Aeromonas hydrophila *motY* is essential for polar flagellum function, and requires coordinate expression of *motX* and Pom proteins

Raquel Molero, Markus Wilhelms, Belén Infanzón, Juan M. Tomás and Susana Merino

Correspondence
Juan M. Tomás
jtomas@ub.edu

Departamento de Microbiología, Facultad de Biología, Universidad de Barcelona, Diagonal 645, 08071 Barcelona, Spain

By the analysis of the *Aeromonas hydrophila* ATCC7966^T genome we identified *A. hydrophila* AH-3 *MotY*. *A. hydrophila* *MotY*, like *MotX*, is essential for the polar flagellum function energized by an electrochemical potential of Na⁺ as coupling ion, but is not involved in lateral flagella function energized by the proton motive force. Thus, the *A. hydrophila* polar flagellum stator is a complex integrated by two essential proteins, *MotX* and *MotY*, which interact with one of two redundant pairs of proteins, PomAB and PomA₂B₂. In an *A. hydrophila* *motX* mutant, polar flagellum motility is restored by *motX* complementation, but the ability of the *A. hydrophila* *motY* mutant to swim is not restored by introduction of the wild-type *motY* alone. However, its polar flagellum motility is restored when *motX* and *-Y* are expressed together from the same plasmid promoter. Finally, even though both the redundant *A. hydrophila* polar flagellum stators, PomAB and PomA₂B₂, are energized by the Na⁺ ion, they cannot be exchanged. Furthermore, *Vibrio parahaemolyticus* PomAB and *Pseudomonas aeruginosa* MotAB or MotCD are unable to restore swimming motility in *A. hydrophila* polar flagellum stator mutants.

Received 8 March 2011
Revised 27 June 2011
Accepted 4 July 2011

INTRODUCTION

Flagellum-mediated motility is a widespread means of locomotion among bacteria. The bacterial flagellum is usually powered by a reversible membrane-embedded motor at the base of the flagellum structure, which uses energy from either the proton or the sodium ion gradient to drive rotation of the flagellum filament (McCarter, 2001; Yorimitsu & Homma, 2001; Blair, 2003). The flagellum motor is divided into two substructures, the rotor and the stator. The rotor is composed of the FliM, FliN and FliG proteins, which form the C ring structure at the base of the flagellum basal body. These three proteins act as a switch that controls the direction of flagellum rotation, clockwise or counter clockwise. The stator is the stationary component of the motor, and consists of membrane-embedded proteins surrounding the C ring and proton or sodium ion channels that couple the flow of ions to flagellum rotation (Berg, 2003; Blair, 2003). Each stator complex contains at least two different proteins in a 4:2 stoichiometry, which form two specific ion channels. In the proton-driven motor of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, MotA and MotB constitute

the stator complex (Blair & Berg, 1990; Stolz & Berg, 1991; MacNab, 1996), which conducts protons across the inner membrane (Kojima & Blair, 2004). However, the stator of the *Vibrio parahaemolyticus* proton-driven lateral flagella motor requires an additional protein for motor function, called *MotY* (LafY) (Stewart & McCarter, 2003), and the stator complex of the *Shewanella oneidensis* MR-1 proton-driven flagella motor requires two additional proteins, *MotX* and *MotY* (Koerdt *et al.*, 2009). In the sodium-driven motor of alkaliphilic *Bacillus* species, the stator requires two proteins, *MotP* and *MotS* (Ito *et al.*, 2004), whereas the stator complex of the *S. oneidensis* MR-1 sodium-driven motor (Koerdt *et al.*, 2009) and the polar flagellum stator of *Vibrio* species, such as *Vibrio alginolyticus* and *V. parahaemolyticus*, require four proteins: PomA, PomB, *MotX* and *MotY* (Asai *et al.*, 1997; McCarter, 2001; Yorimitsu & Homma, 2001). *MotP*/PomA and *MotS*/PomB proteins are homologous to the proton-driven *MotA* and *MotB*, respectively. *MotA* has four transmembrane domains, which are thought to interact with FliG via a cytoplasmic segment to generate torque (Zhou *et al.*, 1998; Asai *et al.*, 2003). *MotB* has a single N-terminal transmembrane domain that harbours an Asp residue crucial for mediating ion flow and torque generation, as well as a peptidoglycan-binding motif at its C terminus (De Mot & Vanderleyden, 1994; Zhou *et al.*, 1995) that anchors the stator complex to the cell wall (De Mot & Vanderleyden, 1994; Asai *et al.*, 1997). *MotX* and *MotY* do not have paralogous

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; RACE, 5' random amplification of cDNA ends; TEM, transmission electron microscopy.

The GenBank/EMBL/DBJ accession number for the nucleotide sequence of *A. hydrophila* determined in this paper is HQ702752.

proteins in *E. coli* and are components of the T ring (Terashima *et al.*, 2006), which is located beneath the P ring of the polar flagellum basal body in *Vibrio* species. MotY has an N-terminal region essential for association of the stator unit around the rotor (Kojima *et al.*, 2008), and like MotB and PomB has a peptidoglycan-binding motif in its C-terminal region (McCarter, 1994). In *V. alginolyticus*, MotX and MotY are required for the assembly of the PomA/PomB complex in the polar flagellum motor (Okabe *et al.*, 2005; Terashima *et al.*, 2006; Kojima *et al.*, 2008). In contrast to the case in *S. oneidensis* MR-1, neither protein is crucial for the recruitment of the PomAB or MotAB stator complex to the flagellated cell pole (Koerdt *et al.*, 2009).

Although most bacterial flagella motors have one stator complex that specifically connects to a single rotor complex to energize the flagellum, *Pseudomonas aeruginosa* PAO1, *S. oneidensis* MR-1 and *Aeromonas hydrophila* AH-3 polar flagella, as well as *Bacillus subtilis* peritrichous flagella, have two different stator complexes that connect to a single rotor complex. Thus, *P. aeruginosa* PAO1 harbours MotAB and MotCD, which power a single polar flagellum by an unknown mechanism (Doyle *et al.*, 2004; Toutain *et al.*, 2005), while *B. subtilis* and *S. oneidensis* MR-1 harbour stator complexes with different ion specificities (Ito *et al.*, 2004; Paulick *et al.*, 2009). *B. subtilis* MotAB and *S. oneidensis* MotAB–MotXY are proton-driven, whereas *B. subtilis* MotPS and *S. oneidensis* PomAB–MotXY are sodium-driven. In contrast, both *A. hydrophila* polar flagellum stator complexes are sodium-driven, although they have different sensitivities to sodium variations (Wilhelms *et al.*, 2009).

Mesophilic *Aeromonas* are ubiquitous water-borne bacteria, considered opportunistic pathogens of both aquatic and terrestrial animals, some species being associated with gastrointestinal and extraintestinal human disease (Janda & Abbott, 2010). All mesophilic aeromonads have a single polar unsheathed flagellum produced constitutively; however, 60 % of clinical isolates also have lateral inducible unsheathed flagella. The lateral flagella stator is composed of two proteins, LafT and LafU (Canals *et al.*, 2006b), but at least five proteins are involved in polar flagellum rotation: an essential protein, MotX (Canals *et al.*, 2006a); and two redundant non-essential dual protein complexes, PomAB and PomA₂B₂ (Wilhelms *et al.*, 2009). In this study we found a novel essential protein, MotY, involved in polar but not lateral flagella rotation, which we demonstrated is proton-driven. The functionality of *motY* is *motX* dose-dependent, and the homologous proteins from the dual polar stator are not interchangeable or substituted by homologous proteins from other bacteria.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown on Luria–Bertani (LB) Miller broth and LB Miller agar at 37 °C, and *Aeromonas* strains were grown either in tryptic soy broth (TSB) or on agar (TSA) at 30 °C. When required, ampicillin

(50 µg ml⁻¹), kanamycin (50 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹), rifampicin (100 µg ml⁻¹), spectinomycin (50 µg ml⁻¹), gentamicin (10 µg ml⁻¹) and tetracycline (20 µg ml⁻¹) were added to the different media. Media were supplemented with 0.2 % (w/v) L-arabinose to induce recombinant protein expression under the arabinose promoter on pBAD33 or pBAD33-Gm.

