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Cloning and characterization of *motX*, a *Vibrio alginolyticus* sodium-driven flagellar motor gene

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Running title: a *motX* gene encoding a component of the sodium-driven motor

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Summary

Four motor proteins, MotX, MotY, PomA and PomB, have been identified as constituents of the Na^+-driven flagellum of *Vibrio* species. In this study, the complete *motX* gene was cloned from *Vibrio alginolyticus* and was shown to complement three *mot* mutations, *motX94*, *motX115* and *motX119*, as well as a *V. parahaemolyticus motX* mutant. The *motX94* mutant contains a frameshift at Val86 of MotX, while the *motX115* and *motX119* mutations are substitutions of Ala146 to Val and Gln 194 to amber, respectively. When MotX was overexpressed in *Vibrio* cells, the amount of MotY detected in the membrane fraction increased, and *vice versa*, suggesting that MotX and MotY stabilize mutually by interacting at the membrane level. When a plasmid containing the *motX* gene was introduced into *motY* mutants NMB117 (*motY117*) and VIO542 (*motY542*), the mutations were suppressed. In contrast, *motY* could not recover any swarm-defective *motX* mutants studied. Considering the above evidence, we propose that MotX is more directly involved than MotY with the mechanical functioning of the Na^+-type flagellar motor, and that MotY may stabilize MotX to support its interaction with other Mot proteins.

Introduction

The bacterial flagellum is the only rotary machine for movement in the biological kingdom. This machine is driven by a specific ion flow which is dependent upon the electrochemical potential (1-3). Protons and sodium ions are the two known coupling ions, and are used for fueling to separate types of such motors. The motor itself is embedded in the cytoplasmic membrane at the base of the flagellar structure, and is composed of a stator and a rotor. In Gram-negative bacteria, the rotor is composed of more than ten protein components, forming a rod and four ring structures: the L, P, MS, and C rings. It is speculated that the stator complexes (the force-generating units) are arranged around the base of the flagellum where they interact with the rotor protein FliG, a component of the C ring (4, 5).

In a proton-driven motor, the force-generating unit is composed of MotA and MotB (6, 7). MotA has four transmembrane segments and one large cytoplasmic loop (8). MotB has a single N-terminal transmembrane segment, in which an important Asp residue is speculated to convey the protons (9), and a peptidoglycan-binding motif at the C-terminus (10, 11). The four MotA and one MotB transmembrane segments, predicted to produce α-helical structures, are thought to form an ion channel (12-14). One of the most prominent hypotheses is that H^+ flow through this channel induces structural changes in MotA which result in alteration of the force-generating unit's interaction with the rotor component FliG, thereby generating rotary power (15, 16).

In a sodium-driven motor, the force-generating unit is thought to be composed of PomA, PomB, MotX, and MotY (17-21). PomA and PomB are homologous to MotA and MotB, respectively. PomA is required to stabilize PomB, and direct interaction between PomA and PomB to form a complex with stoichiometry 2:1 has been demonstrated (22, 23). PomA and PomB have been purified from *Vibrio alginolyticus*, and when reconstituted into proteoliposomes, the PomA/B complex catalyzed sodium influx while PomA alone did not (23).

PomA appears to stably dimerize to form an ion channel with PomB, and both halves of the PomA homodimer function cooperatively to conduct sodium ions (24). We proposed that the periplasmic loop of PomA is important for dimer formation and function (25, 26). The heteromultimeric complex is thought to act as a sodium channel.

A hybrid motor consisting of the proton-type MotA and sodium-type subunits PomB, MotX, and MotY can work as a sodium-driven motor in *Vibrio* cells (27). Furthermore, although the proton-type MotB does not function with either MotA or PomA in *Vibrio* cells, it has been shown that PomB can be replaced with the chimeric protein MomB, which is made from the N-terminal segment of MotB and the C-terminal segment of PomB (28). PomA and PomB may not play a primary role in ion selectivity.

