FEBS Letters 584 (2010) 1268–1272

journal homepage: www.FEBSLetters.org

Proton-conductivity assay of plugged and unplugged MotA/B proton channel by cytoplasmic pHluorin expressed in *Salmonella*

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ARTICLE INFO

Article history:
Received 11 December 2009
Revised 17 February 2010
Accepted 17 February 2010
Available online 21 February 2010

Edited by Renee Tsolis

Keywords:
Intracellular pH
pHluorin
Bacterial flagellar motor
Stator complex
Proton conductance
Chemotaxis

ABSTRACT

MotA and MotB form the proton-channel complex of the proton-driven bacterial flagellar motor. A plug segment of *Escherichia coli* MotB suppresses proton leakage through the MotA/B complex when it is not assembled into the motor. Using a ratiometric pH indicator protein, pHluorin, we show that the proton-conductivity of a *Salmonella* MotA/B complex not incorporated into the motor is two orders of magnitude lower than that of a complex that is incorporated and activated. This leakage is, however, significant enough to change the cytoplasmic pH to a level at which the chemotaxis signal transduction system responds.

1. Introduction

The flagellum of *Escherichia coli* and *Salmonella enterica* is a rotary motor powered by the proton motive force across the cytoplasmic membrane. MotA and MotB form the stator complex of the motor in the cytoplasmic membrane, which functions as a proton channel to couple proton flow to torque generation [1]. A highly conserved aspartic acid residue of *Salmonella* MotB, Asp-33, plays an important role in the proton-relay mechanism [2–4].

Cell growth is not impaired by over-expression of either *E. coli* MotA/B or MotA alone [5,6]. In contrast, an in-frame deletion of residues 51–70 within the periplasmic domain of *E. coli* MotB, a region which is highly conserved among the MotB family, causes considerable proton leakage, thereby arresting cell growth [7]. This result suggests that the deleted region acts as a plug that interferes with proton channel formation, resulting in suppression of undesirable proton flow through the channel when the MotA/B complex is not assembled into the motor. Interestingly, however, neither motility nor cell growth is significantly impaired by in-frame deletion of residues 51–100 in *Salmonella* MotB, which contains the putative plug segment [8,9]. Therefore, it is not clear how the proton-conductivity of the *Salmonella* MotA/B complex is regulated.

In this study, we expressed pHluorin in *Salmonella* cells to study the effect of varying copy numbers of MotA/B and its unplugged variant on intracellular pH change to assay the proton-conductivity of the plugged and unplugged MotA/B complex. We show that the plug segment of *Salmonella* MotB suppresses proton leakage. However, the suppression is not very tight. Therefore, over-expression of the intact MotA/B complex results in a small decrease in the cytoplasmic pH that is sufficient to bias the motor towards CW rotation.

2. Materials and methods

2.1. Bacteria, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella* strains MMPH001 (Δ*araBAD*::*pHluorin*) and YVM021 (Δ*fliC*::*pHluorin*) were constructed using the λ Red homologous recombination system [10]. MMPH001, which is wild-type for motility and chemotaxis, expresses the *pHluorin* gene from the *araBAD* promoter on the chromosome. The *fliC* gene is replaced with the *pHluorin* gene in the YVM021 strain, in which the expression of pHluorin is under control of the *fliC* promoter. L-broth, T-broth, tryptone semi-solid agar plates and motility medium were prepared as described previously [11].

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doi:10.1016/j.febslet.2010.02.051

Table 1
Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source or reference
Salmonella		
SJW1103	Wild type for motility and chemotaxis	[17]
SJW2241	ΔmotA-motB	[18]
MMPH001	ΔaraBAD::pHluorin	This study
MMPH001-46	ΔaraBAD::pHluorin, fliCΔ(205–293)	This study
YVM021	ΔfliC::pHluorin	This study
Plasmids		
pTrcc99A	Cloning vector	GE Healthcare
pKK223-3	Cloning vector	GE Healthcare
pBAD24	Cloning vector	[19]
pKSS13	pKK223-3/MotA+MotB	S. Sugiyama
pKSS13(D33N)	pKK223-3/MotA+MotB(D33N)	S. Kojima
pNSK9	pTrc99A/MotA+MotB	[13]
pTSK30	pTrc99A/MotA+MotB(Δ51–100)	[9]
pTSK30(L119P)	pTrc99A/MotA+MotB(Δ51–100/L119P)	[9]
pTSK30(L119E)	pTrc99A/MotA+MotB(Δ51–100/L119E)	[9]
pYC20	pBAD24/MotA+MotB	This study
pYC109	pBAD24/MotA+MotB(Δ52–71)	This study

2.2. Immunoblotting

Immunoblotting with polyclonal anti-MotA and anti-MotB antibodies was carried out as described before [12].

