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Co-Overproduction and Localization of the *Escherichia coli* Motility Proteins MotA and MotB

M. LISA WILSON† AND ROBERT M. MACNAB*

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

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The motility genes *motA* and *motB* of *Escherichia coli* were placed under control of the *Serratia marcescens trp* promoter. After induction with β-indoleacrylic acid, the levels of MotA and MotB rose over about a 3-h period, reaching plateau levels approximately 50-fold higher than wild-type levels. Both overproduced proteins inserted into the cytoplasmic membrane. Growth and motility were essentially normal, suggesting that although the motor is a proton-conducting device, MotA and MotB together do not constitute a major proton leak. Derivative plasmids which maintained an intact version of *motB* but had the *motA* coding region deleted in various ways were constructed. With these, the levels of MotB were much lower, reaching a peak within 30 min after induction and declining thereafter; pulse-chase measurements indicated that a contributing factor was MotB degradation. The low levels of MotB occurred even with an in-frame internal deletion of *motA*, whose translational initiation and termination sites were intact, suggesting that it is the MotA protein, rather than the process of MotA synthesis, that is important for MotB stability. Termination at the usual site of overlap with the start of *motB* (ATGA) was not an absolute requirement for MotB synthesis but did result in higher rates of synthesis than when translation of *motA* information terminated prematurely. Even in the total absence of MotA, the MotB that was synthesized was found exclusively in the cytoplasmic membrane fraction. In wild-type cells, MotA was estimated by immunoprecipitation to be in about fourfold excess over MotB; a previous estimate of 600 ± 250 copies of MotA per cell then yielded an estimate of 150 ± 70 copies of MotB per cell.

The bacterial flagellar motor is a rotary, switchable device energized by the transmembrane proton motive force. Among the 40 or so genes that are necessary for flagellar assembly and function, two (*motA* and *motB*) fall into a unique category: in their absence, the flagellum, although it looks normal, cannot rotate. Thus, whereas the null phenotype of all other flagellar genes is nonflagellate (Fla$^-$), the null phenotype of these two genes is flagellate but paralyzed (Mot$^-$) (1, 9). Restoration of *motA* or *motB* function by placing the genes under control of a regulatable promoter results in restoration of function to existing flagella as well as to those assembled subsequently (3, 5). MotA and MotB are thought to exist in the vicinity of the flagellar basal body and—together with three flagellar switch proteins (FliG, FliM, and FliN)—to constitute the motor. For a review see, for example, reference 14.

What is actually known of the cellular location of the Mot proteins? They are normally present in amounts too low to be detected biochemically, but programmed expression of the genes cloned onto lambda hybrid phage (19) enabled the *motA* and *motB* gene products to be identified (as proteins with apparent molecular masses of 31 and 39 kilodaltons [kDa], respectively) and indicated that they are located in the cytoplasmic membrane (17), a location consistent with their function; a membrane location was also noted subsequently with plasmid-encoded MotA (22) and MotB (6).

As further evidence for a membrane location, circlets of studlike features seen in freeze-fracture images of the cell membrane have been tentatively identified as consisting of MotA and MotB (13). The deduced sequences of both proteins (8, 21) indicate that MotA is quite hydrophobic (with at least four predicted membrane-spanning regions) but MotB is less so (with one strongly predicted membrane-spanning region); protease-sensitivity and *phoA*-fusion experiments (7) confirm that MotB spans the membrane once, with the N terminus in the cytoplasm and the bulk of the protein residing in the periplasm.

Even when overproduced at levels 50-fold over normal and in the absence of MotB, MotA is membrane associated (22). MotB, however, has been reported to remain in the cytoplasm when it alone is overproduced (21), suggesting that it may require MotA or other flagellar components for insertion into the membrane in appreciable amounts.

We were therefore interested to know whether increasing the levels of both MotA and MotB would alter the situation. In addition to the issue of stabilization of MotB in the membrane, we were also interested in possible interaction of the *motA* and *motB* genes at the translational level, since they are adjacent within the *mocha* operon, with a four-base overlap (ATGA) of their coding regions.

We report here an analysis of plasmids with *motA* and *motB* in their normal relationship with each other, but under an inducible promoter, and also of plasmids with *motB* intact but with in-frame and out-of-frame fusions and internal deletions of *motA*. Under all circumstances, MotB was found in the membrane fraction. However, its stable existence was dependent on the presence of MotA.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, enzymes, and chemicals. The strains and plasmids used are listed in Table 1. Growth conditions, including those for overproduction of proteins whose genes are under control of the *Serratia marcescens trp* promoter, have been described (22). Enzymes and chem-

* Corresponding author.
† Present address: Scully, Scott, Murphy and Presser, Garden City, NY 11530.

