flgK* (encoding HAP1). For the wild-type strain and the *flhC* mutant, both the silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern (gel; A, D) and the corresponding autoradiogram (autorad; B, C) are shown, with the gels demonstrating that the loads and protein compositions are similar in the pre- and postlabeled lanes; for the remaining temperature-sensitive mutants, only the autoradiograms are shown. Proteins of known genetic origin (cf. Table 2) are so indicated. Those indicated by their apparent molecular mass (in kilodaltons) are of unknown origin but were seen consistently and are believed to be flagellar proteins, based on their labeling patterns in master regulatory mutants (see text). Unmarked proteins were not seen consistently; some may be contaminants such as porins, but others (e.g., the one sometimes seen just above the 38-kDa FlgI protein) appear to be flagellar proteins, based on their labeling patterns in master regulatory mutants. In the wild-type strain, proteins are approximately equally labeled (balanced pattern) in the pre- and postlabeled samples, except for two proteins with molecular mass values of 26 and 23 kDa, which are more heavily labeled in the prelabeled sample (early pattern). In the *flhC* mutant, all proteins are much more heavily labeled in the postlabeled sample (late pattern). In the other mutants, different proteins show different labeling patterns; for example, the FlgF rod protein appears late in the *fliI*, *flgI*, and *flgF* mutants but balanced in the *flgK* mutant; the FlgI P-ring protein appears late in the *flgI* mutant but more or less balanced in the other three.

the 26-kDa protein, appeared balanced. All other proteins appeared late, including the 23-kDa protein, which in all other backgrounds (except master operon mutants) had appeared early.

(v) Mutants in genes of unknown function (*flhA* and *fliI*). Most proteins appeared late in these mutants, except for the 23- and 26-kDa proteins (which appeared early) and the M-, P-, and L-ring proteins (which appeared balanced).

(vi) Rod mutants (*flgB*, *flgC*, *flgF*, and *flgG*). No *flgB* mutant was available. In the other classes of rod mutants, the 23- and 26-kDa proteins appeared early. The M-, P-, and L-ring proteins appeared balanced (or sometimes, in the case of the P-ring protein, somewhat late). The rod proteins themselves showed a complex pattern (see above), and all other components appeared late.

(vii) P-ring mutants (*flgI*). Aside from the 23- and 26-kDa proteins (which appeared early) and the M- and L-ring proteins (which appeared balanced), all proteins appeared late.

(viii) L-ring mutants (*flgH*). No *flgH* mutant was available.

(iv) Rod modification mutant (*flgD*). The 23- and 26-kDa proteins appeared early. Others appeared balanced, except hook protein, flagellin, and the remaining proteins of unknown genetic origin, which appeared late.

(x) Hook mutant (*flgE*). The 23- and 26-kDa proteins appeared early. Others appeared balanced, except for the remaining proteins of unknown genetic origin, which appeared late.

(xi) HAP1 mutant (*flgK*). The 23- and 26-kDa proteins appeared early. Others (including HAP1 itself) appeared balanced, except for flagellin and the remaining proteins of unknown genetic origin, which appeared late.

DISCUSSION

The morphogenesis of the bacterial flagellum is interesting from many points of view: the currently recognized flagellar structure has a fairly complex morphology, and that of the complete structure is likely to be considerably more extensive and complex. The organelle spans the entire cell envelope, extending from the cytoplasm through the cytoplasmic membrane, the periplasm, the outer membrane, and into the extracellular environment; it must therefore be able to target its various components to these locations. Many of its external components are exported by a flagellum-specific apparatus in which the proteins are believed to be piped down the central core of the nascent structure. And the flagellar motor is capable of performing its functions of rotation and switching even while assembly of the flagellar filament (and probably even earlier components) is still in progress.

We have used temperature-sensitive mutants to distinguish events that occur before and after any given stage of flagellar assembly. A prerequisite for the success of this approach was that partial structures blocked at the restrictive temperature would proceed to complete assembly at the permissive temperature. Fortunately, this was observed to occur: in no mutant (except for master regulatory mutants) did all proteins appear late (Table 2 and Fig. 4). A number of illuminating new insights were obtained, principal among them being (i) that the overall assembly process cannot be regarded entirely as a linear one, since export of P- and L-ring monomers proceeds independently of assembly of the rest of the basal body, (ii) that some substructures, notably the rod, are only metastable, and (iii) that there are a number of previously unknown flagellar components, including ones that participate in the earliest events in the assembly process. Our data also support the findings of Suzuki, Homma, Ikeda, and co-workers concerning the later stages of flagellar assembly.

Identification of additional flagellar proteins. Flagellar genes are under control of the *flhD-flhC*, or master, operon. Fla^{ts} mutants with defects in these master genes have made possible the identification of several additional HBB components (Table 2); these proteins copurified with the HBB complex, showed late labeling patterns in master regulatory mutants, and showed different labeling patterns in various flagellar mutants. None have the requisite combination of molecular mass and pI to correspond to the components of other proposed substructures of the flagellum such as the switch or the Mot complex, and so their genetic origin is unknown at this time.