Motility assays (swarming and swimming). Freshly grown bacterial colonies were transferred with a sterile toothpick onto the centre of a soft agar plate (1 % tryptone, 0.5 % NaCl, 0.25 % agar). The plates were incubated face up for 24–48 h at 25 °C, and motility was assessed by examining the migration of bacteria through the agar from the centre towards the periphery of the plate. Moreover, swimming motility was assessed by light microscopy observations in liquid medium. When required, the specific inhibitor for the sodium-driven flagella motor, amiloride (1–2 mM, Sigma), or the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (5–20 µM, Sigma) at pH 8.5, were added to the motility assay media.

Transmission electron microscopy (TEM). Bacterial suspensions were placed on Formvar-coated grids and negative-stained with 2 % uranyl acetate, pH 4.1. Preparations were observed on a Hitachi 600 transmission electron microscope.

DNA techniques. DNA manipulations were carried out according to standard procedures (Sambrook *et al.*, 1989). DNA restriction endonucleases and *E. coli* DNA polymerase Klenow fragment were obtained from Promega. T4 DNA ligase and alkaline phosphatase were obtained from Invitrogen and GE Healthcare, respectively. PCR was performed using BioTaq DNA polymerase (Ecogen) in a Perkin Elmer GeneAmp PCR System 2400 thermal cycler. Colony hybridizations were carried out by colony transfer onto positively charged nylon membranes (Roche), followed by lysis according to the manufacturer's instructions. Probe labelling with digoxigenin, and hybridization and detection (GE Healthcare), were carried out as recommended by the suppliers.

Nucleotide sequencing and computer sequence analysis. Plasmid DNA for sequencing was isolated with a Qiagen Plasmid Purification kit, as recommended by the suppliers. dsDNA sequencing was performed by using the Sanger dideoxy chain-termination method (Sanger *et al.*, 1977) with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Custom-designed primers used for DNA sequencing were purchased from Sigma-Aldrich.

The DNA sequence was translated in all six frames, and the deduced amino acid sequences were inspected in the GenBank, EMBL and Swiss-Prot databases by using the BLASTX, BLASTP or PSI-BLAST network service at the National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 1997). The protein family profile was obtained using the protein families database Pfam at the Sanger Center (Bateman *et al.*, 2002). Determination of possible terminator sequences was done by using the Terminator program from the Genetics Computer Group package (Madison, WI, USA). Other online sequence analysis services were also used.

Mapping the *A. hydrophila* AH-3 *motY* and *motX* transcription start sites by 5' random amplification of cDNA ends (RACE)

PCR. Amplification of the *A. hydrophila* AH-3 *motY* and *motX* cDNA 5' ends was performed using the 5' RACE System version 2.0 (Invitrogen). Total RNA was isolated from *A. hydrophila* AH-3 and 5 µg was pretreated with RNase-free DNase I, Amplification Grade (Invitrogen), according to the manufacturer's protocol for each sample. Absence of contaminating DNA in the RNA samples was confirmed by PCR. First-strand cDNA was synthesized using the entire volume of DNase-digested total RNA (5 µg), the *motY* internal primer GSP1-MotY (5'-GGAAGTTGACCGAGGAGAG-3') or the

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype*	Reference or source
A. hydrophila strains		
AH-3	<i>A. hydrophila</i> wild-type, serogroup O:34	Merino <i>et al.</i> (1991)
AH-405	AH-3, spontaneous Rif ^r	Altarriba <i>et al.</i> (2003)
ATCC7966 ^T	<i>A. hydrophila</i> wild-type	Seshadri <i>et al.</i> (2006)
AH-3::motY	AH-405, motY::Km ^r	This work
AH-4461	AH-405, motX::Km ^r	Canals <i>et al.</i> (2006a)
AH-4441	AH-405, fliM::Km ^r	Canals <i>et al.</i> (2006a)
AH-4442	AH-405, fliA::Km ^r	Canals <i>et al.</i> (2006a)
AH-4443	AH-405, fliA::Km ^r	Canals <i>et al.</i> (2006a)
AH-5503	AH-405, lafK::Km ^r	Canals <i>et al.</i> (2006b)
AH-5503::motY	AH-5503, motY::Cm ^r	This work
AH-4473	AH-405 ΔpomAB, ΔpomA ₂ B ₂	Wilhelms <i>et al.</i> (2009)
AH-4452	AH-405 ΔpomAB, pomB ₂ ::Cm ^r	Wilhelms <i>et al.</i> (2009)
AH-4471	AH-405 ΔpomA ₂ B ₂	Wilhelms <i>et al.</i> (2009)
AH-4471::pomB	AH-405 ΔpomA ₂ B ₂ , pomB::Km ^r	This work
E. coli strains		
DH5α	F ⁻ endA hdsR17(r _K ⁻ m _K ⁺) supE44 thi-1 recA1 gyrA96 φ80lacZ	Hanahan (1983)
XL1-Blue	endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 lac (F' proAB lacIZΔM15 Tn10)	Stratagene
MC1061λpir	thi thr1 leu6 proA2 his4 argE2 lacY1 galK2 ara14 xyl5 supE44 λpir	Rubirés <i>et al.</i> (1997)
Plasmids		
pLA2917	Cosmid vector, Tc ^r Km ^r	Allen & Hanson (1985)
pRK2073	Helper plasmid, Sp ^r	Rubirés <i>et al.</i> (1997)
pGEMT	Cloning vector, Ap ^r	Promega
pBAD33	Arabinose-induced expression vector, ori _{p15} P _{BAD} Cm ^r	Guzman <i>et al.</i> (1995)
pBAD33-Gm	Arabinose-induced expression vector, ori _{p15} P _{BAD} Gm ^r	Jimenez <i>et al.</i> (2009)
pACYC184	Plasmid vector, Cm ^r Tc ^r	Sambrook <i>et al.</i> (1989)
pFS100	pGP704 suicide plasmid, pir-dependent, Km ^r	Rubirés <i>et al.</i> (1997)
pCM100	pGP704 suicide plasmid, pir-dependent, Cm ^r	Yu <i>et al.</i> (2004)
pLA-MOTY	pLA2917 with AH-3 motY, Tc ^r	This work
pFS-POMB	pFS100 with an internal fragment of the AH-3 pomB gene, Km ^r	Canals <i>et al.</i> (2006a)
pFS-MOTY	pFS100 with an internal fragment of the AH-3 motY gene, Km ^r	This work
pCM-MOTY	pCM100 with an internal fragment of the AH-3 motY gene, Cm ^r	This work
pBAD33-MOTY	pBAD33 with AH-3 motY gene, Cm ^r	This work
pBAD33-MOTX	pBAD33 with AH-3 motX gene, Cm ^r	This work
pBAD33-MOTYX	pBAD33 with AH-3 motY and motX genes under P _{BAD} control, Cm ^r	This work
pBAD33Gm-MOTY	pBAD33-Gm with AH-3 motY gene under P _{BAD} control, Gm ^r	This work
pBAD33Gm-MOTX	pBAD33-Gm with AH-3 motX gene under P _{BAD} control, Gm ^r	This work
pBAD33Gm-POMB ₂	pBAD33-Gm with AH-3 pomB ₂ gene under P _{BAD} control, Gm ^r	This work
pBAD33Gm-POMB	pBAD33-Gm with AH-3 pomB gene under P _{BAD} control, Gm ^r	This work
pBAD33Gm-POMABvp	pBAD33-Gm with <i>V. parahaemolyticus</i> pomAB genes under P _{BAD} control, Gm ^r	This work
pBAD33Gm-MOTABpa	pBAD33-Gm with <i>P. aeruginosa</i> motAB genes under P _{BAD} control, Gm ^r	This work
pBAD33Gm-MOTCDpa	pBAD33-Gm with <i>P. aeruginosa</i> motCD genes under P _{BAD} control, Gm ^r	This work
pACYC-POMAB	pACYC184 with pomAB genes, Tc ^r	Wilhelms <i>et al.</i> (2009)
pACYC-POMA ₂ B ₂	pACYC184 with pomA ₂ B ₂ genes, Tc ^r	Wilhelms <i>et al.</i> (2009)