MotX and MotY, which are unique components of sodium-driven motors, were first identified in *Vibrio parahaemolyticus* (19, 20). Both proteins have a putative single transmembrane domain, two cysteine residues, and a conserved tetrapeptide CQLV in the putative periplasmic region. The predicted pI is basic for both proteins, 9.8 for MotY and 8.6 for MotX. MotY has a peptidoglycan-binding motif in the C-terminal region which is also observed in both MotB and PomB. It was suggested that MotX forms a sodium-conducting component of the torque generator, as overexpression of MotX in *E. coli* kills the cells and this effect can be blocked by amiloride, a known sodium-channel inhibitor. When both MotX and MotY are produced in *E. coli*, MotY localizes to the cell membrane in the presence of MotX. However, MotY localizes to the cytoplasm when MotX is not co-expressed. From these observations, it was inferred that MotX interacts with MotY (19). However, those experiments have been performed only in *E. coli* and not in the native host cell. The function of MotX and MotY has remained unclear, although it is thought that they may be essential for the recognition of sodium ion by the motor. In this report, we present the cloning of the *V. alginolyticus motX* gene and characterization of the resulting MotX protein.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. *V. alginolyticus* VIO542 (*motY542*) is derived from VIO5 (21). *V. alginolyticus* NMB85 (*motY85*), NMB94 (*motX94*), NMB115 (*motX115*), NMB117 (*motY117*) and NMB119 (*motX119*) were isolated from YM4 (29). *V. parahaemolyticus* LM4170 (*lafX313*::*lux motX118*::mini-Mu *lac* (Tet^S)) (19) and LM4171 (*lafX313*::*lux motY141*::mini-Mu *lac* (Tet^S)) (20) were kindly donated by L. McCarter. *Escherichia coli* DH5α (F^- λ^- *recA1 hsdR17 endA1 supE44 thi-1 relA1 gyrA96* Δ(*argF-lacZYA*)U169 *f* 80d*lacZ*ΔM15) (30) was used for all plasmid constructions. The plasmid pCR2.1 was purchased from Invitrogen (CA). The plasmids pSU21 and pSU41 are broad host range vectors having the replication origin of the plasmid p15A, a multiple cloning site downstream of the lactose promoter-operator, and either a chloramphenicol or a kanamycin resistance gene, respectively (31). *V. alginolyticus* and *V. parahaemolyticus* cells were cultured at 30 °C in VC medium (0.5% polypeptone, 0.5% yeast extract, 0.4% K_2HPO_4, 3% NaCl, 0.5% glucose) or VPG medium (1% polypeptone, 0.4% K_2HPO_4, 3% NaCl, 0.5% glycerol). When necessary, chloramphenicol and kanamycin were added at final concentrations of 2.5 and 100 μg/ml, respectively. *E. coli* cells were cultured at 37 °C in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl).

DNA manipulations and sequencing. Routine DNA manipulations were carried out according to standard procedures (32). Restriction endonucleases and other enzymes for DNA manipulations were purchased from Takara Shuzo (Kyoto, Japan). Nucleotide sequences were determined using the dye terminator cycle sequencing kit (Perkin-Elmer Co.) and an ABI PRISM sequencer (model 310 or 377; PE Applied Biosystems).

Transformation of *Vibrio* cells. Transformation of *Vibrio* cells was carried out by electroporation as described previously (33). The cells were subjected to osmotic shock and

washed thoroughly with 20 mM $MgSO_4$. Electroporation was carried out with the Gene Pulser electroporation apparatus (Japan Bio-Rad Laboratories, Tokyo, Japan) with an electric field of 5.0 to 7.5 kV/cm.

PCR products. Oligonucleotide primers for PCR were synthesized by Sawady Technology Co. (Tokyo, Japan). PCR amplification was carried out with the TaKaRa Taq kit (Takara Shuzo, Kyoto, Japan) and Thermal Cycler (Perkin-Elmer Japan, Chiba, Japan) as recommended by the distributors.

Construction of plasmids. The 1-kb PCR product containing *motX* was cloned into the vector pCR2.1 and the resultant plasmid was named pMO100. The 1-kb *Eco*RI fragment of pMO100 was subcloned into the multicloning site of the plasmid vector pSU21 and named pMO200. The plasmid pMO401 was constructed by cloning a 700-bp *Cla*I-*Bam*HI fragment of PCR products into the multicloning site of the vector pSU41. For the reaction, we used pMO200 as the template and VamotX.Cla1 (5'-TCATCGATGTTTGGTTTCTTAGATACC-3') and VamotX.BamH1 (5'-GACGGATCCGTTGTTTGCCTTAATAGGACG-3') as end primers (the restriction sites are underlined). When we constructed pMO401, we obtained a plasmid containing three point mutations in *motX* (A7L, A8G and I62T) which was named pMO400. Site-directed mutagenesis was performed by a two-step PCR using the end primers and a pair of mutant primers as described previously (34). The mutant primers used were VamotX.A146VFW (5'-ACCTAAATGTGCGTATTCAT-3') and VamotX.A146VRV (5'-ATGAATACGCACATTTAGGT-3') (changed bases are underlined) for pMO406 (A146V).