TABLE 1. Bacterial strains and plasmids

Strain no.	Relevant genotype	Reference
Escherichia coli		
AW330	Wild type	1
W1485	Wild type	2
AW448	*motA*	1
AW507	*motB*	1
MS1569	Δ(*motA-cheA*)	20
UH869	*minA minB*	10
Plasmids		
pGD2	p_{mocha} *motA*$^+$ *motB*$^+$	8
pLW2	p_{trp} *motA*$^+$ *motB*	M. L. Wilson, unpublished data
pLW3	p_{trp} *motA*$^+$ *motB*	22
pLW4	p_{trp} *motA motB*	22
pLW5[a]	p_{trp} *motA*$^+$ *motB*$^+$	This study

[a] Deletion derivatives of pLW5 are described in Fig. 4 and in the text.

icals were obtained from the sources described previously (22). Phosphorylated *Bgl*II linker was from Takara Shuzo Co., Kyoto, Japan.

Construction of pLW5 and deletion derivatives. (i) Plasmid pLW5. Plasmid pLW2 (22) contains *motA* under the control of the *S. marcescens trp* promoter, while pGD2 (8) contains both *motA* and *motB* under control of the natural *mocha* promoter. The *Mlu*I-*Pvu*II fragment of pLW2 was replaced by the *Mlu*I-*Hpa*I fragment of pGD2 to yield pLW5, which has *motA* and *motB* intact in their normal arrangement but under p_{trp} control. *motA* is preceded by a 5′ fragment (sufficient to encode 40 residues) of a chemotaxis gene, *cheY*, such that a small out-of-frame *cheY* fusion protein is the first product of the *trp*-controlled operon.

(ii) Plasmid pLW51. A unique *Bam*HI site exists in a linker segment at the fusion point of pLW5, just upstream of the *motA* ribosomal binding site, and a unique *Bgl*II site exists within *motA*. pLW5 was digested with *Bam*HI and *Bgl*II, filled by using DNA polymerase Klenow fragment, and blunt-end ligated; this resulted in preservation of the reading frames of *cheY* and *motA*, generating an in-frame CheY-MotA fusion.

(iii) Plasmid pLW52. pLW5 was digested as for pLW51 but was ligated by using the compatible sticky ends; this altered the reading frame of *motA* to give an out-of-frame fusion that terminated prematurely.

(iv) Plasmid pLW53. pLW5 contains unique *Bst*EII and *Mlu*I sites within *motA*. It was digested with these enzymes, filled, and blunt-end ligated via an octanucleotide *Bgl*II linker, CAGATCTG; this preserved the reading frame of *motA*, generating an in-frame internal deletion of *motA*.

(v) Plasmid pLW54. pLW54 was made similarly to pLW53 but without the linker; this created an out-of-frame internal deletion of *motA* that terminated prematurely.

Electrophoretic techniques. Preparative and analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously (22).

Recombinant DNA techniques and DNA sequencing. Standard recombinant DNA techniques were carried out as described previously (22). Direct plasmid DNA sequencing was performed by the dideoxy-chain termination method (18) by using a custom-synthesized primer directed at a sequence about 30 base pairs downstream of the *cheY* translational start and about 90 base pairs upstream of the *Bam*HI linker site of pLW5 (see Results).

Radiolabeling. Plasmid-transformed minicells labeled with L-[^{35}S]methionine were obtained as described previously (15). For pulse-chase measurements, minicells (1 ml at an optical density of 1 at 650 nm) were incubated for 20 min at 37°C followed by addition of L-[^{35}S]methionine (15 μCi; 1,460 Ci mmol^{-1}) to initiate the pulse. After 2 min, the chase was begun by adding a 10,000-fold excess of unlabeled methionine. Samples were withdrawn at intervals and frozen on dry ice. The samples were later thawed on ice and spun for 2 min in a microcentrifuge (Beckman Instruments, Inc., Fullerton, Calif.), and the pellet was resuspended in SDS sample buffer and analyzed by SDS-PAGE. The gels were stained with Coomassie blue and then treated with salicylate for fluorographic enhancement (30% methanol for 10 to 20 min, 0.5 M sodium salicylate plus 0.25 M NaOH for 30 min, 30% methanol rinse) and dried for autoradiography.

Cell fractionation. Cells were lysed by modifying the methods described by Witholt and Boekhout (23). Cells were harvested in mid-exponential phase, and spheroplasts were prepared by resuspending the cells at room temperature to 40 mg ml^{-1} dry weight in 200 mM Tris hydrochloride, pH 8.0, successively adding 0.005 volumes of 100 mM EDTA (pH 8), 1 volume of 200 mM Tris hydrochloride and 1 M sucrose (pH 8), and 0.01 volumes of 10 mg of lysozyme ml^{-1} in water. After 5 min, the spheroplasts were lysed either by twofold dilution into 10 mM EDTA followed by chilling and sonication (three 10-s bursts, model W140D with microtip; Branson Sonic Power Co., Danbury, Conn.) or by 11-fold dilution into 10 mM EDTA followed by DNase treatment. The soluble and membrane fractions were separated by centrifugation (Beckman Airfuge, A-95 rotor, 10 min, 30 lb/in^2), and the pellet was resuspended in the original lysis volume.