*Ap^r, ampicillin-resistant; Cm^r, chloramphenicol-resistant; Gm^r, gentamicin-resistant; Km^r, kanamycin-resistant; Rif^r, rifampicin-resistant; Sp^r, spectinomycin-resistant; Tc^r, tetracycline-resistant.

motX internal primer GSP1-MotX (5'-TGTTCATCCAGTTGAG-CAG-3'), and the ThermoScript RT-PCR system (Invitrogen) at 57 °C for 45 min. Reverse transcriptase was deactivated at 85 °C for 5 min, and 1 μl RNase H was then added and incubated at 37 °C for 20 min. Purification of cDNA with S.N.A.P. columns, as well as tailing of purified cDNA using terminal deoxynucleotidyltransferase and dCTP, was done according to the 5' RACE System version 2.0

instructions. Confirmation of cDNA was performed after each step by PCR with nested primers. Tailed cDNA was amplified by primary PCR using 10 μM of each primer, the 5' RACE abridged anchor primer (AAP) and GSP2-MotY (5'-CAGTCGCGGTACTGGAAG-3') or GSP2-MotX (5'-CCTGCAAATTGCTGCTGT-3'). The PCR program applied was 94 °C for 1 min, followed by 35 cycles of 94 °C for 45 s, 58 °C for 30 s and 72 °C for 1 min, and an extension at 72 °C

for 5 min. Nested PCR was performed using primary PCR product diluted 1:100 as template and 10 mM of each nested primer abridged by the universal amplification primer (AUAP) and GSP3-MotY (5'-CATCGAAGTCTGGTAGAA-3') or GSP3-MotX (5'-CAAATTGCTGCTGTGCAT-3'). The PCR program applied was 94 °C for 1 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 1 min, and an extension at 72 °C for 5 min. PCR products were analysed by agarose gel electrophoresis, and amplified bands were excised from the gel, purified, and sequenced with the GSP3-MotY or GSP3-MotX primer.

Construction of defined mutants. The single defined insertion *motY* and the AH-4471::*pomB* mutants were obtained using a method based on suicide plasmid pFS100 (Rubirés *et al.*, 1997). Briefly, a *motY* internal fragment of *A. hydrophila* AH-3 was amplified by PCR (5'-GCCTGGATCAATCCGTCT-3' and 5'-GCCATCTTCGTTTCGTT-3'), ligated into pGEM-T Easy (Promega) and transformed into *E. coli* XL1-Blue. The DNA insert was sequenced, recovered by *EcoRI* restriction digestion, and ligated into *EcoRI*-digested and phosphatase-treated pFS100. The recombinant pFS-MOTY plasmid was transformed into *E. coli* MC1061 (λ pir) and selected for kanamycin resistance (Km^r). Triparental mating with the mobilizing strain HB101/pRK2073 was used to transfer the recombinant plasmid into the *A. hydrophila* AH-405 rifampicin-resistant (Rif^r) strain to obtain the defined insertion mutant AH-3::*motY*, selecting for Rif^r and Km^r. To obtain the AH-4471::*pomB* mutant, the recombinant plasmid pFS-MOTB (Canals *et al.*, 2006a) was transferred by triparental mating with the mobilizing strain HB101/pRK2073 into the *A. hydrophila* AH-4471 rifampicin-resistant mutant (AH-405Δ*pomA*₂B₂) (Wilhelms *et al.*, 2009) to obtain the AH-4471::*pomB* mutant (AH-405Δ*pomA*₂B₂::*pomB*), selecting for Rif^r and Km^r. The correct construction of mutants was verified by Southern blot hybridization.

The double mutant *lafK-motY* was obtained using a method based on suicide plasmid pCM100 (Yu *et al.*, 2004). The AH-3 *motY* internal fragment ligated into pGEM-T Easy (Promega) was recovered by *EcoRI* digestion, blunt-ended, and ligated into *EcoRV*-digested and phosphatase-treated pCM100. The recombinant pCM-MOTY plasmid was transformed into *E. coli* MC1061 (λ pir) and selected for chloramphenicol resistance (Cm^r). Triparental mating with the mobilizing strain HB101/pRK2073 was used to transfer the recombinant plasmid into the *A. hydrophila* A405 *lafK*::Km^r (AH-5503) rifampicin- and kanamycin-resistant (Rif^r, Km^r) strain (Canals *et al.*, 2006b) to obtain the double mutant AH-5503::*motY*, selecting for Rif^r, Km^r and Cm^r. The correct construction was verified by Southern blot hybridization.

Plasmid construction. Plasmid pBAD33-MOTY, containing the complete *motY* gene, and plasmid pBAD33-MOTX, containing the complete *motX* gene from *A. hydrophila* AH-3 under the arabinose promoter (P_{BAD}) on pBAD33 (Guzman *et al.*, 1995), were obtained by PCR amplification of genomic DNA. Oligonucleotides 5'-AAAC-CCTGCAGCAAAACAG-3' and 5'-AAGATCCGGATGGTTACAA-3' generated a band of 1191 bp containing the *motY* gene, and oligonucleotides 5'-GCTCTAGAGGGGTTGCCCATATAAAG-3' and 5'-TCCCCTGGGCTCCCTTTGTTCATTGCTG-3' generated a band of 1136 bp (the *XbaI* site is underlined and the *SmaI* site is underlined and in bold type) containing the *motX* gene. The amplified band containing the *motY* gene was ligated into pGEM-T Easy (Promega) and transformed into *E. coli* XL1-Blue. The DNA insert was recovered by *SacI* (plasmid restriction site) and *EcoRV* (restriction site downstream of *motY* stop codon) restriction digestion, and ligated into the *SacI*/*SmaI*-digested pBAD33 vector to construct the pBAD33-MOTY plasmid (Fig. 1). The amplified band containing the *motX* gene was digested with *SmaI* and *XbaI*, and ligated into the *SmaI*/*XbaI*-digested pBAD33 vector to construct the pBAD33-MOTX plasmid (Fig. 1).

Both plasmids were introduced into *E. coli* DH5α (Hanahan, 1983) and sequenced. Plasmid pBAD33Gm-MOTY, containing the complete *motY*, and pBAD33Gm-MOTX, containing the complete *motX* gene from *A. hydrophila* AH-3 under the arabinose promoter (P_{BAD}) on pBAD33-Gm (Jimenez *et al.*, 2009), were obtained by restriction digestion. The DNA insert of pGEM-T Easy containing *motY* was recovered by *EcoRI* (plasmid restriction site) and *EcoRV* restriction digestion, and ligated into the *EcoRI*/*SmaI*-digested pBAD33-Gm vector to construct the pBAD33Gm-MOTY plasmid. The amplified band containing the *motX* gene was digested with *SmaI* and *XbaI*, and ligated into the *SmaI*/*XbaI*-digested pBAD33-Gm vector to construct the pBAD33Gm-MOTX plasmid. Plasmid pBAD33-MOTYX, containing the complete *motY* and *motX* genes, both under the arabinose promoter on pBAD33, was obtained by PCR amplification of pBAD33-MOTX with the oligonucleotides XBA-PBADF3 (5'-AAAATCTAG-ACGTCACACTTTGCTATGC-3'), which contains a *XbaI* site (underlined), and PBADR (5'-GGAGACCCACACTACCAT-3'), which is located downstream of the pBAD33 polylinker. The amplified band containing the *motX* gene under P_{BAD} control was *XbaI*-digested, ligated to the pBAD33-MOTY recombinant plasmid digested with the same endonuclease, and phosphatase-treated (Fig. 1). Products of ligation were introduced into *E. coli* DH5α, and some recombinant plasmids were *StuI*/*PstI*-digested to differentiate plasmids containing both genes in the same transcription direction, pBAD33-MOTYX, and genes in the opposite direction, pBAD33-MOTYXc. The selected plasmids were sequenced.