Given the straightforward nature of the experimental protocol, where the mature HBB complex is isolated and then simply subjected to gel electrophoresis, it is likely that these proteins are actual components rather than precursors or degradation products. However, they may not all be independent of each other; for example, the fact that the 23- and 26-kDa proteins have similar molecular masses, similar pIs (not shown), and similar and highly unusual labeling patterns suggests they may be related. Identification of the corresponding genes and further characterization of the proteins is needed to answer these questions.

Unincorporated flagellar proteins do not exist as large pools and are subject to degradation. Mutant proteins appeared late, as did most proteins known to assemble after a given mutant protein. This implies that at the restrictive temperature the proteins either were not synthesized or were synthesized but failed to assemble. In the case of the mutant gene, temperature-sensitive failure of synthesis seems much less probable than temperature-sensitive failure of product function, and we think that it can be ruled out as a general explanation. For proteins following the block, inhibition of gene expression is thought to occur only in the case of flagellin and the HAPs, not with the hook or basal-body proteins (21, 30).

Assuming that a protein has been synthesized, why does it fail to assemble? For a mutant protein, this could be because it is a poor substrate for transport to its assembly destination or because it is a poor substrate for the assembly process itself. For a protein following a block, it could be because the pathway for transport to its assembly destination is blocked or because its site of assembly is blocked.

A late pattern, however, also implies that the protein did not survive to assemble at the permissive temperature; i.e., it was degraded. Mutant proteins might be especially susceptible to degradation, but since unincorporated wild-type proteins in most cases failed to survive, this cannot be the major factor. Apparently the cell can recognize, and target for degradation, perfectly normal flagellar proteins simply on the basis of their failure to reach their natural site within that macromolecular assembly. Whether the degradation takes place in the cytoplasm or in another compartment was not established and may vary from protein to protein.

Another way of stating this situation is that large pools of unincorporated flagellar proteins do not generally exist. This applies even to wild-type cells, as can be seen from the balanced patterns of most proteins in Fig. 4B. This is perhaps not surprising: it would be extravagant of the cell to maintain large pools, considering how many different flagellar proteins there are and the small numbers of subunits of each (with the notable exception of flagellin) that get incorporated into flagellar structure (C. J. Jones, R. M. Macnab, H. Okino, and S.-I. Aizawa, J. Mol. Biol., in press).

The main exceptions to the statement that unincorporated proteins fail to survive as free pools are the L- and P-ring proteins and the 23- and 26-kDa proteins. They will be discussed in the context of the assembly pathway itself.

Early assembly stages involve pools of the 23- and 26-kDa proteins. In the absence of large pools, all proteins preceding a block are expected to show a balanced labeling pattern; those following a block are expected to show a late pattern (Fig. 3). These patterns were observed for most proteins, but the 23- and 26-kDa proteins consistently showed distinctly early labeling patterns, with prelabeling-to-postlabeling ratios of as much as 25:1.

In the simplest type of model involving subunit pools, component proteins would enter the pool upon synthesis and be incorporated into structure with the same probability as subunits that had been in the pool for some time. Such a model cannot account for the observed labeling ratio, as the following considerations demonstrate (Fig. 5). The label composition of a large pool after the temperature shift will not be perturbed by the small amounts incorporated into structure and will be determined solely by the time since the shift, progressively changing (for the prelabeled culture) from 100% to 50% over one generation. Proteins assembled before the shift will be 100% labeled, while proteins assembled after the shift will have a mean extent of labeling of 75%. (The latter figure assumes a linear increase of biomass; an exponential increase reduces the figure to 72%.) Proteins whose assembly precedes the block will therefore have a mean extent of labeling of 87.5%, or 12.5% for the corresponding postlabeled culture, and so the labeling ratio will be 7:1. For proteins whose assembly follows the block, the ratio will be 3:1.

Since the observed ratio for the 23- and 26-kDa proteins is much higher than these figures, we conclude that subunits synthesized some time ago are used preferentially over newly synthesized subunits. There must therefore be some slow process by which these proteins are converted from being assembly incompetent to being assembly competent; possible examples of such a process would be covalent modification, insertion into the membrane, or nucleation of the initial assembly structure. If the process were to discriminate heavily enough in favor of material synthesized some time ago, an early labeling pattern could arise even if the pool size was not large and even if the protein was assembled after the block.

In M-ring mutants, the labeling patterns of the 23- and 26-kDa proteins appeared balanced or late, indicating that they require the M ring for their long-term stability. We do