Motility analysis. Motility was assayed by measurement of swarm rate on soft agar as described previously (22).

Preparation of antibody against MotB. Total membranes from induced W1485(pLW5) cells were subjected to preparative SDS-PAGE and stained with Coomassie blue, and the MotB band was excised and electroeluted in SDS-PAGE running buffer (10 mA, 6 h). Stain was removed by three extractions with an equal volume of H_2O-saturated *n*-butanol. The solution was then diluted into 5 volumes of phosphate-buffered saline and concentrated by centrifugation (4,000 × *g*; 25 min) through a Centricon concentration unit (YM membrane; Amicon Corp., Lexington, Mass.; 10-kDa cutoff); it was diluted again with 2.5 volumes of phosphate-buffered saline supplemented with 0.1% SDS, and the concentration step was repeated. The final concentrate was used for subcutaneous injections, essentially as previously described (22). Preabsorption of anti-MotB antiserum used cell extracts of a Δ*motB* strain, MS1569, as previously described (22).

Immunoblotting and immunoprecipitation. Immunoblotting was carried out as described previously (22) with anti-MotB antibody at a 1:200 dilution.

For immunoprecipitation experiments, cells were grown at 34°C in M9 (16) supplemented with glycerol (0.5% [vol/vol]), Casamino Acids (1.0%), thymine (40 μg ml^{-1}), thiamine (1 μg ml^{-1}), and biotin (0.5 μg ml^{-1}); washed with M9 containing (instead of Casamino Acids) all 20 amino acids except methionine (each at 50 μg ml^{-1}); and grown in that medium for 30 min; L-[^{35}S]methionine (100 μCi; 1,460 Ci mmol^{-1}) was added, and incubation was continued for an additional 30 min. Total membranes were prepared essentially as described above and solubilized in Tris-buffered saline–5 mM EDTA–10 mM $MgSO_4$–0.5% Triton X-100. After 5 min at room temperature, the mixture was centrifuged (Beckman Airfuge, A-95 rotor, 10 min, 30 lb/in^2) and

the supernatant, containing primarily inner-membrane proteins (19), was diluted with an equal volume of Tris-buffered saline and used as the extract for immunoprecipitation. Preabsorbed serum (equivalent of 5 μl of the original antiserum) was added to 200 μl of extract, and the mixture was incubated for 75 min at 4°C with mixing. Precipitates were collected by addition of 5 mg of protein A-Sepharose CL 4B beads (washed twice in Tris-buffered saline–0.1% Triton X-100) and incubation for 75 min at 4°C with mixing. The beads were then washed three times at high stringency (0.1% SDS, 0.05% Triton X-100, 0.3 M NaCl, 10 mM Tris hydrochloride [pH 8.6]) and analyzed by SDS-PAGE and autoradiography.

Densitometry. Autoradiographs were analyzed by densitometry as described previously (22).

RESULTS

Construction of a plasmid to overexpress both *motA* and *motB*. A plasmid (pGD2), containing *motA* and *motB* in their normal mutual arrangement and with their natural (*mocha*) promoter, had been constructed previously (8). We had also constructed pLW3, in which the *mocha* promoter was replaced by the *trp* promoter from *S. marcescens*, and had found that cells induced with β-indoleacrylic acid (IAA) overproduced MotA (22); the insert in pLW3 terminated just beyond *motA* and we now wished to construct a related plasmid with the insert continuing through the end of *motB*. The construction of such a plasmid, pLW5, is described in Materials and Methods. It contained functional copies of *motA* and *motB*, as judged by complementation of mutants AW448 (*motA*) and AW507 (*motB*) in swarm assays. Complementation did not require induction with IAA, indicating that high levels of MotA and MotB are not necessary for full function.

To establish the presence of the proteins biochemically, minicells were isolated and plasmid-encoded protein synthesis was allowed to proceed in the presence of L-[^{35}S]methionine. With plasmid pLW3, a radiolabeled band corresponding to MotA (molecular mass = 31 kDa) was seen as a major product (Fig. 1), as has been described previously (22). With pLW5, both MotA and a band with a molecular mass of 39 kDa were seen. This value, although higher than the value (34 kDa) deduced from the *motB* gene sequence (21), agrees with that reported for MotB synthesized from lambda hybrid phage (17, 19). Neither the 31- nor the 39-kDa band was present with the control plasmid, pLW4.

The molar stoichiometry of MotA relative to MotB under these conditions, estimated by densitometry and the known numbers of sulfur-containing residues in the two proteins (8, 21), was about 3.5:1.

Co-overproduction of MotA and MotB. We next attempted to co-overproduce MotA and MotB, using optimum induction conditions described previously for MotA (22). Following IAA addition to early-exponential-phase cultures of cells transformed with pLW5 or control plasmids, samples were withdrawn periodically and fractionated into soluble components (cytoplasm and periplasm) and membrane components (including high-molecular-weight aggregates). Bands corresponding to both MotA and MotB first became detectable on Coomassie blue-stained SDS-PAGE gels of the membrane fraction of pLW5-transformed cells about 2 to 3 h after addition of IAA (Fig. 2b); sedimentation equilibrium centrifugation of the membrane fraction confirmed that both proteins were present in the cytoplasmic membrane (data not shown), as had previously been demonstrated for MotA (22).