Detection of the Mot proteins. Overnight cultures of cells in VC medium were diluted 50-fold with VPG medium. At mid-log phase, cells were harvested, resuspended in TMN50 (50 mM Tris-HCl [pH 7.5], 5 mM Glucose, 5 mM $MgCl_2$, 50 mM NaCl, 250 mM KCl), and sonicated on ice twice for 10 s, using a Sonifier 250 (Branson Ultrasonics Co., Danbury, CT). Undisrupted cells were removed by low speed centrifugation (12,000 x *g* for 5 min at 4 °C), and

the membrane fraction was recovered from the supernatant by centrifugation at 70,000 x *g* for 10 min. The membrane pellet was suspended in TMN50 and a sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 1% SDS, 0.1% bromophenol blue) containing β-mercaptoethanol. The suspension was boiled for 5 min and the proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Japan, Tokyo) using a semi-dry blotting apparatus (Biocraft, Tokyo, Japan). The PVDF membranes were then incubated with the first antibody. The bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit IgG and the ECL assay system (Amersham Pharmacia Biotech).

Antibody. An anti-MotX antibody (MotX192) was raised against a synthetic peptide corresponding to the fragment L192-W211 of the amino acid sequence deduced from the *motX* gene. The anti-MotY antibody (MotY264) was raised against a synthetic peptide corresponding to MotY fragment G264-R283. The anti-MotX and anti-MotY antibodies were obtained from Sawady Technology (Tokyo, Japan) and Biologica (Nagoya, Japan), respectively.

Results

Cloning of *V. alginolyticus motX* gene. We previously reported the cloning of the promoter region of *V. alginolyticus motX* (18). To clone the entire region of the *motX* structural gene, we amplified the region containing it by PCR using an oligonucleotide primer corresponding to the *motX* promoter region and mixed oligonucleotide primers corresponding to the *hmp* gene, which in *V. alginolyticus* is adjacent to *motX* (Fig. 1) (19). The resulting 1 kb DNA fragment was amplified by PCR and cloned into vector pCR2.1, yielding plasmid pMO100.

The fragment was sequenced (Fig. 2A) and the sequence of the chromosomal *motX* gene was also determined directly to verify that of the cloned fragment. As previously described, the *motX* promoter region contains both the σ^{54}-consensus and σ^{28}-consensus sequences (18). According to the ORF, the sequence codes for 212 amino acids. A putative rho-independent transcription terminator was found at the end of the gene. Thus, *motX* would be transcribed as a monocistronic message from the chromosome. The predicted amino acid sequence is 94 % and 85 % identical to the *motX* gene products of *V. parahaemolyticus* and *V. cholerae*, respectively (Fig. 2B). The ORF contains a potential transmembrane region near the amino terminus and a relatively large periplasmic region at the carboxy-terminus, according to a hydropathy profile and the positive inside rule as reported previously for *V. parahaemolyticus* (19).

Characterization of *V. alginolyticus mot* mutants. The *pom* (or *mot*) mutants of *V. alginolyticus*, which possess non-functional yet intact polar flagella, were isolated from the lateral flagella-deficient strain YM4 (29) after the treatment with ethyl methanesulfonate. Of the *mot* mutants, NMB94, NMB115 and NMB119 were unable to swarm when transformed with the plasmid carrying *motY*, *pomA* or *pomB*. However, as shown in Fig. 3A, they were all able to swarm when transformed with plasmid pMO200 carrying the *motX* gene. Therefore, we concluded that the above three mutants resulted from mutations in the *motX* gene. In addition, pMO200 also restored the swarming capability of the *V. parahaemolyticus motX* mutant, LM4170 (Fig. 3B). The *motX* chromosomal region of the three mutants was sequenced. In NMB94 (*motX94*), a two-base deletion (T488 and G489) was found (Fig. 2A), which shifted the reading frame from Cys87 onwards. The NMB115 (*motX115*) and NMB119 (*motX119*) mutants were found to possess substitutions of Ala146 (GCG) to Val (GTG) and of Gln194 (CAG) to amber (TAG), respectively.

Characterization of mutant MotX. The A146V mutant NMB115 appeared not to swarm (Fig. 3), but upon prolonged incubation did exhibit motility a little (data not shown).