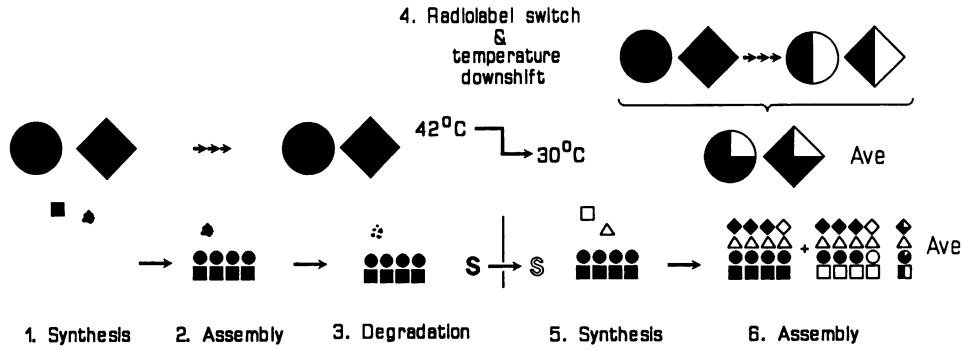

FIG. 5. Effect of large stable pools of unincorporated monomers on labeling patterns during a temperature-shift growth experiment (cf. Fig. 3). Radiolabeled proteins are indicated in black; unlabeled proteins are indicated in white; only the prelabeled culture is illustrated. Large circles and diamonds indicate stable pools of the corresponding monomers, with the extent of labeling indicated by sectoring; squares and triangles indicate proteins which (as in Fig. 3) cannot exist stably unless incorporated into structure. The experiment proceeds through the same stages as in Fig. 3, except that degradation of unincorporated proteins occurs in some cases (squares and triangles) but not others (circles and diamonds). Ave, Average label composition at end of experiment. See text for further explanation.

not know whether the M ring likewise requires these proteins for its stabilization.

M-ring assembly precedes rod assembly. The simplest structure detected by Suzuki and co-workers was the rivet (Fig. 2). This structure could have arisen in two different ways: preassembled substructures or modules, say the M ring–S ring complex and the rod, could have come together; alternatively, one substructure (such as the M ring) could have assembled first and then other substructures (such as the rod) could have progressively assembled onto it. Our results indicate that assembly proceeds by the latter type of pathway, since the labeling pattern of the M ring appeared balanced in all rod mutants tested, whereas the patterns of all rod proteins appeared late in the M-ring mutants.

Only *fliG* mutants yielded a late labeling pattern for the M-ring protein. Whether this means that the FliG switch components are assembled prior to the M ring, or whether they coassemble, cannot be answered from the available data. A mutant defective in another switch gene, *fliN*, yielded a balanced pattern for the M-ring protein, indicating that these two switch proteins play different roles in the assembly process.

One can present an independent argument that the M ring is unlikely to exist in the membrane as an independent entity, since it would presumably create a pore permitting catastrophic leakage of ions, and molecules up to the size of proteins, from the cell. The FliG switch protein, or the 23- and 26-kDa proteins, may prevent such a pore from forming until the flagellum-specific export apparatus is assembled.

If the S ring is a distinct structure rather than just a morphological subset of the M ring, we would imagine that its assembly would occur after the M ring but before the rod. No proteins with a labeling pattern corresponding to this were detected, and so the origin of the S ring and the process by which it assembles remain uncertain.

The FlhA and FliI proteins may play a role in the flagellum-specific export pathway. Suzuki and co-workers had established that the *flhA* and *fliI* genes were necessary for formation of the rivet structure. A more precise description of their role is now possible: they are necessary for rod assembly but not for M-ring assembly. This conclusion is based on the fact that in *flhA* and *fliI* mutants, the M-ring protein showed a balanced labeling pattern, whereas all four rod proteins showed late labeling patterns. Since the rod is the earliest known structure whose components are exported by the flagellum-specific pathway, we suggest that the FlhA and FliI proteins play some role in this pathway.

Rod assembly. FlgB, FlgC, FlgF, and FlgG are rod components and (together with the hook protein and the HAP1 protein) constitute a family of proteins with structural similarities at the primary sequence level (Homma et al., in press; Homma et al., submitted). FlgG comprises the distal zone of the rod (39), following which the flagellar structure continues in a segmented fashion with the hook, a HAP1 zone, a HAP3 zone, the filament, and a HAP2 zone (Fig. 1 and 2).

The proximal end of the rod consists of the FlgB, FlgC, and FlgF proteins (and possibly other components [Jones et al., in press]), probably also in successive zones. Do the data enable us to state the order in which these zones occur? The labeling data for the rod proteins indicated a considerable amount of interdependence in terms of stable incorporation, with only the complete rod structure (in association with the P ring; see below) appearing to be truly stable. With this caveat, we tentatively conclude that FlgB is the most proximal protein, since it appeared early to balanced in other rod mutants; also that FlgC is proximal to FlgF, since the stability of FlgF was more dependent on the distal protein FlgG than was the stability of FlgC. Thus, the order of the proteins is tentatively concluded to be FlgB-FlgC-FlgF-FlgG.

The late appearance of all four rod proteins in P-ring mutants indicates that the P ring is important for rod stability, but it cannot be essential in this regard, since Suzuki and co-workers detected rivet structures in P-ring mutants. In the absence of information concerning the frequency with which these rivets were observed, we suspect that they may have been exceptional examples of rod structures that succeeded in completing assembly without the P ring to stabilize them. Another relevant observation is that the rod remains stable indefinitely in mutants lacking the P and L rings, under special conditions where hook assembly has occurred (25, 38). Thus the hook, as well as the P ring, contributes to the stabilization of the rod; in the wild-type cell, of course, both stabilizing structures are present.