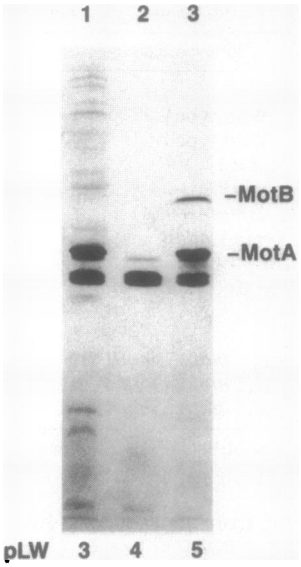


FIG. 1. Synthesis of MotA and MotB in minicells under noninducing conditions. The minicell-producing strain UH869 was transformed by the plasmids indicated, and minicells were labeled by L-[^{35}S]methionine, subjected to SDS-PAGE, and autoradiographed. MotA can be seen with plasmid pLW3, both MotA and MotB can be seen with pLW5, and neither can be seen with the control plasmid pLW4. The light and heavy bands just below MotA are the precursor and mature forms of β-lactamase.

The amounts of MotA and MotB increased for several hours and remained at a high level even after overnight growth; there was about twice as much MotA as MotB, assuming they have similar stain affinities. At no time was either MotA or MotB detected in the soluble fraction (Fig. 2a).

Growth rates and motility (as judged by swarm assay) were essentially normal under conditions of simultaneous overproduction of MotA and MotB (data not shown).

Antibody directed against MotB. Overproduction of MotB protein enabled us to raise a polyclonal antibody (see Materials and Methods). The crude serum was used to probe membranes containing excess MotA and MotB (W1485 [pLW5]) or excess MotA only (W1485[pLW3]). The immunoblot revealed that specific antibodies against MotB were present along with antibodies (also present in the preimmune serum) to numerous other antigens present in both strains (data not shown). Preadsorption with a cellular extract from a Δ*motB* strain (MS1569) removed nearly all these contaminating antibodies (Fig. 3a). By using this preadsorbed serum, MotB could be detected in the membranes of wild-type strain AW330 but not those of a stable *motB* mutant (AW507) or, unexpectedly, a *motA* mutant (AW448) (Fig. 3b).

The antibody also enabled detection of the homologous protein in wild-type *Salmonella typhimurium* (data not shown).

Construction of plasmids encoding MotB but not MotA. A previous study had demonstrated that MotA inserts into the cell membrane, even when overproduced (22). The data presented above demonstrate that this is also true of both MotA and MotB when co-overproduced. We wished next to examine whether MotA was playing a role in the synthesis or localization of MotB. We were also interested to know whether the process of *motA* translation (specifically, initiation at the *motA* start codon and termination at the ATGA overlap with the *motB* start codon) was important for the

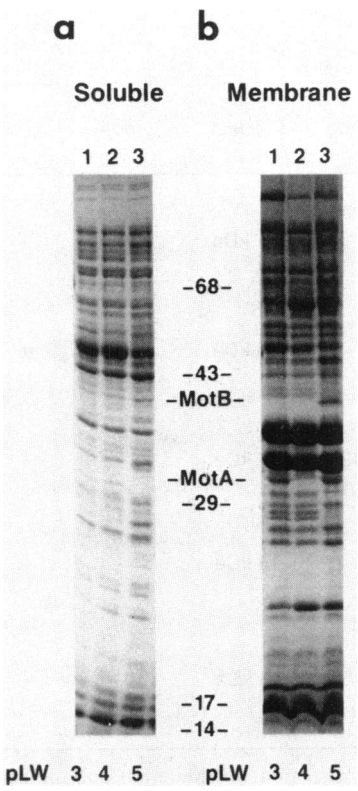


FIG. 2. Co-overproduction and localization of MotA and MotB. Strain W1485 was transformed with the plasmids indicated and grown for 3 h in the presence of the inducer IAA. Cells were then fractionated into soluble and membrane components (see text), analyzed by SDS-PAGE, and stained by Coomassie blue; lanes contain material from the same number of cells. With plasmid pLW5, both MotA and MotB can be seen in the membrane fraction; with plasmid pLW3, only MotA can be seen, again in the membrane fraction; with the control plasmid, neither protein can be seen. Neither MotA nor MotB can be seen in the soluble fractions. The positions of molecular mass markers are indicated.

