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NOTES

An Intergenic Stem-Loop Mutation in the *Bacillus subtilis ccpA-motPS* Operon Increases *motPS* Transcription and the MotPS Contribution to Motility†

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A stem-loop mutation between *ccpA* and *motP* in the *Bacillus subtilis ccpA-motPS* operon increased *motPS* transcription and membrane-associated MotPS levels, motility, and number of flagella/cell when MotPS is the sole stator and the MotPS contribution to motility at high pH, Na^+, and viscosity when MotAB is also present.

Hetero-oligomeric "Mot" complexes composed of MotA and MotB or their homologues form rings around individual bacterial flagella. The Mot complexes are stators for the flagellar rotor and also constitute ion channels that couple the energy of transmembrane ion gradients of either H^+ or Na^+ to rotation (2, 12, 20). Some bacteria have dual flagellar and/or Mot systems that are adaptive to different swimming modes, e.g., in liquid versus on surfaces, or certain physical-chemical conditions, e.g., salinity, pH, and viscosity (1, 11, 14, 15). In *Bacillus subtilis*, a single flagellar rotor system is powered by two Mot complexes that are coupled to fluxes of different cations, such that MotAB is H^+ coupled and MotPS is Na^+ coupled (10, 11). MotAB is dominant in laboratory strains of *B. subtilis*, which are only slightly motile when *motAB* is disrupted (11). However, a variant with increased motility (up-motile mutant) that exhibited robust MotPS-dependent swimming on soft agar plates when MotPS was the sole stator, was isolated; motility was highest at elevated viscosity, pH, and NaCl concentrations (11). Here, we clarified the molecular basis for the up-motile phenotype affecting MotPS-dependent motility, the effect of the mutation on the number of flagella/cell, and its effect on the contribution of MotPS to the motility of *B. subtilis* possessing a wild-type *motAB* locus.

The *motPS* genes are downstream of the *ccpA* gene, which encodes a central regulator of carbon metabolism, forming a putative *ccpA-motPS* operon (6, 7, 16). Coordinated expression of *ccpA* and *motPS* could represent a multipronged response to alkali stress, since both Na^+-coupled MotPS-dependent motility and increased metabolic production of acids are adaptive to high pH (11, 13). Sequence analysis of the up-motile mutant (AB::Tn-M) selected in a *motAB* mutant strain (AB::Tn) showed no mutations in the *ccpA* and *motPS* coding sequences (strains are listed in Table S1 in the supplemental material). However, a point mutation (G→A) was found at the 33rd nucleotide following the stop codon of the *ccpA* gene, within a stem-loop structure in the intergenic region between *ccpA* and *motP* that has the potential to serve as an intrinsic transcriptional terminator. The mutation is predicted by the Mfold program (22) to change the free energy (ΔG) of the RNA secondary structure from −18.5 kcal/mol to −12.3 kcal/mol. This mutation was confirmed to be sufficient to confer the up-motile phenotype after deletion of the native *ccpA-motPS* operon from *B. subtilis* AB::Tn using the method described previously by Horton (8), producing strain AB::TnΔCPS. Upon introduction of the mutant or wild-type *ccpA-motPS* operon into the *amyE* locus of this strain, the mutant *ccpA-motPS* locus supported the same up-motile phenotype as the original up-motile strain on soft agar plates, whereas the strain expressing the wild-type locus did not (shown in Fig. S1 in the supplemental material together with a diagram of the mutation site; primers and details of strain construction are available on request).

The levels of MotP and MotS in the membranes of the up-motile AB::Tn-M strain that lacks *motAB*, its AB::Tn parent strain, and the wild-type strain were analyzed by Western blots of sodium dodecyl sulfate–10% polyacrylamide gels (17) carried out using a chemiluminescence protocol according to the manufacturer's instructions (Amersham Biosciences). The polyclonal anti-MotP or anti-MotS antibodies used for detection were raised in rabbits against synthetic peptides corresponding to residues 88 to 100 of MotP (SLSDHARKHGLL) and to residues 1 to 14 of MotS (MKLRRERFERRNGS), with an additional cysteine added to the C terminus to facilitate conjugation to keyhole limpet hemocyanin (Operon Biotechnologies, Inc., Tokyo, Japan); a purified immunoglobulin G fraction (Melon Gel IgG Spin purification kit; Pierce Biotechnology, Inc., IL) was used. The intensity of the MotP and MotS bands was comparable in the wild-type and AB::Tn sam-