Plasmids pBAD33Gm-POMB₂ and pBAD33Gm-POMB, containing the complete *pomB*₂ and *pomB* from *A. hydrophila* AH-3, respectively; plasmid pBAD33Gm-POMABvp, containing the *pomAB* from *V. parahaemolyticus*; and plasmids pBAD33Gm-MOTABpa and pBAD33Gm-MOTCDpa, containing the *motAB* and *motCD* from *P. aeruginosa*, respectively, all of them under the control of the arabinose promoter (P_{BAD}) on pBAD33-Gm (Jimenez *et al.*, 2009), were obtained by PCR amplification of genomic DNA. Oligonucleotides 5'-AGCGATCCCAA-ATCCATAG-3' and 5'-ACGCGTCGACAGCTCTTGACGAGCTTTT-3', which contain a *Sall* site (italic type), generated a band of 1348 bp containing *A. hydrophila pomB*₂, and oligonucleotides 5'-TCCCCCGGATCCAGGCCATGTTCCATC-3' and 5'-ACGCGTCGACATACCGGCTAACGAGACCA-3' generated a band of 1244 bp containing the *A. hydrophila pomB* (*SmaI* site underlined and in bold type, and *Sall* site in italic type). The amplified band containing *pomB*₂ was *PvuII*- (restriction site 125 bp upstream of the *pomB*₂ start codon) and *Sall*-digested, and the amplified band containing *pomB* was *SmaI* and *Sall* restriction-digested. Digested bands were ligated into the *SmaI*/*Sall*-digested pBAD33Gm vector to construct the pBAD33Gm-POMB₂ and pBAD33Gm-POMB plasmids, respectively. Oligonucleotides 5'-GGAA-TTCTGGCTCTAAACGGGTCTG-3' and 5'-GCTCTAGATGCAGGT-AAGGCTTCGAT-3' generated a band of 2003 bp containing the *pomAB* from *V. parahaemolyticus*, oligonucleotides 5'-GGAATTCCTGCA-GGAATGAGGAGA-3' and 5'-GCTCTAGATGCCAGACTTTCATTG-C-3' generated a band of 1835 bp containing the *motAB* from *P. aeruginosa*, and oligonucleotides 5'-GGAATTCGAGGGAGCCTAGC-AGTCC-3' and 5'-GCTCTAGACAGCTACCGGCACATTCT-3' generated a band of 2018 bp containing the *motCD* from *P. aeruginosa* (*XbaI* site underlined, and *EcoRI* site underlined and in bold type). The amplified band containing the genes was *EcoRI* and *XbaI* restriction-digested and ligated into the *EcoRI*/*XbaI*-digested pBAD33Gm vector to construct the pBAD33Gm-POMABvp, pBAD33Gm-MOTABpa and pBAD33Gm-MOTCDpa plasmids, respectively. All plasmids were independently introduced into *E. coli* DH5α and sequenced.

RT-PCR. Total RNA was isolated, by RNeasy Protect Bacteria reagent (Qiagen) and RNeasy Mini kit (Qiagen), from *E. coli* DH5α and the *A. hydrophila* AH-3::*motY* mutant, with and without plasmid pBAD33-MOTY, grown at 37 °C in LB or 25 °C in TSB, respectively, and supplemented with 0.2% L-arabinose, as well as from *A. hydrophila*

Fig. 1. Diagram of pBAD33-MOTYX and pBAD33-MOTYXc plasmid construction. White squares containing a grey arrow show PCR-amplified fragments of *A. hydrophila* AH-3 genomic DNA. Grey arrows show the DNA-encoded fragments. Small black arrows show oligonucleotides XBA-PBADF3 and PBADR from the pBAD33 plasmid vector. The bent arrow represents the arabinose promoter (P_{BAD}).

wild-type AH-3. Chloramphenicol was added when required. To ensure that RNA was devoid of contaminating DNA, the preparation was treated with RNase-free Turbo DNase I (Ambion). First-strand cDNA synthesis was carried out using the ThermoScript RT-PCR system (Invitrogen) and random primers on 3–5 µg DNase-digested total RNA, according to the manufacturer's instructions. PCR without reverse transcriptase was also performed to confirm the absence of contaminating DNA in the RNA sample. PCR, second-strand synthesis and subsequent DNA amplification were carried out using the AccuPrime Taq DNA polymerase (Invitrogen) and the oligonucleotide pair 5'-TCCGTGAGTATGCAGTTACA-3' and 5'-CATGTTGATCACCACACG-3' to amplify the *motY* DNA fragment of 821 bp. Oligonucleotides 5'-TCAACTGGATTGAGCAGGG-3' and 5'-GCTGGTCGCCTTCTGATG-3' were used to amplify the *motX* DNA fragment of 450 bp. Amplicons were analysed by agarose gel electrophoresis with ethidium bromide staining. *A. hydrophila* and *E. coli* ribosomal 16S primers were used as a control for the cDNA template.

Isolation of the *A. hydrophila* polar flagellar basal bodies. The isolation of the *A. hydrophila* polar flagellar basal bodies was carried out from an overnight culture in TSB (1000 ml) at 25 °C, as described by Terashima *et al.* (2006). Briefly, after cultivation, the cells were harvested in a sucrose solution (0.5 M sucrose, 50 mM Tris/HCl, pH 8.0) and converted into spheroplasts by adding lysozyme and EDTA to final concentrations of 0.1 mg ml⁻¹ and 2 mM, respectively. After lysis of spheroplasts with 1% (w/v) Triton X-100, 5 mM MgSO₄ and 0.1 mg DNase I ml⁻¹ were added to reduce viscosity, and then 5 mM EDTA was added. Unlysed cells and cellular debris were recovered by centrifugation at 17 000 g for 20 min. PEG 6000 and NaCl were added to the lysate to final concentrations of 2%

and 100 mM, respectively, and flagella were collected by centrifugation at 27 000 g for 30 min. The pellet was suspended in TET buffer [10 mM Tris/HCl, pH 8.0, 5 mM EDTA, 0.1% (w/v) Triton X-100]. To remove cellular debris, the suspension was centrifuged at 1000 g for 15 min at 4 °C, and the supernatant was centrifuged at 100 000 g for 30 min. To dissociate the flagella into monomeric flagellin, the pellet was suspended in TET buffer and diluted 30-fold in 50 mM glycine-HCl (pH 3.5) containing 0.1% (w/v) Triton X-100 and shaken for 60 min at room temperature. After treatment, the mixture was centrifuged at 1000 g for 15 min at 4 °C, and the supernatant was centrifuged at 150 000 g for 40 min and the pellet suspended in TET buffer.

RESULTS

Identification of a novel polar flagellum stator protein in mesophilic *Aeromonas*

Previous work demonstrated that *A. hydrophila* AH-3 has a sodium-driven polar flagellum whose rotation is performed by one essential stator protein, MotX, and one of two redundant sodium-driven stator proteins, PomAB and PomA₂B₂ (Wilhelms *et al.*, 2009). The *A. hydrophila* ATCC7966^T genome sequence (Seshadri *et al.*, 2006) contains polar flagellum stator genes homologous to those described for *A. hydrophila* AH-3 (Altarriba *et al.*, 2003) as well as an ORF, AHA_2642, annotated as *motY*, based on homologies to the corresponding gene in *Vibrio* species.