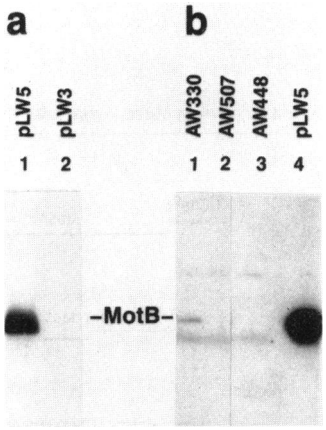


FIG. 3. Specificity of anti-MotB antiserum. Blots were probed with preabsorbed anti-MotB antiserum and ^{125}I-protein A and autoradiographed. (a) Induced levels of MotB in total membranes prepared from W1485 cells transformed with pLW5 (overproducing MotA and MotB) and pLW3 (overproducing MotA). (b) Detection of cellular levels of MotB in total membranes from AW330 (wild type), AW507 (*motB*), and AW448 (*motA*); W1485(pLW5), induced for MotA and MotB overproduction, is shown for comparison.

synthesis of MotB, even where no functional MotA was being made.

Deletion plasmids constructed to examine this question were named pLW51, pLW52, pLW53, and pLW54 (Fig. 4; see Materials and Methods for details of their construction). They were initially identified by screening for loss of *motA* complementation and retention of *motB* complementation. Predicted restriction site and fragment size data were verified (data not shown), and the correctness of the constructions was confirmed by DNA sequencing through the ligation site. Properties of these plasmids and the products they were expected to encode are given in Fig. 4.

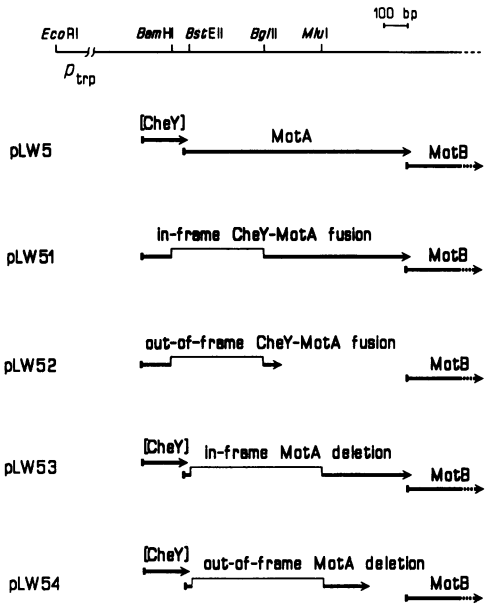
Properties of deletion plasmids encoding MotB but not MotA. All four deletion plasmids were expressing the *motB* gene at functional levels, as judged by complementation (Fig. 4). In minicell experiments, MotB was produced in amounts readily detectable by autoradiography, even under noninducing conditions (Fig. 5). pLW51 also produced a protein with a molecular mass of 25 kDa, the value expected for the in-frame CheY-MotA fusion protein; the presence of a MotA domain was confirmed by immunoblotting with anti-MotA antibody (22). The fusion or deletion products of the other three plasmids were too small to be detected in the gel system used.

The levels of MotB appeared higher in the in-frame constructions (pLW51 and pLW53) than in the other two, but minicell experiments are not well suited to quantitative comparisons between cultures. The question of relative amounts and rates of synthesis will be addressed below.

We next attempted to overproduce MotB. Cells were transformed with the four deletion plasmids (and pLW5 as a positive control) and induced with IAA as described above, and samples were removed periodically (from 3 to 18 h postinduction) for fractionation and analysis. MotB was not detectable by Coomassie blue stain at any time point with any of the deletion plasmids (Fig. 6), including pLW53, which has the normal translation start and stop sites for *motA*. In the case of pLW51, successful induction was verified by the 25-kDa CheY-MotA fusion protein, which was detected in the membrane fraction in amounts that increased with time; successful induction was also indicated for the positive control pLW5 by the presence of MotA and MotB and for all five plasmids by the presence of various *trp*-related bands.

The anti-MotB antibody enabled us to examine induction of *motB* expression with greater sensitivity. Levels of MotB detectable by immunoblotting were obtained in the membrane fraction with all four plasmids. The amount increased during the first 30 min after addition of IAA (Fig. 7) but thereafter dropped significantly, leveling off by about 50 min at about 50% of the peak value. No MotB was detected in the soluble fractions at any time (data not shown). Both the rates of increase and the peak levels were approximately three- to fourfold higher in the in-frame constructs, pLW51 or pLW53, than in the corresponding out-of-frame ones, pLW52 or pLW54; differences between pairs of plasmids with different translational start sites but the same stop site (pLW51 and pLW53, or pLW52 and pLW54) were small. The rate with pLW5-transformed cells was about twice those for the in-frame constructs and continued for about 3 h to give the high final levels evident, for example, in Fig. 6b.