NMB115 cells in culture medium actually displayed a slow motile phenotype. The plasmid pMO406, which carries the *motX* gene with the A146V substitution, was introduced into NMB94. The transformant, pMO406/NMB94, swarmed comparably to the wild type (data not shown), suggesting that the A146V mutation by itself may not directly affect flagellar function, rather, it may exert its effects on other factors such as protein stability. As shown in Fig. 4, the MotX protein expressed in NMB115 was detected in smaller amounts compared to the wild-type. This may suggest that the mutant protein of A146V MotX is unstable, yet possibly quite functional. NMB94 harboring pMO400 could swarm equally as well as the wild-type, although pMO400 contains three point mutations in *motX* (data not shown). The amount of MotX expressed from plasmid pMO401 was very high, while expression from plasmids pMO406 and pMO400, which carry the mutant *motX* gene, was low (Fig. 4). Those mutations may affect the expression of the gene or the stability of the protein. It is worth noting that the growth rate of cells carrying pMO401 was not affected.

Interaction between MotX and MotY *in vivo*. It has been reported that MotY of *V. parahaemolyticus*, when produced in *E. coli*, is localized to the membrane only when expressed together with MotX, suggesting that the two interact with each other (19). Here, such possible interaction between MotX and MotY was also examined in *V. alginolyticus*. MotX was detected in the membrane fraction of the wild-type strain, but not in the membrane fraction of the *motY* mutant, VIO542. However, MotX was detected in the membrane fraction of the *motY* mutant harboring the plasmid pIO6, which carries *motY* (Fig. 5A). We suggest that MotX, expressed from the chromosome of VIO542, is stabilized in the membrane by MotY expressed from the plasmid. Symmetrically, MotY was detected in the membrane of the *motX* mutant NMB94 when harboring the plasmid pMO401 carrying *motX*, although it could not be detected when harboring the vector plasmid (Fig. 5B). These results suggest that MotX and MotY interact and mutually stabilize each other in the membrane of *V. alginolyticus*.

Multicopy *motX* suppresses *motY* mutation. As described above, MotX and MotY help to stabilize each other. If this stabilization is indeed the main function of one of these two proteins, then one should find that overexression of one should help to overcome a deficiency of the other. This appears to be true for MotY. The non-swarming MotY mutants VIO542, whose mutation site has not been determined and NMB117, which possesses an amber (TAG) mutation at position Gly32, both recovered swarming capacity when transformed with pMO401, a motX-containing plasmid.

On the other hand, plasmid-derived MotY was not capable of rescuing *motX* mutations (data not shown). This suggests, however, that MotY might not be required for mechanical motor function *per se*, but may stabilize or support the structure of MotX in the membrane and permit normal function. On the other hand, MotX, in addition to its role in stabilizing MotY, may be more directly involved in the motor function of the *V. alginolyticus* Na^+-type flagella, potentially assisting in torque generation.

Discussion

We cloned the entire region of the *motX* gene of *V. alginolyticus*. The predicted amino acid sequence of *V. alginolyticus motX* has 94% identity with *V. parahaemolyticus motX*, and 85% identity with *V. cholerae motX*. The putative transmembrane regions have relatively low similarity, as it is the case for MotY among the *Vibrio* spp. (21). If the transmembrane regions were contributing to function or channel formation, one would expect them to display high similarity. In this case, the transmembrane region may not have a special function other than to simply anchor the protein in the membrane. We are in the process of trying to exchange the MotX transmembrane region with that from other membrane proteins.

By using the cloned gene, we identified three *motX* mutants, NMB94, NMB115 and NMB119. Western blot analysis of different mutant proteins showed that NMB115 MotX A146V protein levels are significantly lower compared to wild-type when both proteins are expressed from identical plasmids (Fig. 4). When a plasmid containing *motX* with the NMB115 A146V mutation was introduced back into NMB115 cells, the mutant cells recovered motility similar to wild-type cells. Taken together, these results suggest that NMB115 may produce functional protein, but just not in the amount required to produce a complement of functional flagella. Most likely, the A146V mutation has a negative effect on MotX stability, and a further look at this mutant may help reveal details about the kinetics of MotX and flagellar turnover.

The motor genes of *V. cholerae* and *V. alginolyticus* are homologous, and the polar flagellum of *V. cholerae* is sodium driven (35, 36). Deletion strains defective in *pomA*, *pomB*, *motX*, or *motY* of *V. cholerae*, which are non-motile, became weakly motile when *E. coli motA* and *motB* genes are introduced (37). *E. coli* proton motor components can restore function to a *Vibrio* sodium motor lacking MotX and MotY, but this hybrid motor is driven by the proton-motive force rather than by sodium. We therefore propose that MotX and MotY determine the ion specificity for the sodium motor.