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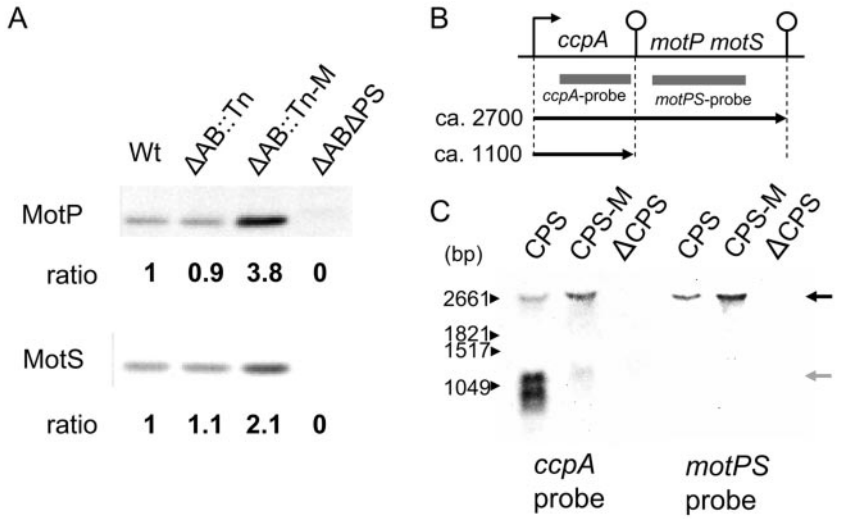

FIG. 1. Western and Northern analyses of MotP and MotS in the wild type, AB::Tn, AB::Tn-M, and ΔABΔPS. The cells were grown in 2× TY medium at 37°C. (A) Western analyses using antibodies against peptides corresponding to regions of MotP and MotS. The strain is indicated above each lane, with Wt representing the wild type. A quantitative imaging system, Pluor-S MAX (Bio-Rad), was used for detection and analysis of a chemiluminescence image. (B) Schematic diagram of the *ccpA-motPS* region of the *B. subtilis* chromosome indicating the probes used for Northern analyses. (C) Results of the Northern analyses, with the strain indicated above each lane and the probe used for the particular blot indicated below the panel. The expected sizes for *ccpA* mRNA and *ccpA-motPS* mRNA are indicated by a gray arrow and a black arrow, respectively.

ples, as expected, while the levels of MotP and MotS were increased 3.8 and 2.1 times, respectively, in the AB::Tn-M samples (Fig. 1A).

Transcript levels for *ccpA* and *motPS* were measured for the *motAB*$^+$ strains CPS and CPS-M that express the wild-type or mutant *ccpA-motPS* locus, respectively, only from the *amyE* locus, with a ΔCPS strain as a negative control. RNA was prepared as described previously (9), and Northern analysis was carried out using digoxigenin RNA probes (DIG RNA labeling kit, SP6/T7; Roche Applied Sciences). Both the *motPS* and *ccpA* probes (Fig. 1B and C) hybridized to a 2.7-kb band in RNA from both CPS and CPS-M (Fig. 1C). This size corresponds to the expected size for *ccpA-motPS* mRNA and was the only band observed with the *motPS* probe. The amount of the 2.7-kb mRNA in CPS-M cells was about twofold higher than that in CPS cells. In addition to the 2.7-kb *ccpA* band, a significant amount of *ccpA*-containing mRNA in the CPS strain was found in two bands around 1.1 kb in size, the expected size for a monocistronic *ccpA* transcript. A much weaker 1.1-kb mRNA signal was observed in the CPS-M mRNA (Fig. 1C, left). These results indicated that *ccpA* and *motPS* form an operon, since both *ccpA* and *motPS* probes hybridized to the 2.7-kb transcript. Consistent with a transcriptional termination function for the intergenic stem-loop, transcription of *ccpA* alone occurred at a higher level than transcription of the entire operon in the wild-type strain, whereas the level of the polycistronic *ccpA-motPS* mRNA is increased in the up-motile mutant, and little monocistronic *ccpA* mRNA was detected.