The AHA_2642 deduced amino acid sequence exhibits 48 % identity and 70 % similarity to *Vibrio cholerae* MotY. The *A. hydrophila* AH-3 genomic library was screened by colony blotting using an AHA_2642 DNA probe, leading to the identification of clone pLA-MOTY (Canals *et al.*, 2006a), which carries the entire *motY* gene. *A. hydrophila* AH-3 MotY is predicted to be 291 aa in length and exhibits 99 % identity/100 % similarity to *A. hydrophila* ATCC7966^T MotY, and 44–46 % identity/64–66 % similarity to the

MotY of various *Vibrio* species, which is involved in sodium-type motor formation (Fig. 2a). *A. hydrophila* AH-3 MotY harbours a signal peptide with a cleavage site between amino acids 19 and 20, and a C-terminal OmpA family domain (peptidoglycan-binding motif) between amino acids 183 and 279. Putative Shine–Dalgarno and σ^{28} promoter (CTAAA-N₁₅-GCCGATAC) sequences were found at 9 and 30 bp upstream of the TTG start codon, respectively (Fig. 2b). Downstream of *motY* is a gene

Fig. 2. Characteristics of *A. hydrophila* MotY. (a) Sequence alignment of *A. hydrophila* AH-3 MotY (AH-3), *A. hydrophila* ATCC7933^T AHA_2642 (ATCC7966), *V. parahaemolyticus* MotY (Vp), *V. cholerae* MotY (Vch) and *P. aeruginosa* PA3526 (Pa). The deduced amino acid sequences were aligned using CLUSTAL W. White letters with a black background indicate identical amino acid residues, and black letters with a grey background indicate similar amino acid residues. The black circles show cysteine residues conserved in MotY, which establish a disulfide bridge important for MotY N-terminal region stability. The inverted black triangle shows the MotY arginine residue that is important for motility and is conserved in OmpA/MotB-like proteins. (b) Promoter sequences of *A. hydrophila* AH-3 *motY* and *motX* determined using the 5' RACE System version 2.0 (Invitrogen). RBS indicates Shine–Dalgarno sequences upstream of the *motY* and *motX* start codons TTG and ATG, respectively. Asterisks show the location of the transcriptional start sites; -10 and -35 show sequences for σ^{28} binding.

predicted to encode a lactoylglutathione lyase. In addition, the exact location of the *A. hydrophila* AH-3 *motY* and *motX* promoters was determined by RACE assays (Fig. 2b). Primary PCR of tailed cDNA failed to give the expected amplification bands, but nested PCR using primers AUAP (abridged universal amplification primer) and GSP3-MotY or GSP3-MotX showed unique DNA bands of approximately 400 and 410 bp, respectively. The DNA sequences of the amplified bands indicated that they were tailed with G residues, and the *motY* and *motX* transcription start sites were located -18 nt and -18 or -19 nt upstream, respectively, from their translation start sites.

In order to analyse the presence of *motY* in mesophilic *Aeromonas*, we performed dot blot hybridization experiments against total genomic DNA of 50 different strains using an internal AHA_2642 DNA probe. The probe hybridized to the chromosomal DNA of all mesophilic *Aeromonas* strains tested, independently of their ability to produce lateral flagella.

***A. hydrophila* MotY is essential for polar flagellum movement**

To investigate the possible role of MotY in the motility of *A. hydrophila* AH-3, a defined insertion mutant in *motY* was created (AH-3::*motY*), and motility assays in liquid medium and on soft agar plates were performed. The mutant's ability to form polar and lateral flagella was also analysed. Motility assays in liquid medium by light microscopy showed that the *motY* mutation abolished swimming motility and also caused a decrease of radial expansion in soft agar motility (76 % reduction) in relation to the wild-type (Fig. 3a). The radial expansion of the *motY* mutant was similar to that observed in the *motX* stator mutant and in the *fliM*, *fliH* and *fliA* non-polar flagellum mutants (Canals *et al.*, 2006a). TEM analysis showed polar and lateral flagella in the *motY* mutant (Fig. 3b), as in the *motX* mutant, as previously described (Canals *et al.*, 2006a). Since the *A. hydrophila* polar flagellum is constitutive and lateral flagella are inducible in semisolid media or on the surface, these results suggest that the non-motile phenotype in liquid medium is a consequence of the inability of the polar flagellum to rotate in the *motY* and *motX* mutants.

In order to analyse whether AH-3::*motY* soft agar motility was only produced by lateral flagella rotation, an *A. hydrophila* AH-3 double mutant, *lafK-motY* (AH-5503::*motY*), was created and soft agar motility was analysed. No radial expansion was observed, although the mutant showed a polar flagellum (Fig. 3). Furthermore, the motility of the AH-3::*motY* and a non-polar flagellum mutant, *fliH*::Km (AH-4442) (Wilhelms *et al.*, 2009), was assayed in soft agar with the sodium-driven flagellar inhibitor amiloride or the protonophore CCCP. These assays showed that the motility of both mutants was strongly inhibited with an increase in the CCCP concentration, whereas no decrease in radial expansion was

induced by amiloride treatment (Fig. 4). The results suggest that AH-3::*motY* soft agar motility is produced by lateral flagella movement, which is energized by the proton motive force, and that MotY is not involved in lateral flagella movement.

Complementation studies using *A. hydrophila* AH-3::*motY* and *lafK-motY* (AH5503::*motY*) mutants

We introduced the pLA-MOTY cosmid into the AH-3::*motY* and *lafK-motY* (AH-5503::*motY*) mutants by triparental mating. Transconjugants failed to swim or show a radial expansion identical to that of the mutants (Fig. 5a). Since pLA-MOTY complementation *in trans* was unable to restore swimming motility, we examined whether it was produced by a low level of *motY* expression. We cloned the *motY* gene into the pBAD33 arabinose-expression vector (pBAD33-MOTY), as described in Methods (Fig. 1), and transferred it to the AH-3::*motY* mutant by triparental mating. Transconjugants grown in 0.2 % L-arabinose failed to swim in liquid medium and soft agar; therefore, the motility defect was not rescued, as happened above with pLA-MOTY (Fig. 5a). We also cloned the *motY* gene into the pBAD33-Gm arabinose-expression vector (pBAD33Gm-MOTY) and transferred it to the double mutant *lafK-motY* (AH-5503::*motY*). As observed above, when introduced into the AH-3::*motY* mutant, the plasmid was unable to restore polar flagella motility in 0.2 % L-arabinose, and this was the case even when L-arabinose concentrations were increased.

In order to determine whether the inability to complement the AH-3::*motY* mutant was caused by the non-transcription of the *motY* gene from pBAD33-MOTY, we analysed *motY* transcription in DH5 α and AH-3::*motY* transconjugants by RT-PCR from cells grown with 0.2 % L-arabinose. Transcripts of *motY* were found in both transconjugant strains containing the pBAD33-MOTY plasmid, but no transcripts were detected in strains without the recombinant plasmid (Fig. 5b). Analysis of *motX* and *motY* transcription in the wild-type strain AH-3 grown in liquid medium showed a higher level of *motY* transcription in relation to *motX* (Fig. 5b).

In *V. alginolyticus*, MotX and MotY are components of the T ring (Terashima *et al.*, 2006), and MotX rapidly degrades in the absence of MotY (Yagasaki *et al.*, 2006). Therefore, we investigated whether coordinated expression levels of the *motY* and *motX* genes were able to restore the swimming motility of the AH-3::*motY* mutant. We cloned *motY* and *motX* into the pBAD33 vector (pBAD33-MOTYX and pBAD33-MOTYXc, respectively), each under the control of the P_{BAD} promoter, as described in Methods (Fig. 1), and transferred them independently to the AH-3::*motY* mutant. The motility of transconjugants grown with 0.2 % L-arabinose was analysed. The introduction of pBAD33-MOTYX, which contain both genes in the same transcriptional direction, or pBAD33-MOTYXc, which contain the genes in opposite directions, restored motility

Fig. 3. (a) Motility of *A. hydrophila* strains: wild-type (AH-3), the non-polar flagellum mutant AH-4442 (*flhA*), the non-functional polar flagellum mutant AH-4461 (*motX*), the *motY* mutant and the double mutant AH-5503::*motY* (*lafK::motY*), grown for 20 h at 25 °C on soft agar. (b) TEM of the *A. hydrophila* AH-3 *motY* mutant and AH-5503::*motY* (*lafK::motY* double mutant) grown overnight at 25 °C on liquid medium and soft agar plates. Bacteria were gently placed onto Formvar-coated copper grids and negatively stained using 2% uranyl acetate. Bars, 1 μ m.

in liquid medium and soft agar to the *motY* mutant (Fig. 5a). Deletion of a 901 bp *StuI*–*SmaI* fragment of pBAD33-MOTYX, containing the *motX* gene, rendered the plasmid unable to restore polar flagellum motility in the AH-3::*motY* mutant, as happens with pBAD33-MOTY alone.