Pulse-chase experiments indicated that degradation was a contributing factor to the decline in MotB levels. The effect was most pronounced with pLW51, which had the highest



	Gene preceding *motB*			Complementation	
	Start	Stop	Product	*motA*	*motB*
pLW5	*motA*	0	31 kDa	+	+
pLW51	*cheY*	0	25 kDa	−	+
pLW52	*cheY*	-495	7 kDa	−	+
pLW53	*motA*	0	13 kDa	−	+
pLW54	*motA*	-153	8 kDa	−	+

FIG. 4. Plasmid pLW5 and deletion derivatives made from it; for details of their construction, see Materials and Methods. [CheY] is a severely truncated chemotaxis protein encoded by pLW5. pLW51 has an in-frame deletion between the 5′ coding region of *cheY* and the 3′ coding region of *motA*, so that translation proceeds from the normal *cheY* start to the normal *motA* stop; pLW52 has a similar deletion but out of frame, so that translation of the *motA* region terminates prematurely. pLW53 has an in-frame deletion within *motA*, so that translation should start and stop normally, even though the product is severely defective; pLW54 has a similar deletion but out of frame, so that translation of the *motA* region should start normally but terminate prematurely. The start of the coding region of each mutant MotA protein is indicated (as the initiation codon of either *cheY* or *motA*), as is the end of the coding region (expressed as the number of base pairs upstream of the *motB* initiation codon); the deduced sizes of their products are also shown. The ability of the plasmids to complement transformed *motA* and *motB* mutants, as judged by swarm assay, is shown on the right.

initial rate and peak level of the four deletion plasmids. Whereas radiolabel in the CheY-MotA fusion protein and other plasmid-encoded proteins quickly rose to a peak value and then remained constant, that in MotB peaked about 2 min after commencing the chase and declined thereafter, with a half-life of about an hour (Fig. 8). With plasmid pLW5, in contrast, MotB radiolabel (like that of MotA) remained constant after reaching its peak value. Neither in the case of the induction nor the chase experiments did MotB levels decay to zero, perhaps because of the presence of low levels of chromosomally encoded MotA.

Expression of *motB* and location of its product in the absence of an intact *motA* gene. The biochemical experiments described thus far involved hosts with a chromosomal copy of *motA*. We therefore repeated the immunoblotting experiment described above by using AW448 (1), a *motA* mutant which has no detectable MotA antigen. Cells were harvested after 30 min of induction with IAA (the observed optimal time for MotB production; Fig. 7) and analyzed. Immunoblotting (Fig. 9) showed that MotB was synthesized and was essentially all located in the membrane. Thus MotA is not required for the association of MotB with the membrane.

As before (Fig. 7), the amount of MotB produced from pLW51 exceeded that from pLW52 (where *motA* translation was terminating prematurely), in this case by about 5- to 10-fold.

Relative amounts of MotA and MotB in wild-type cells. In a variety of studies, including the present one (e.g., Fig. 1), the

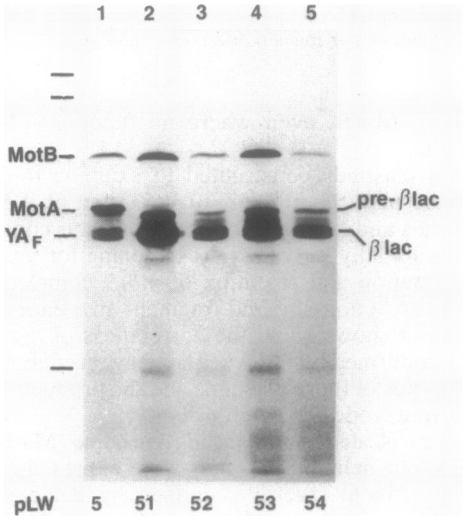


FIG. 5. Synthesis of MotB under noninducing conditions in minicells transformed with the plasmids indicated. pLW5 encodes MotA as well as MotB, while the other plasmids are pLW5 derivatives with *motB* intact but with in-frame (pLW51 and pLW53) or out-of-frame (pLW52 and pLW54) deletions of *motA* (see text and Fig. 4). Both MotA and MotB were detected with plasmid pLW5; MotB was detected with the four derivative plasmids. With pLW51, the in-frame CheY-MotA fusion protein (YA_F) is seen. pre-βlac, Precursor form of β-lactamase; βlac, mature form.