To better define the influence of the stem-loop element on transcription, *ccpA-lacZ* and *motPS-lacZ* fusions were generated in the wild-type (*motAB*$^+$) strain using the pMutin4 integration plasmid (18) to fuse *lacZ* to the *ccpA* gene upstream of the stem-loop or to *motS* downstream of either a wild-type stem-loop (*motPS-lacZ*) or an up-motile mutant stem-loop (*motPS-lacZ*-M) (Fig. 2A). The resulting strains grew comparably at 37°C in 2× TY medium (10) (Fig. 2B). Samples were taken at different points during growth for measurements of β-galactosidase activity (4). The most striking feature of the expression patterns was that *motPS-lacZ* transcriptional activity was significantly lower than that of *ccpA-lacZ*, whereas the transcriptional activity of *motPS-lacZ*-M was close to that of *ccpA-lacZ*, with a 3.6-fold increase relative to the *motPS-lacZ* fusion (Fig. 2C). These results are consistent with the observation that the up-motile mutation results in increased levels of *ccpA-motPS* polycistronic mRNA.

A microscopic examination was carried out on negatively stained preparations (21) of four motile strains (wild type, AB::Tn-M, ΔPS, and CPS-M) and three nonmotile strains

TABLE 1. Number and length of flagella of *B. subtilis mot* strains at pH 7.0

Strain	Stator(s)[c]	No. of flagella/cell[a]		Length of flagella (μm)[b]	
		Range of values	Avg	Range of values	Avg
Wild type	MotAB, MotPS	9–11	9.9	6.9–8.3	7.7
AB::Tn	MotPS	4–7	5.8	5.4–7.6	6.7
AB::Tn-M	MotPS-M	10–13	11.6	6.8–8.3	7.3
AB::TnΔPS	None	2–3	2.2	3.5–4.7	4.3
ΔAB	MotPS	4–6	5.0	6.2–7.6	6.9
ΔPS	MotAB	6–8	6.8	5.8–7.5	6.8
CPS-M	MotAB, MotPS-M	11–14	12.6	7.0–8.3	7.6

[a] Flagella were counted in five cells. The standard deviations of the average values shown were less than 2 for all values.

[b] Measurements were made for cells. The standard deviations of the average values shown were between 0.6 and 1.1.

[c] MotPS designates expression from a wild-type *ccpA-motPS* locus, and MotPS-M designates expression from an up-motile mutant locus.