Since in *V. alginolyticus* the overexpression of *motX* in a *motY* mutant is able to restore polar flagellum motility (Okabe *et al.*, 2001), we introduced the pBAD33-MOTX plasmid into the AH-3::*motY* mutant and the pBAD33Gm-MOTX plasmid into the double mutant *lafK::motY*. The overexpression of *A. hydrophila* *motX* in the AH-3::*motY* mutant was unable to restore swimming motility (Fig. 5a).

***A. hydrophila* hybrid polar flagellum stators are not functional**

Previously, we demonstrated that *A. hydrophila* has two redundant sodium-dependent polar stators, PomAB and PomA₂B₂, which allow bacteria to swim in liquid medium and soft agar (Wilhelms *et al.*, 2009). Since both polar flagellum stators are sodium-dependent, and subunits PomA/PomA₂ and PomB/PomB₂ are homologous proteins, they might constitute functional natural hybrid motors. To analyse this we transferred the *pomB*, *pomB*₂, *pomAB* and *pomA*₂B₂ genes to three mutants: AH-4473, unable to express *pomAB*-A₂B₂ (Wilhelms *et al.*, 2009);

Fig. 4. Radial expansion at 25 °C in soft agar plates containing no amiloride or 1, 1.5 and 2 mM amiloride, or containing no CCCP or 5, 10 and 20 µM CCCP of *A. hydrophila* lateral flagella mutant AH-5503 (*lafK*), polar flagellum mutant AH-4442 (*flhA*) and the *motY* mutant. Motility was determined by measuring the diameter (in mm) of the zone of expansion from the point of inoculation after 48 h growth. Error bars, SD from five different experiments.

AH-4452, which expresses *pomA*₂ alone (Wilhelms *et al.*, 2009); and AH4471:: *pomB*, which expresses *pomA* alone (see Methods). The three mutants had a polar flagellum, but were unable to swim in liquid medium and showed reduced radial expansion in soft agar in relation to the wild-type, AH-3 (Fig. 6). Plasmids pACYC-POMAB and pACYC-POMA₂B₂, containing the *A. hydrophila* AH-3 *pomAB* and *pomA*₂B₂ genes, respectively, have previously been transferred to the AH-4473 (*pomAB*-A₂B₂) and AH-4452 (*pomAB*-B₂) mutants, and both are able to restore swimming motility in liquid medium and soft agar in these mutants (Wilhelms *et al.*, 2009). The transfer of one of these two plasmids into the AH4471:: *pomB* (*pomA*₂B₂-B) mutant also restored the swimming phenotype, as in the AH-4473 and AH-4452 mutants (Fig. 6). However, plasmid pBAD33GM-POMB₂, containing *A. hydrophila* AH-3 *pomB*₂, was able to complement swimming motility in liquid medium and radial expansion in soft agar in the AH-4452 (*pomAB*-B₂) mutant when L-arabinose was added to the medium, but was unable to perform this complementation in AH4471:: *pomB* (*pomB*-A₂B₂) and AH-4473 (*pomAB*-A₂B₂) mutants under identical growth conditions (Fig. 6). In a similar way, plasmid pBAD33GM-POMB, containing *A. hydrophila* AH-3 *pomB*, was able to restore motility in AH4471:: *pomB* (*pomB*-A₂B₂), but was unable to do so in the AH-4452 (*pomAB*-B₂) and AH-4473 (*pomAB*-A₂B₂) mutants (Fig. 6). These results suggest that homologous proteins of *A. hydrophila* polar flagellum stators are not interchangeable.

Complementation of *A. hydrophila* Δ*pomAB*Δ*pomA*₂B₂ by *V. parahaemolyticus* *pomAB* and *P. aeruginosa* *motAB* or *motCD*

Since *A. hydrophila* PomA₂/PomB₂ stator proteins are homologous to *V. parahaemolyticus* PomA/PomB [65 % identity and 81 % similarity for PomA₂ (E=3e-81), and 59 % identity and 74 % similarity for PomB₂ (E=2e-95)], and *A. hydrophila* PomA/PomB stator proteins are homologous to *P. aeruginosa* MotA/MotB [50 % identity and 70 % similarity for PomA (E=4e-57), and 28 % identity and 50 % similarity for PomB (E=2e-27)], as well as to *P. aeruginosa* MotC/MotD [50 % identity and 69 % similarity for PomA (E=2e-56), and 29 % identity and 50 % similarity for PomB (E=2e-27)], we examined whether *V. parahaemolyticus* *pomAB* or *P. aeruginosa* *motAB* or *motCD* can complement the *A. hydrophila* Δ*pomAB*Δ*pomA*₂B₂ mutant (AH-4473). Plasmids containing *V. parahaemolyticus* *pomAB* (pBAD33Gm-POMABvp) and *P. aeruginosa* *motAB* (pBAD33Gm-MOTABpa) or *motCD* (pBAD33Gm-MOTCDpa) were independently transferred to the AH-4473 mutant. Transconjugants were tested for motility in liquid medium and soft agar in the presence of L-arabinose, and all of them were unable to swim in liquid medium and showed a radial expansion in soft agar identical to that of the mutant.

DISCUSSION

The *A. hydrophila* AH-3 polar flagellum has a complex stator energized by the electrochemical potential of Na⁺ as

Fig. 5. (a) Motility of the *A. hydrophila* wild-type (AH-3), *motY* mutant, and the *motY* mutant harbouring plasmids pLA-MOTY (1), pBAD33-MOTX (2), pBAD33-MOTY (3) and pBAD33-MOTXY (4) grown for 28 h at 25 °C on soft agar. Mutants complemented with pBAD33 plasmids were grown with 0.2 % L-arabinose. (b) PCR and RT-PCR amplicons of *A. hydrophila* *motX*, *motY* and *rrsA* (16S rRNA) internal fragments obtained from *A. hydrophila* AH-3 genomic DNA (lanes 1, 2 and 3, respectively) and 5 µg total RNA (lanes 6, 7 and 11, respectively). Also shown are RT-PCR amplicons of *motY* and the *rrsA* internal fragment obtained from 5 µg total RNA of the *A. hydrophila* AH-3 *motY* mutant containing plasmid pBAD33 (lanes 5 and 10, respectively), or containing plasmid pBAD33-MOTY (lanes 4 and 9, respectively). Lane 8, DNA molecular marker Ecoladder I (Ecogen). Strains containing pBAD33 derivatives were grown with 0.2 % L-arabinose.

coupling ion. We previously described this polar stator as a complex composed of an essential protein, MotX, and two sets of redundant proteins, PomAB and PomA₂B₂, which are non-essential to drive motility in liquid medium or soft agar (Canals *et al.*, 2006a; Wilhelms *et al.*, 2009). Although these redundant pairs of proteins constitute complexes that function as a sodium channel, they have different sensitivities to variations in sodium concentration (Wilhelms *et al.*, 2009). However, the stator of the sodium-driven motors of other Gram-negative bacteria such as *Vibrio* spp. and *S. oneidensis* possess four proteins: MotX, MotY, PomA and PomB (Terashima *et al.*, 2006; Paulick *et al.*, 2009).

By the analysis of the *A. hydrophila* ATCC7966^T genome we identified *A. hydrophila* AH-3 MotY, which exhibits 44–46 % identity and 64–66 % similarity to MotY of different *Vibrio* species. Furthermore, MotY shows an N-terminal signal peptide, a C-terminal OmpA family domain involved in peptidoglycan binding, and two cysteine residues, which are critical to intramolecular sulfide bond formation

between MotX and MotY of *Vibrio* spp. (Yagasaki *et al.*, 2006). Both *motX* and *-Y* of *A. hydrophila*, although located in different chromosomal regions, are under the control of σ^{28} promoters, as determined by RACE assays with high identity (Fig. 2b). Since MotY homologues are also reported to form part of certain proton-driven flagella systems, such as the lateral flagella of *Vibrio* spp. and the polar flagellum of *S. oneidensis* and *P. aeruginosa* (Stewart & McCarter, 2003; Doyle *et al.*, 2004; Paulick *et al.*, 2009), we investigated whether *Aeromonas* MotY was involved in polar/lateral flagella rotation. By using a *motY* mutant and a double mutant *lafK-motY*, we were able to demonstrate that MotY is only essential for *A. hydrophila* polar flagellum rotation in the presence of MotX. Therefore, the *A. hydrophila* polar flagellum stator is a complex integrated by two essential proteins, MotX and MotY, which interact with one of two redundant pairs of proteins, PomAB and PomA₂B₂.