The stabilizing effect of the P ring on the rod is somewhat surprising, considering that the outer cylinder (i.e., the structure consisting of the P and L rings) is thought to permit completely free rotation of the rod (4). Stabilization must be

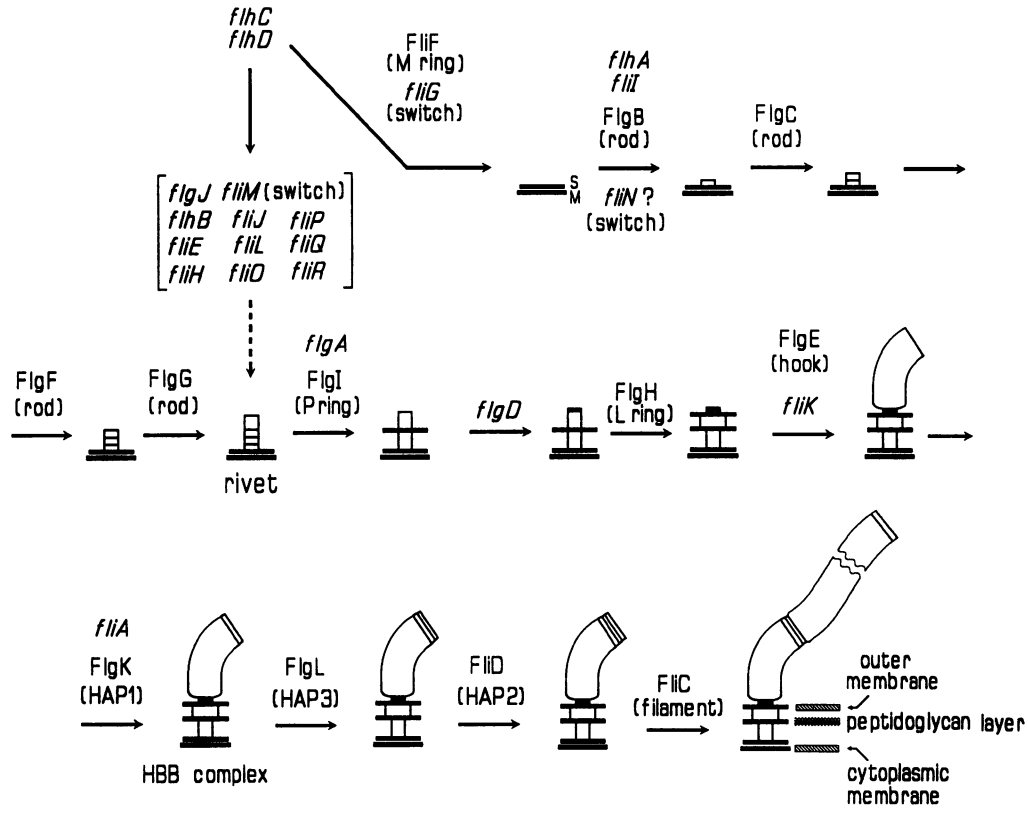

FIG. 6. Morphogenetic pathway for the flagellum of *S. typhimurium*. This pathway combines the data of Suzuki, Homma, Ikeda, and co-workers (Fig. 2) with data obtained in this study and utilizes gene/product/morphology correspondences and other information obtained in recent years. The *flhD-flhC* operon enables expression of all other operons. The set of genes in brackets were necessary for detection of the rivet structure (45, 46); since no temperature-sensitive mutants defective in these genes were obtained, it is not known at what point prior to the rivet these genes participate. The S ring is arbitrarily shown as assembling with the M ring in this diagram.

propagated over considerable distances, since the length of the rod exceeds the direct reach of the P ring, which is located in the vicinity of the junction between the FlgG portion and the proximal portion (39, 44). Independent evidence for propagated effects in the rod comes from a mutant whose rod is prone to shear at the junction between the proximal and distal zones (39), yet whose defect lies in the M- ring protein, which is located about 20 nm away.

It is interesting to recall that although the rod appears to be a rather vulnerable structure during the process of its assembly, when complete it is strong enough to bear the torsional load of a 10-μm filament rotating against the viscous resistance of the external environment.

Export and assembly of the P- and L-ring proteins. The rod proteins (Homma et al., in press), hook and hook-associated proteins (Homma et al., submitted), and flagellin (27, 34) all appear to be exported by a flagellum-specific pathway. The P- and L-ring proteins, however, which have conventional signal sequences at their N termini and undergo peptide cleavage (16, 19, 26), presumably use the primary cellular export pathway (40), in which case they would have no contact with other flagellar proteins until they nucleate onto the rod.

This export pathway explains why the labeling patterns of the P- and L-ring proteins were balanced in most mutants tested. Our interpretation—that subunits of these proteins can exist in a stable state in the absence of other flagellar structures—does not contradict the scheme of Suzuki and co-workers (Fig. 2) in which assembly of the P- and L-ring structures follows rivet assembly. Although there is no direct information concerning the state of the P- and L-ring proteins in the absence of the rod, we assume they are monomeric, since assembly of P- and L-ring subunits around the rod is a much more plausible process than threading of preexisting rings onto it. Since the L- and P-ring proteins were independently stable, we conclude that they exist in separate pools rather than a single pool of heterodimers; this is consistent with the observation that L-ring mutants can still assemble the P ring onto the rod (25, 38, 45, 46).