It has been reported that overexpression of MotX, but not MotY, kills *E. coli* (19), and that this was exacerbated by addition of Na^+ to the medium. Furthermore, amiloride, which is a known inhibitor of sodium channels in animal cells as well as Na^+-driven flagellar motors, protected against the deleterious effects of MotX induction. This would suggest that MotX is involved in sodium metabolism and may be a component of the motor's sodium channel. However, in this study, when MotX was over-produced in *Vibrio* cells, the growth of the cells was not affected. This discrepancy between the *Vibrio* and *E. coli* results may be explained by differences in expression levels, by an inhibition in channel formation in *Vibrio* cells, or that MotX affects only the sodium channels of *E. coli*. On the other hand, PomA and PomB were recently purified as a complex and it was shown that they could catalyze sodium influx in reconstituted proteoliposomes (23). This is not inconsistent with the hypothesis that MotX acts

to modulate an ion channel composed of PomA and PomB. If this hypothesis were true, the sodium influx allowed by the PomA/B complex should be enhanced by the addition of MotX. However, MotX and MotY did not co-purify with the PomA/B complex, implying that their interaction with the PomA/B complex is weak.

MotX and MotY seem to stabilize each other in *V. alginolyticus* (Fig.5). It has been shown that MotY of *V. parahaemolyticus* is stabilized in the presence of MotX when overexpressed in *E. coli*. In addition, MotY was localized in the membrane in the presence of MotX, and found in the cytoplasm in its absence. However, MotX is stable without MotY in *E. coli*, a contrast with *Vibrio* that may be due to differences in their expression systems. Alternatively, the level of expression of MotX, which was high enough in *E. coli* to be detected by Coomassie blue staining, may affect the stability of MotX. In the present work, MotX was overexpressed in *Vibrio*, but not at a level detectable by Coomassie blue staining.

Here, we also demonstrate that overexpression of MotX could suppress *motY* mutations (Fig.6), and propose that MotX may interact with the PomA/B complex even in the absence of MotY. Therefore, the role of MotY might be limited to anchoring or stabilizing MotX. The putative transmembrane region of MotX is reasonably hydrophobic and predicted to form an α-helical structure. In contrast, the putative transmembrane region of MotY, as suggested previously (20), is not as hydrophobic and unlikely to form an α-helix. MotY by itself can be recovered as a cytoplasmic protein, without MotX (19), and because of this we strongly suspect that MotY is not a membrane-embedded protein, even though a transmembrane region was predicted from the sequence (20, 21). A reliable biochemical study will be necessary to absolutely determine the localization of MotX and MotY, and is sure to help elucidate their true functions.

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Figure legends

Fig. 1. (A) Map of the chromosomal region flanking the *motX* gene. The arrows show the PCR primers used to clone *motX*. (B) Restriction maps of the plasmids. The white boxes represent the vector part of pSU21 and pSU41, and the arrows indicate the direction of transcription from the *lac* promoter. The inserted fragments are indicated by solid lines. Abbreviations: B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; Sp, *Sph*I; X, *Xho*I; C, *Cla*I; EV, *Eco*RV.

Fig. 2. (A) The nucleotide and deduced amino acid sequences of *V. alginolyticus motX*. The putative Shine-Dalgarno sequence is underlined. The σ^{54}- and σ^{28}-consensus sequences are indicated by double underlines. Chavrons indicate inverted repeats. The C-terminal sequence corresponding to the synthetic peptide used for antibody production is enclosed by a box. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number (AB070443). (B) Alignment of MotX of *V. alginolyticus*, *V. parahaemolyticus* and *V. cholerae* . The putative transmembrane region (TM) is enclosed by a box. Va, *V. alginolyticus*; Vp, *V. parahaemolyticus*; Vc, *V. cholerae*.

Fig. 3. Swarming abilities of various *motX* mutants harboring *V. alginolyticus motX*. Fresh colonies of NMB94, NMB115, NMB119 (A), and LM4170 (B) harboring pSU41 (vector) or pMO200 (*motX*) were inoculated in triplicate on 0.3% agar-VC plates containing chloramphenicol and incubated at 30 ºC for 6 h (A) and 9 h (B).