In *Vibrio* spp. and *S. oneidensis* MR-1, *motY* mutants are fully complemented by their wild-type *motY* (Yagasaki *et al.*,

Fig. 6. Motility of *A. hydrophila* wild-type (AH-3), and AH-4473 ($\Delta pomAB-A_2B_2$), AH-4452 ($\Delta pomAB-B_2$) and AH-4471::*pomB* ($\Delta pomB-A_2B_2$) mutants, as well as mutants complemented with plasmids pBAD33Gm-POMB (1), pBAD33Gm-POMB₂ (2) and pACYC-POMAB (3) grown for 28 h at 25 °C on soft agar. Strains with pBAD33 derivatives were grown with 0.2 % L-arabinose. The AH-4473 mutant ($\Delta pomAB-A_2B_2$) with plasmid pACYC-POMA₂B₂ shows the same motility phenotype as with plasmid pACYC-POMAB.

2006; Koerdt *et al.*, 2009). In *A. hydrophila* *motY* and *lafK-motY* mutants, complementation assays using pLA-MOTY and pBAD33-MOTY or pBAD33Gm-MOTY in the presence of L-arabinose showed that these plasmids were unable to restore polar flagellum movement (Fig. 5a), even when grown with different L-arabinose concentrations, although RT-PCR analysis showed that *motY* was transcribed from pBAD33-MOTY in the *motY* mutant grown with 0.2 % L-arabinose (Fig. 5b). However, the pLA-MOTX recombinant plasmid was able to fully restore swimming motility in the *A. hydrophila* AH-3 *motX* mutant (AH-4461) (Canals *et al.*, 2006a). Since *V. alginolyticus* MotX and MotY are components of the T ring (Terashima *et al.*, 2006), and MotY stabilizes MotX and anchors it to the peptidoglycan layer (Okabe *et al.*, 2002), we investigated whether coordinated expression of the *motY* and *motX* genes was able to restore the swimming motility of the AH-3::*motY* mutant. Thus, pBAD33-MOTYX and pBAD33-MOTYXc, containing *A. hydrophila* *motY* and *motX*, respectively, both under arabinose promoter control but transcribed in the same direction (pBAD33-MOTYX) or in opposite directions (pBAD33-MOTYXc), were transferred

to the AH-3::*motY* mutant. Both recombinant plasmids were able to restore swimming motility in liquid medium and soft agar to the AH-3::*motY* mutant when grown in the presence of L-arabinose (Fig. 5a). The pBAD33-MOTYX *motX* deletion abolished swimming motility in the AH-3::*motY* mutant strain. These results show that *A. hydrophila* *motY* overexpression was unable to complement the AH-3::*motY* mutant; however, coordinated expression of MotX and MotY was able to restore the function of the polar flagellum motor. In *Vibrio*, it has been reported that the MotX–MotY interaction is the key to the correct folding and function of MotX, since MotY stabilizes the complex and anchors it to the peptidoglycan layer, and also regulates MotX solubility. Therefore, the MotX–MotY interaction is another level of control of the amount of MotX in the periplasm (Okabe *et al.*, 2005; Yagasaki *et al.*, 2006). Several features of *A. hydrophila* MotX–Y are in agreement with or contradict the results for *Vibrio*:

(1) *A. hydrophila* MotX–Y seems to be located in the purified hook-basal flagella bodies, as in *Vibrio* (Terashima *et al.*, 2006). The *A. hydrophila* polar flagellum displays a T ring (Fig. 7).

Fig. 7. TEM of *A. hydrophila* AH-3 polar flagellum basal bodies. The hook-basal bodies were gently placed onto Formvar-coated copper grids and negatively stained using 2 % uranyl acetate. Bar, 25 nm.

(2) Overexpression of *A. hydrophila* *motX* did not restore *motY* mutant motility, although this effect has been described by the above authors in *Vibrio* (Okabe *et al.*, 2001).

(3) The presence of *A. hydrophila* *motY* alone is not able to restore the lack of polar motility in the corresponding mutant; it has to be coordinated with *motX* expression. In the case of *Vibrio*, the presence of *motY* alone restores the motility of the corresponding mutant independently of the amount of MotX (Yagasaki *et al.*, 2006).

Accordingly, our data for *A. hydrophila* suggest that a non-coordinated or early expression of *motY* with respect to *motX* abolishes T ring formation and therefore polar flagellum motility. However, the early expression of MotX does not interfere with T ring formation.

In addition to MotX–MotY, the *A. hydrophila* polar flagellum motor has two redundant sodium-dependent polar flagellum stators, PomAB and PomA₂B₂, of which the subunits PomA/PomA₂ and PomB/PomB₂ are homologous proteins, having four and one transmembrane regions (TMs), respectively. Furthermore, PomA/PomA₂ show 30 % identity and 53 % similarity, whereas PomB/PomB₂ show 23 % identity and 43 % similarity. Both polar flagellum stators are sodium-dependent, and only one of them is essential for polar flagellum motility in liquid medium and soft agar (Wilhelms *et al.*, 2009). The *Vibrio* homologous proteins, PomA and PomB, constitute a multimeric complex composed of four PomA and two PomB proteins, where the cooperation of PomA TM3 and the PomB TM, which are in close proximity over their entire length, is required for coupling of Na⁺ to drive the

flagellar rotation (Yakushi *et al.*, 2004). The results obtained indicate that homologous proteins of *A. hydrophila* polar flagellum stators are not interchangeable, probably because critical amino acids localized in the PomA or PomA₂ TM3 segment and the PomB or PomB₂ TM involved in PomAB and PomA₂B₂ interaction are different in these two redundant protein pairs. Finally, *V. parahaemolyticus* PomAB, and *P. aeruginosa* MotAB or MotCD cloned independently into plasmid pBAD33, were unable to restore swimming motility to an *A. hydrophila* Δ pomAB Δ pomA₂B₂ mutant (AH-4473) grown with L-arabinose, despite their high similarities. One possible reason is that charged residues in the cytoplasmic regions of *V. parahaemolyticus* PomA and *P. aeruginosa* MotA or MotC are unable to interact with oppositely charged residues of the *A. hydrophila* FliG C-terminal region, interactions that are essential for polar flagellum movement.

ACKNOWLEDGEMENTS

This work was supported by Plan Nacional de I+D (Ministerio de Educación, Ciencia y Deporte and Ministerio de Sanidad, Spain) and by Generalitat de Catalunya (Centre de Referència en Biotecnologia). R.M. has a predoctoral fellowship from Ministerio de Educación, Ciencia y Deporte. We thank Maite Polo for her technical assistance and the Servicios Científico-Técnicos of the University of Barcelona.

REFERENCES

- Allen, L. N. & Hanson, R. S. (1985). Construction of broad-host-range cosmid cloning vectors: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. *J Bacteriol* **161**, 955–962.
- Altarriba, M., Merino, S., Gavin, R., Canals, R., Rabaan, A., Shaw, J. G. & Tomás, J. M. (2003). A polar flagella operon (*flg*) of *Aeromonas hydrophila* contains genes required for lateral flagella expression. *Microb Pathog* **34**, 249–259.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Asai, Y., Kojima, S., Kato, H., Nishioka, N., Kawagishi, I. & Homma, M. (1997). Putative channel components for the fast-rotating sodium-driven flagellar motor of a marine bacterium. *J Bacteriol* **179**, 5104–5110.
- Asai, Y., Yakushi, T., Kawagishi, I. & Homma, M. (2003). Ion-coupling determinants of Na⁺-driven and H⁺-driven flagellar motors. *J Mol Biol* **327**, 453–463.
- Bateman, A., Birney, E., Cerruti, L., Durbin, R., Eddy, S. R., Griffiths-Jones, S., Howe, K. L., Marshall, M. & Sonnhammer, E. L. L. (2002). The Pfam protein families database. *Nucleic Acids Res* **30**, 276–280.
- Berg, H. C. (2003). The rotary motor of bacterial flagella. *Annu Rev Biochem* **72**, 19–54.
- Blair, D. F. (2003). Flagellar movement driven by proton translocation. *FEBS Lett* **545**, 86–95.
- Blair, D. F. & Berg, H. C. (1990). The MotA protein of *E. coli* is a proton-conducting component of the flagellar motor. *Cell* **60**, 439–449.