The stability of the P- and L-ring proteins even when unincorporated makes them unusual among the flagellar proteins. They may be intrinsically stable, or it may be that proteolytic degradation is a less active process in the periplasm and outer membrane than in the cytoplasm (47).

Mutant P-ring proteins, however, were not stable. They may fail to be exported at the restrictive temperature, and get degraded in the cytoplasm, or they may be less able than the wild-type protein to resist degradation in the periplasm. Wild-type P-ring protein, although stable in the absence of rod structure, was not stable in some rod-mutant backgrounds, perhaps because it becomes tied up in dead-end structures with the defective rod proteins.

Several proteins assemble late and are probably internal components. Four of the proteins of unknown genetic origin appeared late in all Fla^{ts} mutants tested, including ones with defects as late in the assembly process as HAP1. Since the composition of the external portion of the flagellum has been well characterized and does not include these proteins, it seems that they must be internal components added late in the assembly process. Possible roles might be in the export

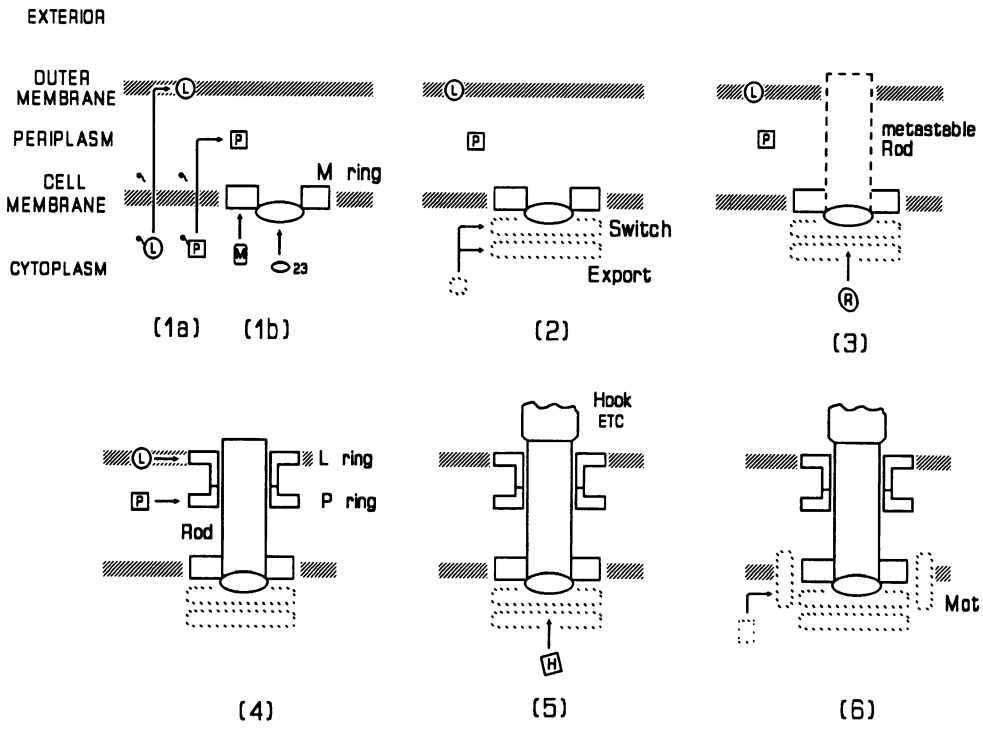

FIG. 7. Major stages in flagellar morphogenesis. Stage 1a: L- and P-ring monomers are exported via the signal peptide-dependent pathway to the outer membrane and periplasm, respectively. Stage 1a proceeds independently of stage 1b (and all others). Stage 1b: A putative complex containing the M ring and the 23- and 26-kDa proteins assembles stably in the cytoplasmic membrane. Stage 2: Peripheral complexes assemble. These include the switch complex and the apparatus for flagellum-specific export of rod, hook, hook-associated, and filament proteins. This stage is poorly understood, and the structures are inferred from indirect evidence. Stage 3: Rod proteins are exported and assemble; the rod is metastable at this point. Stage 4: The P and L rings assemble onto the rod, stabilizing it. Stage 5: Hook protein, HAPs, and flagellin are sequentially exported and assemble. Stage 6: Mot proteins assemble into the membrane around the flagellum and enable it to rotate.

of the last external components to be added (the HAPs and flagellin) or in some aspect of motor function.

An updated flagellar morphogenetic pathway. Figure 6 depicts a morphogenetic assembly scheme which combines our data with those of Suzuki, Homma, Ikeda, and co-workers. It also takes into account correspondences obtained in recent years between genes, proteins, and structural features. Comparison of Fig. 2 and 6 shows that much of the novel information that has been gained applies to the early stages of the process. Of the 21 genes required for the first structure detected by Suzuki and co-workers, 2 master genes have since been shown to precede all structure (21, 30), and 9 are now believed to participate in successively more complex precursors of the rivet, namely, the M ring (and presumably the S ring) and a segmentally growing rod consisting of at least four different proteins.

Eleven genes remain in the "black box" category, where all that can be said is that they are necessary at some stage prior to the rivet (dashed arrow in Fig. 6). With the exception of *fliM*, which encodes a component of the flagellar switch, the functions of these genes are unknown. Should Fla^{ts} mutants defective in these genes become available, it may be possible to position them more precisely in the morphogenetic pathway.