Fig. 4. Detection of MotX. The membrane fractions from cells were prepared as described in Materials and Methods. Thirty micrograms of protein (except for pMO401 (wt) which contained 10 μg protein) from the membrane fraction was subjected to SDS-PAGE and analyzed by immuno-blotting using anti-MotX antibody. From right to left, YM4; NMB115;

NMB94 transformed with pSU41 (vector), with pMO401 (wt), with pMO400 (A7L, A8G, I62T), and with pMO406 (A146V).

Fig. 5. Stability of MotX and MotY. (A) The membranes of NMB94 and VIO542, harboring pSU41 (vector) or pIO6 (*motY*), were prepared and subjected to SDS-PAGE and immuno-blotting using anti-MotX antibody. (B) The membranes of NMB94 and VIO542, harboring pSU41 (vector) or pMO401 (*motX*), were prepared and subjected to SDS-PAGE and immuno-blotting using anti-MotY antibody.

Fig. 6. Suppression of the swarm-defective *motY* mutations by MotX. Fresh colonies of VIO542 and NMB117 harboring pSU41 (vector) or pMO401 (*motX*) were inoculated in triplicate on 0.25% agar-VPG plates containing kanamycin and incubated at 30 °C for 24 h.

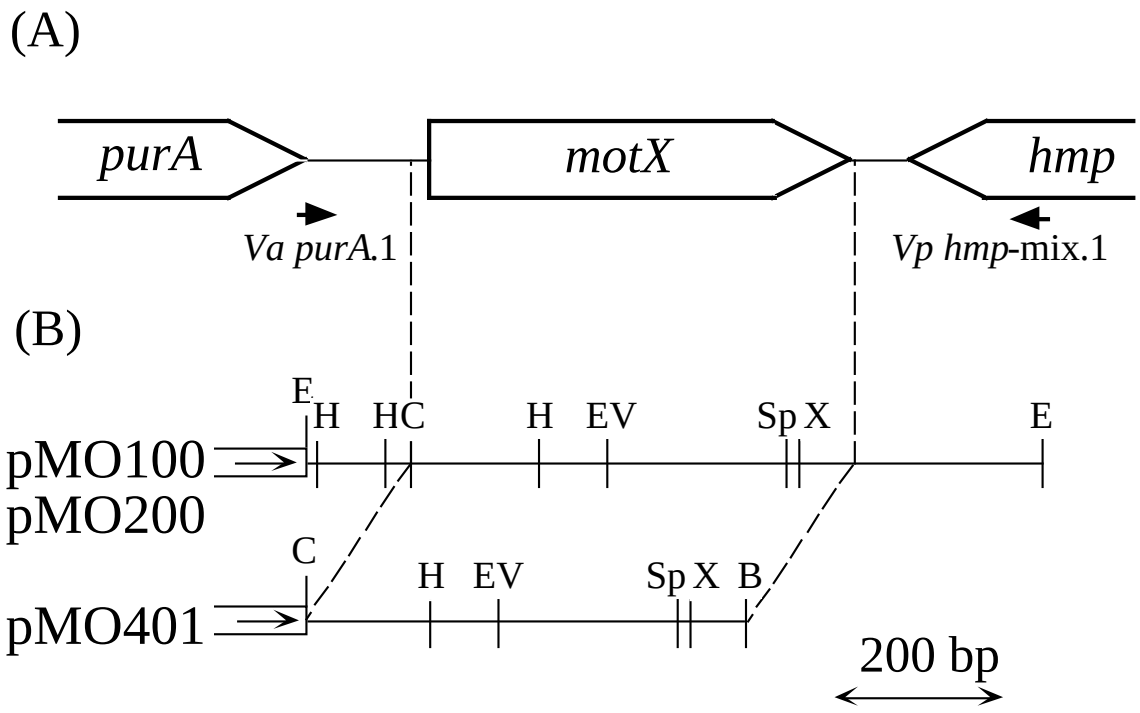

Fig. 1. Okabe et al.