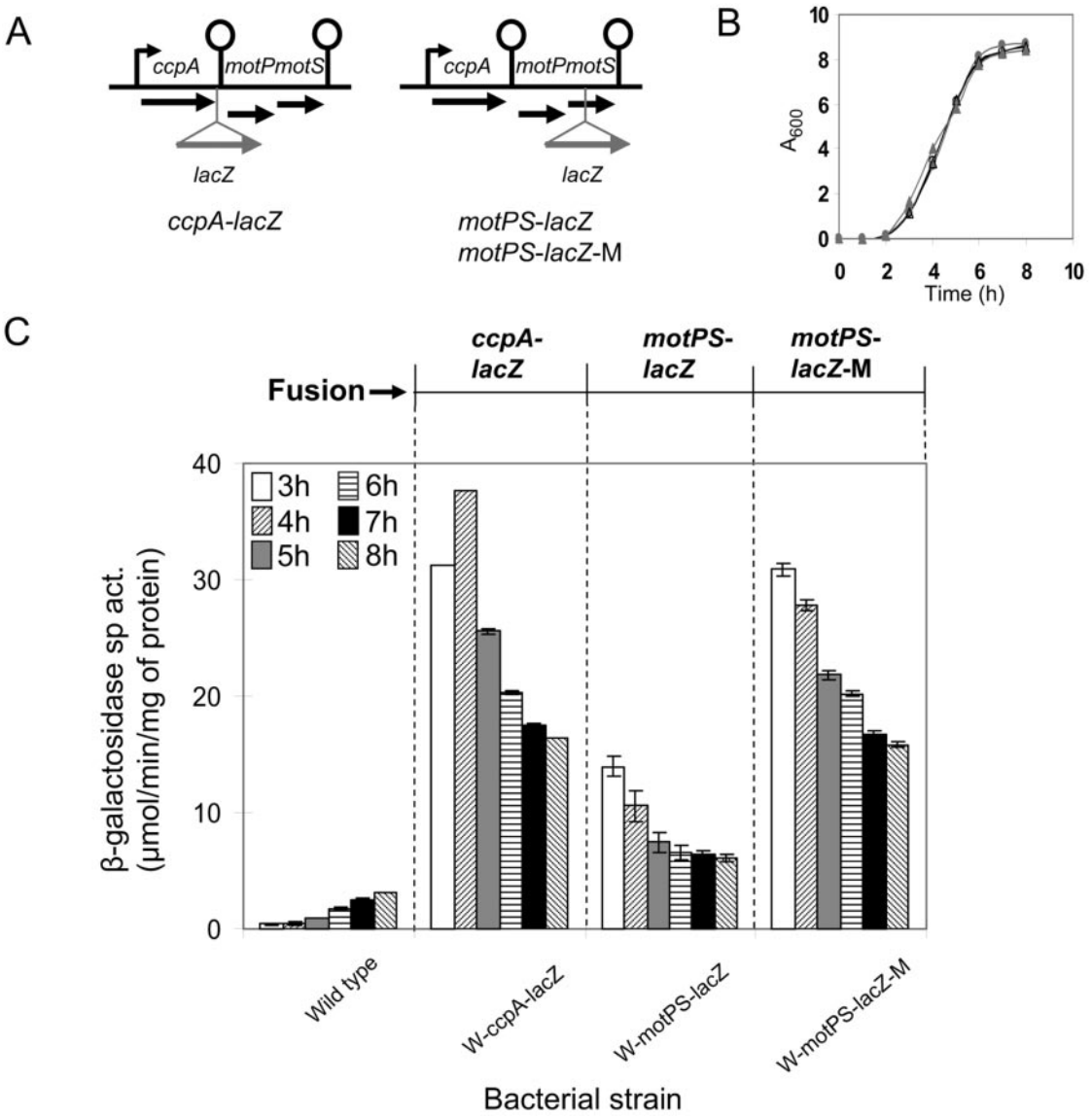

FIG. 2. Expression of *ccpA-lacZ* and *motPS-lacZ* fusions in wild-type and Δ*motAB* strains. (A) Schematic diagram of the integrated pMutin plasmid locus in the *ccpA-motPS* region. Each construct was inserted into the pMutin plasmid upstream of the stem-loop structure at the intergenic region of *ccpA* and *motP*. (B) Growth curves of strains. BR151MA (wild type), ○; W-*ccpA-lacZ*, ●; W-*motPS-lacZ*, △; W-*motPS-lacZ*-M, ▲. Growth in 2× TY medium at 37°C was monitored by A_{600}. (C) β-Galactosidase activity of the *ccpA-lacZ* and *motPS-lacZ* fusion constructs (indicated at the top of each panel) at different times during growth. Activity is expressed as nanomoles *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram protein. The strains used for the experiment are indicated below the panel. The error bars indicate the standard deviations of the values.

(AB::Tn, AB::Tn-ΔPS, and ΔAB). Strains expressing *motPS* from the up-motile mutant *ccpA-motPS* locus had an average of 12 flagella/cell whether or not MotAB was also present, a number of flagella/cell that was similar to that of the wild-type strain (average of 10) and higher than the number of flagella/cell in cells expressing only MotAB (average of 7) or MotPS (average of 5) from the wild-type *ccpA-motPS* locus (Table 1). The inability of the MotPS-only cells to swim in liquid, in contrast to the MotAB-only cells, is probably due to lower numbers of Mot complexes in the former cells, and that number is increased by the up-mutation. The *motAB motPS* double mutant had an average of 2 flagella/cell and was the only strain in which flagellar length was also significantly shorter than that of the wild type (Table 1). Calvio et al. (3) recently identified the *B. subtilis swrA* gene of the dicistronic *swrAB* operon as the locus of the *ifm* mutation that increases flagellar number and results in hypermotility (5). We verified that up-motile strains contained no changes in the *swrAB* sequence. Our results support other evidence that the presence of Mot complexes influences flagellar assembly (10, 19) and indicate that the presence of either the MotAB or MotPS stator is sufficient to allow normal flagellar biogenesis in *B. subtilis*.

Finally, the contribution of MotPS to the swimming speed under different conditions was assessed (10) in three strains