Although export of some components proceeds independently of other events, the description by Suzuki and co-workers—of a linear pathway with progressive buildup of a single structure of ever-increasing complexity—still holds as far as addition of monomeric subunits is concerned. All of the evidence argues against a process in which flagellar substructures assemble independently and then join together. Yet such modular assembly processes are prevalent in some other biological systems; T4 phage, for example, form by joining preassembled head, tail, and tail-fiber substructures (50). The reason that the flagellum does not assemble in this fashion is probably that it is vastly simpler to export protein subunits across the cell envelope than to export entire substructures such as the rod, the hook, or the filament.

Overview of the assembly process. The complete assembly pathway must include structures beyond those shown in Fig. 6, such as the switch, the export apparatus, and components such as the 23- and 26-kDa proteins whose genetic origin is not yet known. We suggest it consists of at least seven major stages (Fig. 7).

The first two proceed independently of each other. One (stage 1a) is the export, via the cell's primary export pathway, of P- and L-ring monomers to the periplasm and the outer membrane, respectively. This can proceed regardless of other flagellar assembly events, since this export pathway does not employ any flagellar structure.

The other earliest stage (stage 1b) is the nucleating event of the entire assembly process, namely, the formation of the central flagellar substructure associated with the cytoplasmic membrane. This includes the M ring (and probably the S ring) and the 23- and 26-kDa proteins. No export is required for this stage, since the components are inserted into the cytoplasmic membrane, a process which in bacteria is believed to occur spontaneously. The resulting structure is not yet capable of carrying out flagellum-specific export.

The next stage (stage 2) consists of assembling components onto the cytoplasmic face of the structure assembled in stage 1b. This stage is poorly characterized, since none of the corresponding structures have yet been detected by electron microscopy. Nonetheless, there is strong reason to suspect such structures exist. For example, three proteins (FliG, FliM, and FliN) responsible for switching the direction of flagellar rotation are part of the flagellar structure, but they also can recognize cytoplasmic proteins such as CheY; genetic and other evidence suggests that they exist as a complex at the cell membrane but not integral to it (29, 41, 51). There is also the flagellum-specific export apparatus; virtually nothing is known about it, but since the pathway for export is thought to be through the nascent flagellar structure itself, an axially central location at the cytoplasmic face of the basal body is likely.

Once the flagellum-specific export apparatus is in place, the first of its substrates, the rod proteins, can be exported through the nascent structure to the periplasm and assemble into a metastable structure (stage 3).

With the metastable rod complete, P- and L-ring subunits (existing meantime as stable pools in the periplasm and outer membrane) form into the rings around the rod and thereby stabilize it (stage 4). There may be additional events in P-ring assembly if, as has been suggested (11), it is actually associated with the peptidoglycan layer. There must also be events involving the penetration of the outer membrane by the rod; this may include the poorly understood process known as rod modification, and may normally occur in concert with the formation of the L ring (although L-ring assembly is not an absolute requirement [25, 38, 45]).

Then the export and assembly of the truly external components—hook, HAP1, HAP3, HAP2, and filament—proceeds in sequential fashion (stage 5).

The MotA and MotB proteins are essential for flagellar rotation. They can insert and enable flagellar rotation when all other assembly is complete (6, 7), to constitute the final stage (stage 6) of the entire process as shown here. However, they can insert into the membrane regardless of the status of the flagellar assembly process (49; M. L. Wilson and R. M. Macnab, submitted for publication), and may be able to associate with the nascent flagellum at a quite early stage (certainly before filament formation [5]). In part the process may be decided by regulation of their synthesis which, like that of flagellin, is normally dependent on the existence of completed HBB structures (21, 30).

Concluding comments. The pathway of flagellar assembly is seen to be a complicated one, whose broad outline has been delineated by this and previous studies. Major questions remain to be answered. Among the most important of these are the identification of structures such as the flagellar switch and the apparatus for flagellum-specific export, characterization of the export process itself, and determination of the mode of attachment of the flagellum to the cell surface so that the motor can propel the cell. A general issue, about which virtually nothing is known, is whether the processes of export and assembly are under control at the level of the whole cell or the individual organelle.