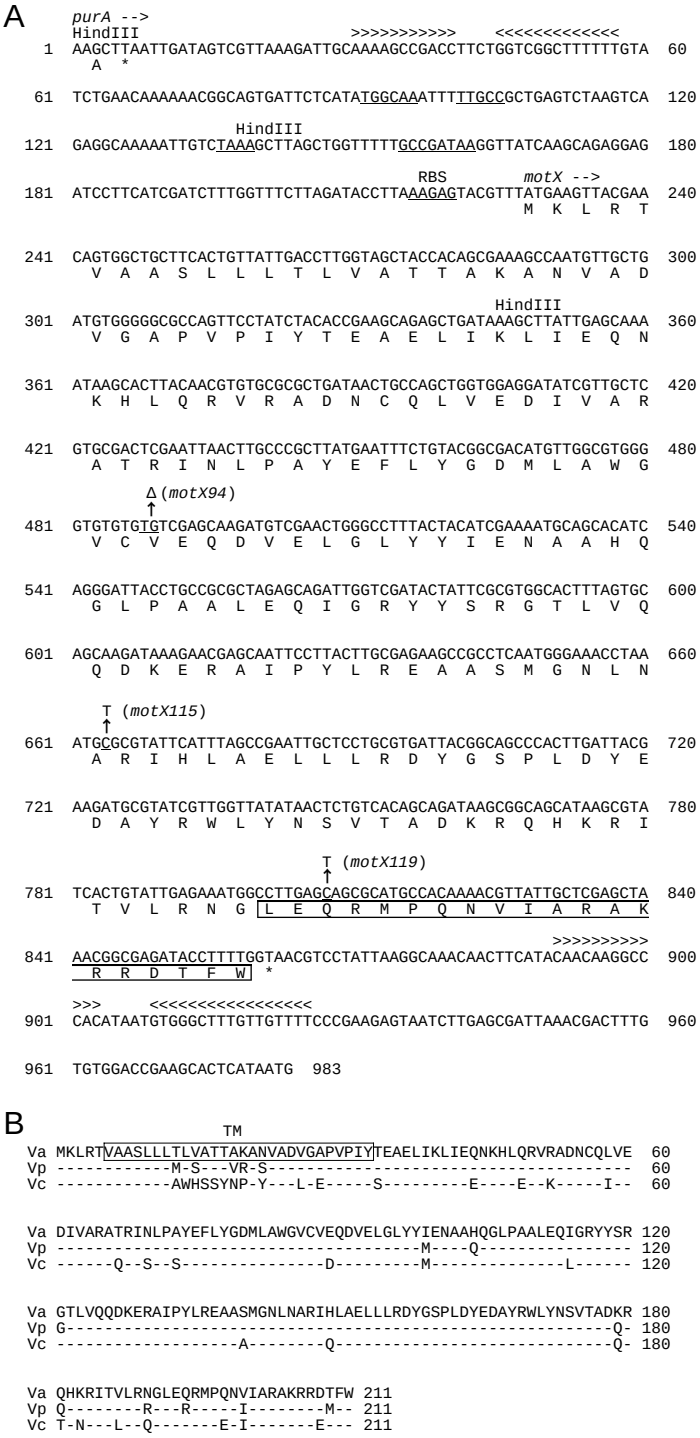

Fig. 2. Okabe et al.