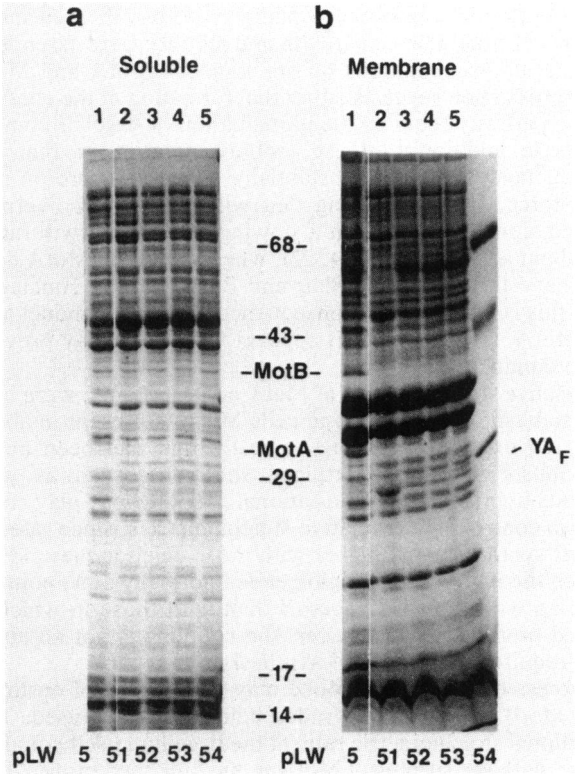


FIG. 6. Attempted overproduction of MotB without overproduction of MotA. Strain W1485 was transformed with the plasmids shown and induced with IAA for up to 18 h. Soluble and membrane fractions were subjected to SDS-PAGE and stained with Coomassie blue. MotB was detected only with pLW5-transformed cells, which also overproduced MotA; both proteins were found in the membrane fraction (cf. Fig. 2). In the case of pLW51-transformed cells, the success of the induction was verified by the presence of the in-frame fusion CheY-MotA protein (YA_F) (cf. Fig. 5). The samples shown here were taken after 3 h of induction, but no evidence of MotB overproduction was obtained at any point.

amount of MotA had been found to exceed that of MotB. However, none of these estimates were obtained with wild-type cells under normal conditions. With antibodies available against both proteins, we were in a position to make such an estimate.

Wild-type cells were grown in the presence of L-[^{35}S] methionine, and their solubilized membranes were precipitated with either MotA or MotB antibody (see Materials and Methods). The immunoprecipitates were washed at high stringency and then analyzed by SDS-PAGE, autoradiography, and densitometry. The ratio of MotA to MotB was calculated to be 3.9 ± 0.5, similar to the value described above for minicell experiments in which the two genes were plasmid encoded under control of the *trp* promoter (Fig. 1).

We had previously estimated the absolute stoichiometry of MotA in wild-type cells to be 600 ± 250 (22). On the basis of this and the above relative stoichiometry, we estimate the absolute stoichiometry of MotB to be about 150 ± 70.

DISCUSSION

Knowledge of the structural and functional relationship between MotA and MotB and between both proteins and the bacterial flagellum is necessary for a full description of the flagellar motor. In this study we have investigated the

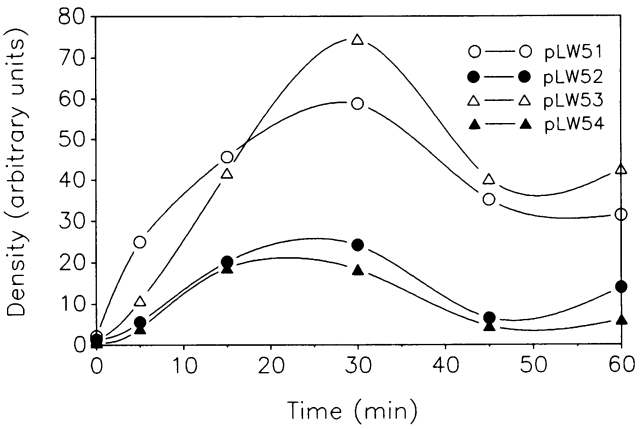


FIG. 7. Time course of induced MotB expression determined by immunoassay. W1485 cells, transformed with the various *motA* deletion plasmids indicated, were induced with IAA, harvested at the times indicated, and fractionated into soluble and membrane material. The membrane material was immunoblotted with anti-MotB antiserum as in Fig. 3. The autoradiographs were then analyzed by densitometry, and the integrated density of the MotB band was plotted in arbitrary units.

synthesis, location, and stability of MotB under conditions in which MotA was either present or absent. We have also investigated whether normal initiation and termination of translation of the *motA* coding region is important for translation of *motB*. The availability of an anti-MotB antibody (Fig. 3) has enabled us to identify the protein with confidence and to detect it when it was present only in small amounts.

MotB is located in the cytoplasmic membrane under all conditions tested. Most of the published evidence indicates that MotB is an integral membrane protein (see introduction). In agreement with this, we have found that MotB inserts into the membrane in large amounts when large amounts of MotA are also present (Fig. 2). In the absence of MotA, MotB produced at low levels still inserts into the membrane (Fig. 9). We cannot say whether large amounts could do so, because in our hands MotB was not overproduced to levels detectable by Coomassie blue stain unless MotA was also being overproduced (Fig. 6). This result differs from that obtained by Stader et al. (21), who, using

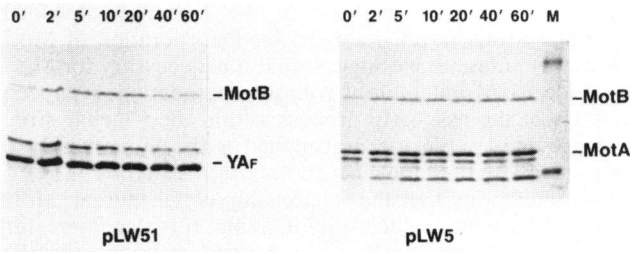


FIG. 8. Pulse-chase analysis of MotB stability in the presence (pLW5) and absence (pLW51) of MotA. Minicells containing these plasmids were pulse-labeled with L-[^{35}S]methionine for 2 min and then chased with a 10,000-fold excess of unlabeled methionine. Samples were withdrawn at the indicated times and subjected to SDS-PAGE and autoradiography. Of all the proteins detected, only MotB gives evidence of being chased and then only in the plasmid where *motA* is absent. Lane M contains molecular mass markers: ovalbumin (43 kDa) and carbonic anhydrase (29 kDa). YA_F, In-frame CheY-MotA fusion synthesized by pLW51.