- Canals, R., Ramirez, S., Vilches, S., Horsburgh, G., Shaw, J. G., Tomás, J. M. & Merino, S. (2006a). Polar flagellum biogenesis in *Aeromonas hydrophila*. *J Bacteriol* **188**, 542–555.
- Canals, R., Altarriba, M., Vilches, S., Horsburgh, G., Shaw, J. G., Tomás, J. M. & Merino, S. (2006b). Analysis of the lateral flagellar gene system of *Aeromonas hydrophila* AH-3. *J Bacteriol* **188**, 852–862.
- De Mot, R. & Vanderleyden, J. (1994). The C-terminal sequence conservation between OmpA-related outer membrane proteins and MotB suggests a common function in both Gram-positive and Gram-negative bacteria, possibly in the interaction of these domains with peptidoglycan. *Mol Microbiol* **12**, 333–334.
- Doyle, T. B., Hawkins, A. C. & McCarter, L. L. (2004). The complex flagellar torque generator of *Pseudomonas aeruginosa*. *J Bacteriol* **186**, 6341–6350.
- Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* **177**, 4121–4130.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Ito, M., Hicks, D. B., Henkin, T. M., Guffanti, A. A., Powers, B. D., Zvi, L., Uematsu, K. & Krulwich, T. A. (2004). MotPS is the stator-force generator for motility of alkaliphilic *Bacillus*, and its homologue is a second functional Mot in *Bacillus subtilis*. *Mol Microbiol* **53**, 1035–1049.
- Janda, J. M. & Abbott, S. L. (2010). The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clin Microbiol Rev* **23**, 35–73.
- Jimenez, N., Lacasta, A., Vilches, S., Reyes, M., Vazquez, J., Aquillini, E., Merino, S., Regué, M. & Tomás, J. M. (2009). Genetics and proteomics of *Aeromonas salmonicida* lipopolysaccharide core biosynthesis. *J Bacteriol* **191**, 2228–2236.
- Koerdt, A., Paulick, A., Mock, M., Jost, K. & Thormann, K. M. (2009). MotX and MotY are required for flagellar rotation in *Shewanella oneidensis* MR-1. *J Bacteriol* **191**, 5085–5093.
- Kojima, S. & Blair, D. F. (2004). Solubilization and purification of the MotA/MotB complex of *Escherichia coli*. *Biochemistry* **43**, 26–34.
- Kojima, S., Shinohara, A., Terashima, H., Yakushi, T., Sakuma, M., Homma, M., Namba, K. & Imada, K. (2008). Insights into the stator assembly of the *Vibrio* flagellar motor from the crystal structure of MotY. *Proc Natl Acad Sci U S A* **105**, 7696–7701.
- MacNab, R. (1996). Flagella and motility. In *Escherichia coli* and *Salmonella*, pp. 123–145. Edited by F. C. Neidhardt. Washington, DC: American Society for Microbiology.
- McCarter, L. L. (1994). MotY, a component of the sodium-type flagellar motor. *J Bacteriol* **176**, 4219–4225.
- McCarter, L. L. (2001). Polar flagellar motility of the *Vibrionaceae*. *Microbiol Mol Biol Rev* **65**, 445–462.
- Merino, S., Camprubí, S. & Tomás, J. M. (1991). The role of lipopolysaccharide in complement-killing of *Aeromonas hydrophila* strains of serotype O:34. *J Gen Microbiol* **137**, 1583–1590.
- Okabe, M., Yakushi, T., Asai, Y. & Homma, M. (2001). Cloning and characterization of *motX*, a *Vibrio alginolyticus* sodium-driven flagellar motor gene. *J Biochem* **130**, 879–884.
- Okabe, M., Yakushi, T., Kojima, M. & Homma, M. (2002). MotX and MotY, specific components of the sodium-driven flagellar motor, colocalize to the outer membrane in *Vibrio alginolyticus*. *Mol Microbiol* **46**, 125–134.
- Okabe, M., Yakushi, T. & Homma, M. (2005). Interactions of MotX with MotY and with the PomA/PomB sodium ion channel complex of the *Vibrio alginolyticus* polar flagellum. *J Biol Chem* **280**, 25659–25664.
- Paulick, A., Koerdt, A., Lassak, J., Huntley, S., Wilms, I., Narberhaus, F. & Thormann, K. M. (2009). Two different stator systems drive a single polar flagellum in *Shewanella oneidensis* MR-1. *Mol Microbiol* **71**, 836–850.
- Rubirés, X., Saigi, F., Piqué, N., Climent, N., Merino, S., Albertí, S., Tomás, J. M. & Regué, M. (1997). A gene (*wbbL*) from *Serratia marcescens* N28b (O4) complements the *rfb-50* mutation of *Escherichia coli* K-12 derivatives. *J Bacteriol* **179**, 7581–7586.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463–5467.
- Seshadri, R., Joseph, S. W., Chopra, A. K., Sha, J., Shaw, J. G., Graf, J., Haft, D., Wu, M., Ren, Q. & other authors (2006). Genome sequence of *Aeromonas hydrophila* ATCC 7966^T: jack of all trades. *J Bacteriol* **188**, 8272–8282.
- Stewart, B. J. & McCarter, L. L. (2003). Lateral flagellar gene system of *Vibrio parahaemolyticus*. *J Bacteriol* **185**, 4508–4518.
- Stolz, B. & Berg, H. C. (1991). Evidence for interactions between MotA and MotB, torque-generating elements of the flagellar motor of *Escherichia coli*. *J Bacteriol* **173**, 7033–7037.
- Terashima, H., Fukuoka, H., Yakushi, T., Kojima, S. & Homma, M. (2006). The *Vibrio* motor proteins, MotX and MotY, are associated with the basal body of Na-driven flagella and required for stator formation. *Mol Microbiol* **62**, 1170–1180.
- Toutain, C. M., Zegans, M. E. & O'Toole, G. A. (2005). Evidence for two flagellar stators and their role in the motility of *Pseudomonas aeruginosa*. *J Bacteriol* **187**, 771–777.
- Wilhelms, M., Vilches, S., Molero, R., Shaw, J. G., Tomás, J. M. & Merino, S. (2009). Two redundant sodium-driven stator motor proteins are involved in *Aeromonas hydrophila* polar flagellum rotation. *J Bacteriol* **191**, 2206–2217.
- Yagasaki, J., Okabe, M., Kurebayashi, R., Yakushi, T. & Homma, M. (2006). Roles of the intramolecular disulfide bridge in MotX and MotY, the specific proteins for sodium-driven motors in *Vibrio* spp. *J Bacteriol* **188**, 5308–5314.
- Yakushi, T., Maki, S. & Homma, M. (2004). Interaction of PomB with the third transmembrane segment of PomA in the Na⁺-driven polar flagellum of *Vibrio alginolyticus*. *J Bacteriol* **186**, 5281–5291.
- Yorimitsu, T. & Homma, M. (2001). Na⁺-driven flagellar motor of *Vibrio*. *Biochim Biophys Acta* **1505**, 82–93.
- Yu, H. B., Rao, P. S., Lee, H. C., Vilches, S., Merino, S., Tomas, J. M. & Leung, K. Y. (2004). A type III secretion system is required for *Aeromonas hydrophila* AH-1 pathogenesis. *Infect Immun* **72**, 1248–1256.
- Zhou, J. D., Fazzio, R. T. & Blair, D. F. (1995). Membrane topology of the MotA protein of *Escherichia coli*. *J Mol Biol* **251**, 237–242.
- Zhou, J., Lloyd, S. A. & Blair, D. F. (1998). Electrostatic interactions between rotor and stator in the bacterial flagellar motor. *Proc Natl Acad Sci U S A* **95**, 6436–6441.

Edited by: P. W. O'Toole