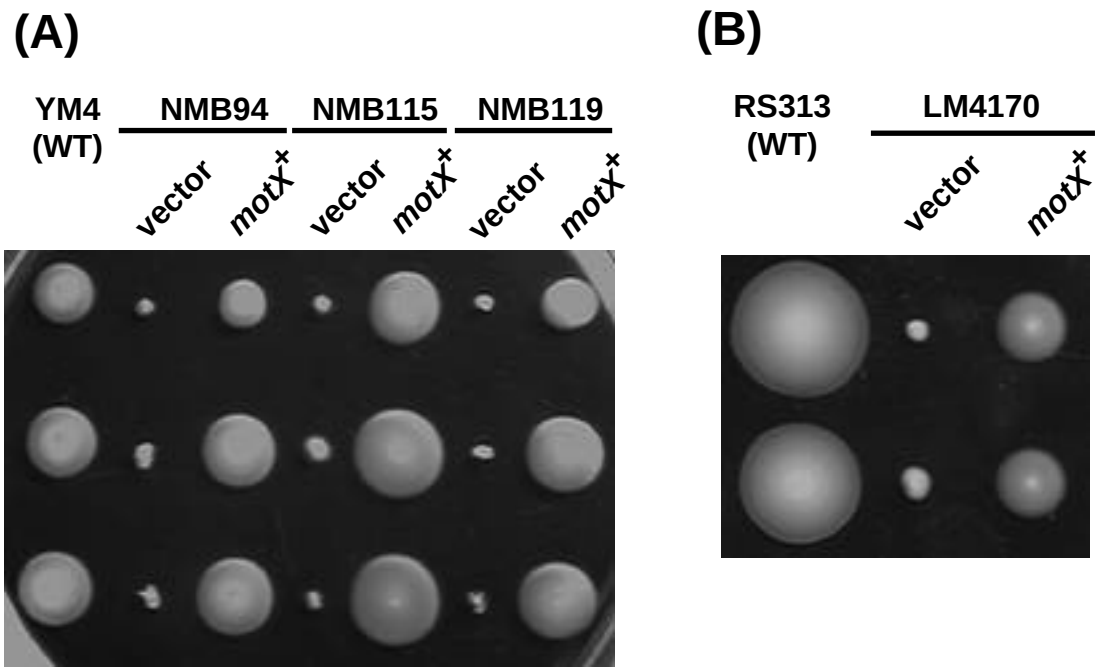

Fig. 3. Okabe et al.

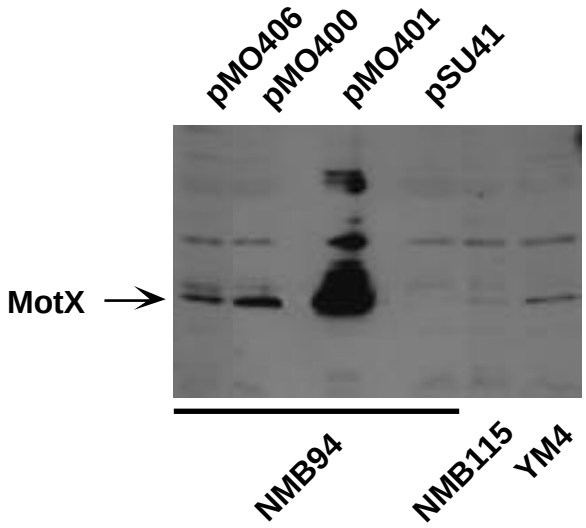

Fig. 4. Okabe et al.

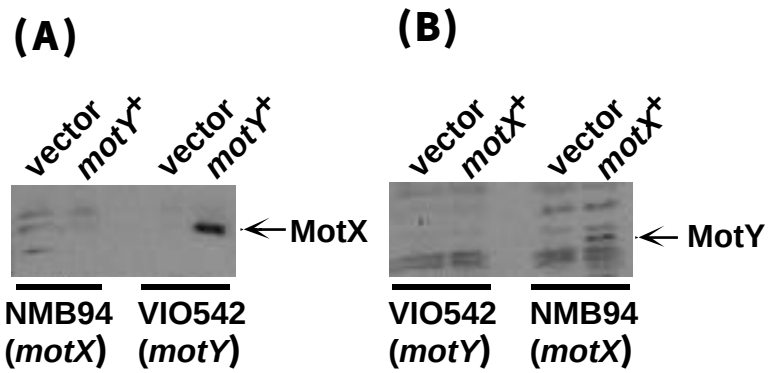

Fig. 5. Okabe et al.

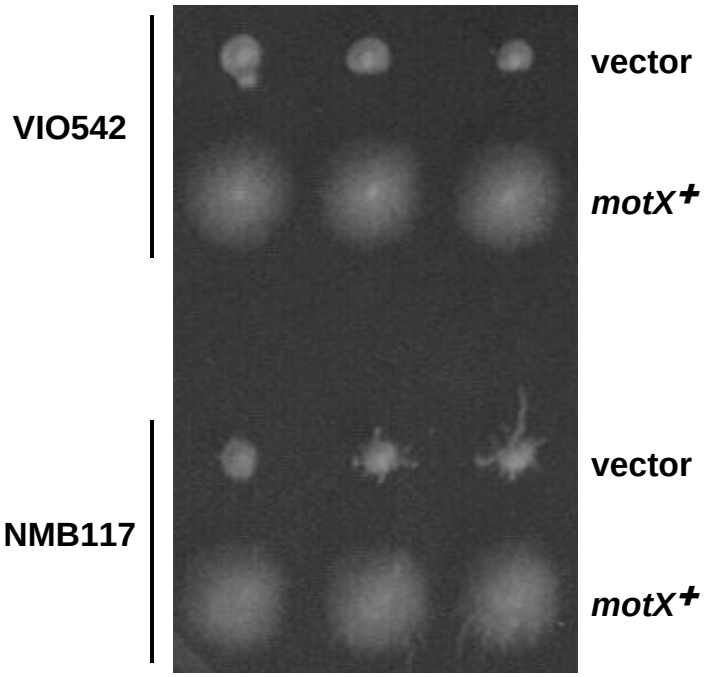

Fig. 6. Okabe et al.