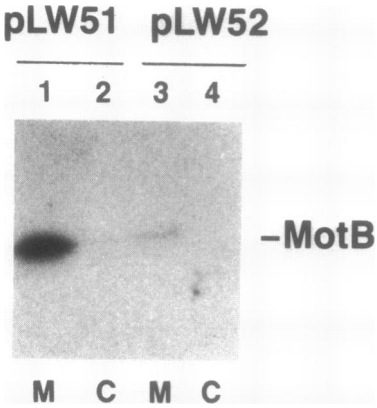


FIG. 9. Partitioning of MotB into the membrane in the absence of MotA. Strain AW448 (*motA*) was transformed with the *motA* deletion plasmids indicated, induced for 30 min with IAA, harvested, and fractionated. Membrane and soluble fractions were then analyzed by immunoblotting with anti-MotB antiserum.

the same promoter and similar induction conditions as in the present study, reported that a 42-kDa protein which they identified as MotB was overproduced in the absence of overproduced MotA and was located in the cytoplasm. We do not know the reason for the discrepancy but are confident (because of our use of an antibody specific for MotB) that we are correctly identifying the protein and that there were not large amounts of it anywhere in the cell unless MotA was also present in large amounts.

MotB requires MotA for its stable existence. MotB was synthesized in the absence of MotA (Fig. 9). However, the levels were unexpectedly low. Immunoblotting failed to detect cellular levels of MotB in a *motA* mutant (Fig. 3b), and Coomassie blue staining failed to detect plasmid-encoded MotB even under inducing conditions (Fig. 6). These results are unlikely to be a consequence of polarity, since the mutant complements a *motB* mutant (1) and the in-frame deletion plasmids should terminate translation of *motA* coding information at the normal point. This leaves the possibilities that *motB* expression is being inhibited or that MotB is unstable in the absence of MotA. The latter possibility is supported by pulse-chase experiments, which indicated that MotB was degraded under conditions where its amounts exceeded those of MotA (Fig. 8). The fact that, upon induction, levels first rose and then declined (Fig. 7) also suggests a cellular response to the presence of MotB. Although MotA is not required for incorporation of MotB into the membrane, we suggest that it is necessary for MotB to reside there indefinitely without degradation. (A recent analysis of the assembly process within the flagellar structure provides extensive precedent for the vulnerability of components that cannot incorporate into structure [11].)

It is interesting that the relationship is not mutual; MotA alone can be overproduced and is stable. It is also interesting that overproduced MotB is not dependent upon other flagellar structures for its stable existence, even though in the functioning organelle at least some such structures are presumed to interact with the Mot proteins.

The simplest hypothesis for the stabilization of MotB by MotA is that the two proteins form a complex; attempts to detect such a complex by coprecipitation with a single antibody (either anti-MotA or anti-MotB) were unsuccessful, but this certainly does not disprove the hypothesis.

The flagellar motor is proton driven and therefore constitutes a proton conductance pathway across the cell membrane. The fact that cell growth and motility were essentially normal under conditions of simultaneous MotA and MotB overproduction suggests either that formation of the conductance pathway requires the participation of flagellar components in addition to these motility proteins or that the conductance does not substantially perturb the proton motive force. It is interesting that wild-type MotA overproduced alone does result in a slowing down of growth rates, by about a factor of two (4, 22), whereas mutant MotA does not have this effect (4). Blair and Berg (4) have concluded that this is likely to be a consequence of proton conductance by MotA, in which case it appears that MotB may prevent such conductance.

Relative stoichiometries of MotA and MotB. We were able to establish that in wild-type cells MotA is present in about fourfold excess over MotB, a result that had been noted previously in various artificial situations, such as with lambda hybrid phage. Translational coupling may play some role in controlling the relative stoichiometries, since rates of MotB synthesis were higher with those deletion plasmids in which the *motA* coding region ended normally at its point of overlap with the start of *motB* than with those in which it ended prematurely. However, the coupling is not an absolute requirement for MotB synthesis.

Excess of MotA over MotB may be a means of ensuring that MotB will not be degraded before it can be used. The functional stoichiometric ratio at the flagellum itself could be unity, with the surplus of MotA in the bulk membrane. If all of the MotB in a wild-type cell is flagellum associated, its stoichiometry would be about 25 per flagellum. This number is somewhat higher than the maximum number of force-generating units inferred from rotation measurements (3, 5) or the number of flagellar studs seen by electron microscopy (13). Interestingly, it is about the same as the estimated number of subunits in the cytoplasmic-membrane-associated M ring of the basal body (12).

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