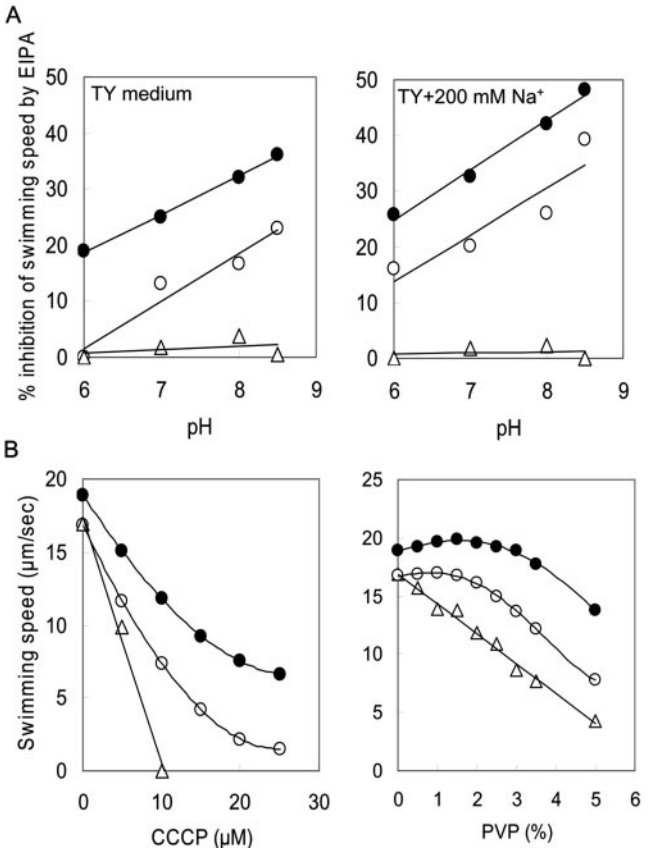

FIG. 3. Effects of the Na^+ channel inhibitor EIPA, the uncoupler CCCP, and PVP-mediated viscosity increase on swimming speed. (A) Swimming speeds were assayed for the wild type (○), CPS-M with the wild-type *motAB* locus and an up-motile *ccpA-motPS* operon expressed from the *amyE* locus (●), and the ΔPS strain that is deleted for *motPS* and wild type for *motAB* (△) after pregrowth without (left) or with (right) 200 mM added Na^+ at pH 6.0, 7.0, 8.0, and 8.5 in the presence or absence of EIPA. The values shown are the percent inhibition by EIPA calculated from data shown in the supplemental material (see Fig. S2 in the supplemental material). (B) The same strains used in A were pregrown overnight in TY medium plus 200 mM NaCl (pH 7.0) at 37°C. A 20-μl sample was used to inoculate 1 ml of fresh medium. After 6 h, each culture was diluted 50-fold with TY medium plus 200 mM NaCl (pH 8.5), with a range of CCCP (left) or PVP (right) concentrations.

that have wild-type *motAB* loci but differ in *motPS* status: wild-type *B. subtilis*; CPS-M, expressing a mutant *ccpA-motPS* operon in the *amyE* locus; and ΔPS, lacking *motPS*. First, cells grown at pH 7.0 in TY medium (which contains 14 to 17 mM Na^+), with or without the addition of 200 mM NaCl, were transferred into TY medium without added NaCl, at different pH values. The effect of the Na^+ channel blocker 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), which selectively inhibits MotPS-dependent swimming (11), was assayed. Indeed, EIPA did not significantly inhibit the motility of a strain lacking *motPS* (ΔPS) under any condition of pH or Na^+ content, whereas inhibition was observed in the *motPS*-containing strains (see Fig. S2 in the supplemental material). The inhibition by EIPA as a function of pH and pregrowth with 200 mM added Na^+ showed that MotPS has a significant role in motility in the up-motile MotPS strain even at pH 6.0 without pregrowth with added Na^+, whereas MotPS expressed from the wild-type locus contributed to swimming at pH 6.0 only if cells were pregrown with added Na^+ (Fig. 3A). At all pH values, the role of MotPS, as assessed by percent EIPA inhibition, was greater in the strain expressing the up-motile *ccpA-motPS* locus. Next, the contribution of MotPS to swimming of the *motAB*$^+$ strains at a low protonmotive force (lowered by protonophore carbonyl cyanide *m*-chlorophenylhydrazone [CCCP]) or elevated viscosity (achieved by the addition of polyvinylpyrrolidone [PVP]) was studied in cells pregrown and assayed in the presence of 200 mM NaCl at pH 8.5, conditions that maximized the MotPS contribution (see Fig. S2 in the supplemental material). Under these conditions, MotPS clearly contributed to swimming in the presence of added CCCP or PVP, with the mutant *ccpA-motPS* locus conferring greater adaptability than the wild-type locus to either low protonmotive force or elevated viscosity (Fig. 3B).

The swimming-speed assays show that MotPS plays a role in the motility profile of wild-type *B. subtilis* with a functional MotAB stator, especially once the organism is exposed to elevated Na^+ levels. The increased impact of the up-motile MotPS phenotype in strains containing the stem-loop mutation is most evident at elevated Na^+, pH, and viscosity or at low protonmotive force, conditions that could select for mutations of this type when "undomesticated strains" are exposed to them in the environment.

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