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LITERATURE CITED

1. **Aizawa, S.-I., G. E. Dean, C. J. Jones, R. M. Macnab, and S. Yamaguchi.** 1985. Purification and characterization of the flagellar hook-basal body complex of *Salmonella typhimurium*. J. Bacteriol. **161**:836–849.
2. **Aswad, D., and D. E. Koshland, Jr.** 1975. Isolation, characterization and complementation of *Salmonella typhimurium* chemotaxis mutants. J. Mol. Biol. **97**:225–235.
3. **Bartlett, D. H., B. B. Frantz, and P. Matsumura.** 1988. Flagellar transcriptional activators FlbB and FlaI: gene sequences and 5′ consensus sequences of operons under FlbB and FlaI control. J. Bacteriol. **170**:1575–1581.
4. **Berg, H. C.** 1974. Dynamic properties of bacterial flagellar motors. Nature (London) **249**:77–79.
5. **Berg, H. C., M. D. Manson, and M. P. Conley.** 1982. Dynamics and energetics of flagellar rotation in bacteria, p. 1–31. *In* W. B. Amos and J. G. Duckett (ed.), Prokaryotic and eukaryotic flagella. Cambridge University Press, Cambridge.
6. **Blair, D. F., and H. C. Berg.** 1988. Restoration of torque in defective flagellar motors. Science **242**:1678–1681.
7. **Block, S. M., and H. C. Berg.** 1984. Successive incorporation of force-generating units in the bacterial rotary motor. Nature (London) **309**:470–472.
8. **Chun, S. Y., and J. S. Parkinson.** 1988. Bacterial motility: membrane topology of the *Escherichia coli* MotB protein. Science **239**:276–278.
9. **Dean, G. E., R. M. Macnab, J. Stader, P. Matsumura, and C. Burks.** 1984. Gene sequence and predicted amino acid sequence of the *motA* protein, a membrane-associated protein required for flagellar rotation in *Escherichia coli*. J. Bacteriol. **159**: 991–999.
10. **DePamphilis, M. L., and J. Adler.** 1971. Purification of intact flagella from *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **105**:376–383.
11. **DePamphilis, M. L., and J. Adler.** 1971. Fine structure and isolation of the hook-basal body complex of flagella from *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **105**:384–395.
12. **Emerson, S. U., K. Tokuyasu, and M. I. Simon.** 1970. Bacterial flagella: polarity of elongation. Science **169**:190–192.
13. **Homma, M., S.-I. Aizawa, G. E. Dean, and R. M. Macnab.** 1987. Identification of the M-ring protein of the flagellar motor of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **84**: 7483–7487.
14. **Homma, M., and T. Iino.** 1985. Locations of hook-associated proteins in flagellar structures of *Salmonella typhimurium*. J. Bacteriol. **162**:183–189.
15. **Homma, M., T. Iino, and R. M. Macnab.** 1988. Identification and characterization of the products of six region III flagellar genes (*flaAII.3* through *flaQII*) of *Salmonella typhimurium*. J. Bacteriol. **170**:2221–2228.
16. **Homma, M., Y. Komeda, T. Iino, and R. M. Macnab.** 1987. The *flaFIX* gene product of *Salmonella typhimurium* is a flagellar basal body component with a signal peptide for export. J. Bacteriol. **169**:1493–1498.
17. **Homma, M., K. Kutsukake, and T. Iino.** 1985. Structural genes for flagellar hook-associated proteins in *Salmonella typhimurium*. J. Bacteriol. **163**:464–471.
18. **Homma, M., K. Kutsukake, T. Iino, and S. Yamaguchi.** 1984. Hook-associated proteins essential for flagellar filament formation in *Salmonella typhimurium*. J. Bacteriol. **157**:100–108.
19. **Homma, M., K. Ohnishi, T. Iino, and R. M. Macnab.** 1987. Identification of flagellar hook and basal body gene products (FlaFV, FlaFVI, FlaFVII and FlaFVIII) in *Salmonella typhimurium*. J. Bacteriol. **169**:3617–3624.
20. **Iino, T.** 1969. Polarity of flagellar growth in *Salmonella*. J. Gen. Microbiol. **56**:227–239.
21. **Iino, T.** 1985. Genetic control of flagellar morphogenesis in *Salmonella*, p. 83–92. *In* M. Eisenbach and M. Balaban (ed.), Sensing and response in microorganisms. Elsevier Science Publishers B.V., Amsterdam.