2.3. Bead assay for motor rotation

Bead assays for motor rotation speed and directional switching were carried out as described [13], using polystyrene beads with diameters of 0.8 μm and 1.5 μm (Invitrogen). The measurements were done at 25 °C.

2.4. Spectroscopy of pHluorin for intracellular pH measurement

Intracellular pH measurements were carried out at an external pH value of 5.5 using ratiometric pHluorin, a pH-sensitive green fluorescent protein [14], as described before [15]. To generate the calibration curve, the 410/470-nm excitation ratios of purified pHluorin were determined at different pH values (Supplementary Fig. 1). The fluorescence-excitation spectra of MMPH001 or YVM021 expressing MotA/B and its mutants were recorded on a fluorescence spectrophotometer (RF-5300PC, Shimazu). The 410/470-nm excitation ratios were calculated and converted to pH values based on the calibration curve.

3. Results

3.1. Effect of the plug segment of MotB on the proton-conductivity of the MotA/B complex

In-frame deletion of residues 51–100 in *Salmonella* MotB does not affect cell growth although the conserved plug segment is missing [8,9]. To confirm this, we measured the growth rate of *Salmonella* cells over-expressing the MotA/B(Δ51–100) complex and observed no growth impairment (Fig. 1A), indicating that a proton channel is not formed. Then, to test whether the plug segment suppresses proton leakage through *Salmonella* MotA/B complex in the similar way to that observed in *E. coli* MotB, an in-frame deletion variant of MotB, MotB(Δ52–71) missing residues 52–71, was co-expressed with MotA from the pBAD24-based plasmid. Cell growth was totally inhibited by induction of MotA/B(Δ52–71) but not by MotA/B (Fig. 1B), even though the expression level of MotA/

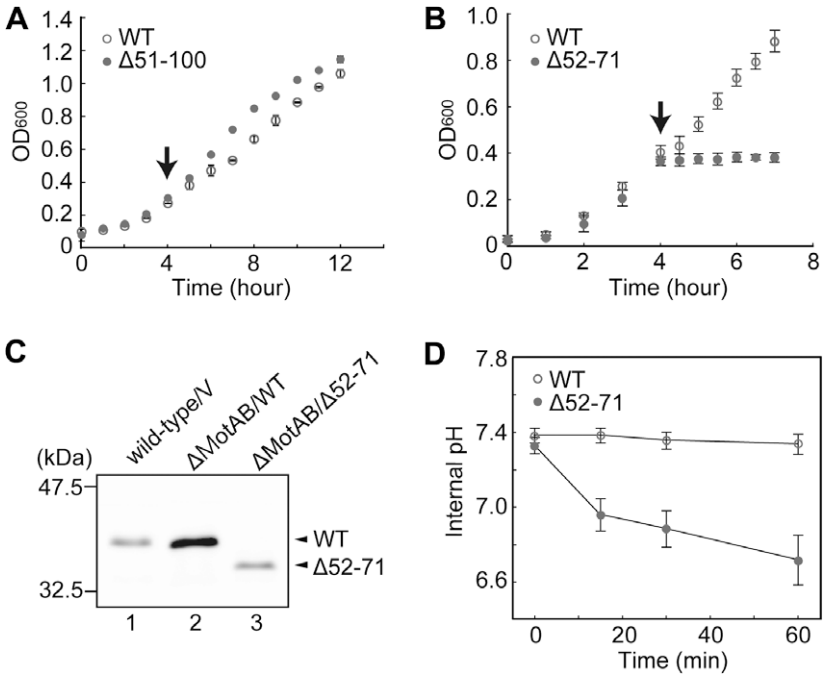

Fig. 1. Effects of deletion of the plug segment of *Salmonella* MotB on cell growth and proton leakage. (A) Growth curve of SJW2241 (Δ*motAB*) harboring pNSK9 (wild-type MotA/B, indicated as WT) (open circles) or pTSK30 (MotA/B(Δ51–100), indicated as Δ51–100) (closed circles). The arrow indicates the time when 1 mM IPTG was added. The optical density at 600 nm (OD_{600}) was measured. (B) Growth curve of SJW2241 expressing wild-type MotA/B (open circles) or MotA/B(Δ52–71) (indicated as Δ52–71) (closed circles). The arrow indicates the time when 1 mM arabinose was added. (C) Immunoblotting, using polyclonal anti-MotB antibody, of total proteins from *Salmonella* cells transformed with the pBAD24-based plasmids: lane 1, SJW1103 (Wild-type) carrying pBAD24 (V); lane2, SJW2241 transformed with pYC20 (WT); lane 3, SJW2241 harboring pYC109 (Δ52–71). (D) Measurement of the intracellular pH of YVM021 cells (Δ*fliC*::*pHluorin*) using the pHluorin probe after induction of MotA/B or MotB(Δ52–71) expression at an external pH of 5.5.