22. **Iino, T., Y. Komeda, K. Kutsukake, R. M. Macnab, P. Matsumura, J. S. Parkinson, M. I. Simon, and S. Yamaguchi.** 1988. New unified nomenclature for the flagellar genes of *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **52**:533–535.
23. **Ikeda, T., S. Asakura, and R. Kamiya.** 1985. "Cap" on the tip of *Salmonella* flagella. J. Mol. Biol. **184**:735–737.
24. **Ikeda, T., M. Homma, T. Iino, S. Asakura, and R. Kamiya.** 1987. Localization and stoichiometry of hook-associated proteins within *Salmonella typhimurium* flagella. J. Bacteriol. **169**: 1168–1173.
25. **Jones, C. J., M. Homma, and R. M. Macnab.** 1987. Identification of proteins of the outer (L and P) rings of the flagellar basal body of *Escherichia coli*. J. Bacteriol. **169**:1489–1492.
26. **Jones, C. J., M. Homma, and R. M. Macnab.** 1989. L-, P-, and M-ring proteins of the flagellar basal body of *Salmonella typhimurium*: gene sequences and deduced protein sequences. J. Bacteriol. **171**:3890–3900.
27. **Joys, T. M.** 1985. The covalent structure of the phase-1 flagellar filament protein of *Salmonella typhimurium* and its comparison with other flagellins. J. Biol. Chem. **260**:15758–15761.
28. **Khan, S., M. Dapice, and T. S. Reese.** 1988. Effects of *mot* gene expression on the structure of the flagellar motor. J. Mol. Biol. **202**:575–584.
29. **Kihara, M., M. Homma, K. Kutsukake, and R. M. Macnab.** 1989. Flagellar switch of *Salmonella typhimurium*: gene sequences and deduced protein sequences. J. Bacteriol. **171**: 3247–3257.
30. **Komeda, Y.** 1982. Fusions of flagellar operons to lactose genes on a Mu *lac* bacteriophage. J. Bacteriol. **150**:16–26.
31. **Komeda, Y., M. Silverman, P. Matsumura, and M. Simon.** 1978. Genes for the hook-basal body proteins of the flagellar apparatus in *Escherichia coli*. J. Bacteriol. **134**:655–667.
32. **Kutsukake, K., T. Iino, Y. Komeda, and S. Yamaguchi.** 1980. Functional homology of *fla* genes between *Salmonella typhimurium* and *Escherichia coli*. Mol. Gen. Genet. **178**:59–67.
33. **Kutsukake, K., T. Suzuki, S. Yamaguchi, and T. Iino.** 1979. Role of gene *flaFV* on flagellar hook formation in *Salmonella typhimurium*. J. Bacteriol. **140**:267–275.
34. **Kuwajima, G., I. Kawagishi, M. Homma, J.-I. Asaka, E. Kondo, and R. M. Macnab.** 1989. Export of an N-terminal fragment of *Escherichia coli* flagellin by a flagellum-specific pathway. Proc. Natl. Acad. Sci. USA **86**:4953–4957.
35. **Macnab, R. M.** 1987. Flagella, p. 70–83. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
36. **Meynell, E. W.** 1961. A phage, ϕ, which attacks motile bacteria. J. Gen. Microbiol. **25**:253–290.
37. **Morrissey, J. H.** 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307–310.
38. **Ohnishi, K., M. Homma, K. Kutsukake, and T. Iino.** 1987. Formation of flagella lacking outer rings by *flaM*, *flaU* and *flaY* mutants of *Escherichia coli*. J. Bacteriol. **169**:1485–1488.
39. **Okino, H., M. Isomura, S. Yamaguchi, Y. Magariyama, S. Kudo, and S.-I. Aizawa.** 1989. Release of flagellar filament-hook-rod complex by a *Salmonella typhimurium* mutant defective in the M ring of the basal body. J. Bacteriol. **171**:2075–2082.
40. **Oliver, D.** 1985. Protein secretion in *Escherichia coli*. Annu. Rev. Microbiol. **39**:615–648.
41. **Ravid, S., and M. Eisenbach.** 1984. Direction of flagellar rotation in bacterial cell envelopes. J. Bacteriol. **158**:222–230.
42. **Silverman, M., P. Matsumura, and M. Simon.** 1976. The identification of the *mot* gene product with *Escherichia coli*-lambda hybrids. Proc. Natl. Acad. Sci. USA **73**:3126–3130.
43. **Stader, J., P. Matsumura, D. Vacante, G. E. Dean, and R. M. Macnab.** 1986. Nucleotide sequence of the *Escherichia coli motB* gene and site-limited incorporation of its product into the cytoplasmic membrane. J. Bacteriol. **166**:244–252.
44. **Stallmeyer, M. J. B., S.-I. Aizawa, R. M. Macnab, and D. J. DeRosier.** 1989. Image reconstruction of the flagellar basal body of *Salmonella typhimurium*. J. Mol. Biol. **205**:519–528.
45. **Suzuki, T., T. Iino, T. Horiguchi, and S. Yamaguchi.** 1978. Incomplete flagellar structures in nonflagellate mutants of *Salmonella typhimurium*. J. Bacteriol. **133**:904–915.
46. **Suzuki, T., and Y. Komeda.** 1981. Incomplete flagellar structures in *Escherichia coli* mutants. J. Bacteriol. **145**:1036–1041.
47. **Talmadge, K., and W. Gilbert.** 1982. Cellular location affects protein stability in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **79**:1830–1833.
48. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.
49. **Wilson, M. L., and R. M. Macnab.** 1988. Overproduction of the MotA protein of *Escherichia coli* and estimation of its wild-type level. J. Bacteriol. **170**:588–597.
50. **Wood, W. B., and R. A. Crowther.** 1983. Long tail fibers: genes, proteins, assembly, and structure, p. 259–269. *In* C. K. Mathews, E. M. Kutter, G. Mosig, and P. B. Berget (ed.), Bacteriophage T4. American Society for Microbiology, Washington, D.C.
51. **Yamaguchi, S., S.-I. Aizawa, M. Kihara, M. Isomura, C. J. Jones, and R. M. Macnab.** 1986. Genetic evidence for a switching and energy-transducing complex in the flagellar motor of *Salmonella typhimurium*. J. Bacteriol. **168**:1172–1179.
52. **Yamaguchi, S., H. Fujita, A. Ishihara, S.-I. Aizawa, and R. M. Macnab.** 1986. Subdivision of flagellar genes of *Salmonella typhimurium* into regions responsible for assembly, rotation, and switching. J. Bacteriol. **166**:187–193.
53. **Yamaguchi, S., T. Iino, T. Horiguchi, and K. Ohta.** 1972. Genetic analysis of fla and mot cistrons closely linked to H1 in *Salmonella abortusequi* and its derivatives. J. Gen. Microbiol. **70**:59–75.