The voltage-gated Na⁺ channel Na_vBP co-localizes with methyl-accepting chemotaxis protein at cell poles of alkaliphilic *Bacillus pseudofirmus* OF4

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Na_vBP, found in alkaliphilic *Bacillus pseudofirmus* OF4, is a member of the bacterial voltage-gated Na⁺ channel superfamily. The alkaliphile requires Na_vBP for normal chemotaxis responses and for optimal pH homeostasis during a shift to alkaline conditions at suboptimally low Na⁺ concentrations. We hypothesized that interaction of Na_vBP with one or more other proteins *in vivo*, specifically methyl-accepting chemotaxis proteins (MCPs), is involved in activation of the channel under the pH conditions that exist in the extremophile and could underpin its role in chemotaxis; MCPs transduce chemotactic signals and generally localize to cell poles of rod-shaped cells. Here, immunofluorescence microscopy and fluorescent protein fusion studies showed that an alkaliphile protein (designated McpX) that cross-reacts with antibodies raised against *Bacillus subtilis* McpB co-localizes with Na_vBP at the cell poles of *B. pseudofirmus* OF4. In a mutant in which Na_vBP-encoding *ncbA* is deleted, the content of McpX was close to the wild-type level but McpX was significantly delocalized. A mutant of *B. pseudofirmus* OF4 was constructed in which *cheAW* expression was disrupted to assess whether this mutation impaired polar localization of McpX, as expected from studies in *Escherichia coli* and *Salmonella*, and, if so, whether Na_vBP would be similarly affected. Polar localization of both McpX and Na_vBP was decreased in the *cheAW* mutant. The results suggest interactions between McpX and Na_vBP that affect their co-localization. The inverse chemotaxis phenotype of *ncbA* mutants may result in part from MCP delocalization.

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INTRODUCTION

Our understanding of the activation and physiological roles of bacterial channels lags far behind our knowledge of their structures (Booth *et al.*, 2003, 2005; Kung & Blount, 2004). Recently, roles have been demonstrated for the voltage-gated sodium channel Na_vBP from alkaliphilic *Bacillus pseudofirmus* OF4 in pH homeostasis, motility and

chemotaxis at alkaline pH (Ito *et al.*, 2004b). The current study probes cell biological properties of Na_vBP that could underpin these physiological roles.

Na_vBP is a member of the bacterial voltage-gated sodium channel superfamily Na_vBac, whose founding member is the NaChBac channel from alkaliphilic *Bacillus halodurans* C-125 (Koishi *et al.*, 2004; Ren *et al.*, 2001). The strong resemblance of Na_vBac channels to one of the six-transmembrane segment repeats of biomedically important eukaryotic voltage-gated channels has led to intense

Abbreviations: CFP, cyan fluorescent protein; IFM, immunofluorescence microscopy; MCP, methyl-accepting chemotaxis protein.

investigative interest in these bacterial channels (Ren *et al.*, 2001). Electrophysiological and mutational analyses have focused largely on NaChBac, either in lipid bilayers or expressed in eukaryotic cells (Blanchet *et al.*, 2007; Chahine *et al.*, 2004; Kuzmenkin *et al.*, 2004; Pavlov *et al.*, 2005; Richardson *et al.*, 2006; Zhao *et al.*, 2004). Since *B. halodurans* C-125, the native host of NaChBac, is not genetically accessible, it has not been possible to evaluate the match between properties determined in non-native settings and conditions under which NaChBac can be shown to function physiologically. In our initial studies, we therefore turned to *B. pseudofirmus* OF4, in which the *ncbA* gene that encodes the NaChBac homologue Na_vBP could be deleted in order to probe the physiological roles of the channel (Ito *et al.*, 2004b).

B. pseudofirmus OF4 grows on non-fermentative carbon sources in a pH range from 7.5 to >11. At pH values above 7.5, robust Na⁺/H⁺ antiporter (exchanger) activity, together with the activity of the proton-coupled ATP synthase, support net uptake of protons in respiring cells, thus achieving a cytoplasmic pH that is lower than the external pH. Antiport action reduces the proton motive force and establishes an inwardly directed sodium motive force. Na⁺-coupled solute uptake and flagellar rotation take advantage of this sodium motive force to support solute uptake and motility (Fujinami *et al.*, 2007; Ito *et al.*, 2004a; Krulwich *et al.*, 2007; Padan *et al.*, 2005). The Na⁺ that enters the cytoplasm during this Na⁺-coupled bioenergetic work completes the Na⁺ cycle and plays an

important role in supporting the ongoing Na⁺/H⁺ antiport activity for pH homeostasis (Fig. 1).

Bioenergetic studies on an *ncbA* mutant of *B. pseudofirmus* OF4 lacking Na_vBP have shown that the channel plays a role in Na⁺ re-entry in support of pH homeostasis at pH 10.5 under conditions of low Na⁺ concentration ([Na⁺]) and low concentrations of solutes whose entry is coupled to Na⁺. The *ncbA* mutant also displays a motility defect at high pH, even though it possesses the *motPS* genes that encode a stator force-generating Na⁺ channel required for flagella rotation (Ito *et al.*, 2004a, b). The *ncbA* mutant exhibits motility only after prolonged growth in liquid culture or on soft agar plates. The third, striking phenotype observed at high pH in both poorly motile and highly motile variant *ncbA* mutants is a high frequency of tumbling (Ito *et al.*, 2004b). The tumbling *ncbA* mutants exhibit an 'inverse chemotaxis', i.e. the mutants move toward repellents and away from attractants.

The parallel electrophysiological studies of the channel in eukaryotic cells have demonstrated that Na_vBP is an Na⁺-specific voltage-gated channel whose activation is potentiated at high pH (Ito *et al.*, 2004b). The electrophysiological characteristics indicate that in the natural alkaliphile host, the role of high external pH (and secondary elevation of cytoplasmic pH) in channel opening is supplemented by additional triggers (Ito *et al.*, 2004b). Such an additional trigger could result from an interaction between the channel and either the chemotaxis signalling machinery or the flagellar motor (Fig. 1). A particularly attractive

Fig. 1. Schematic diagram of the Na⁺ cycle of alkaliphilic *B. pseudofirmus* OF4. Na⁺/H⁺ antiporters, particularly the Mrp antiporter depicted here (Krulwich *et al.*, 2001), catalyse net proton accumulation in the cytoplasm in cells that are extruding protons during respiration. Na⁺ re-entry in support of pH homeostasis is achieved by Na⁺/solute symporters (Krulwich, 1995). Na_vBP has roles in motility, chemotaxis and pH homeostasis at high pH (Ito *et al.*, 2004b). We hypothesized the interaction of Na_vBP with one or more other proteins *in vivo*, specifically either MCPs or flagellar motor proteins.

hypothesis is that Na_vBP interacts with transmembrane chemoreceptors (also known as methyl-accepting chemotaxis proteins, MCPs), accounting for both triggering and channel effects in chemotaxis. The membrane-associated MCPs have periplasmic ligand-binding domains that monitor attractants and repellents on the outside of the cell, and have signalling/adaptation domains in their cytoplasmic segments that communicate with the flagellar motor via a two-component signalling pathway (Szurmant & Ordal, 2004). Signalling is initiated by a signalling complex that is formed at conserved cytoplasmic domains of the MCPs (Wadhams & Armitage, 2004; Zhulin, 2001). In *Escherichia coli* and *Salmonella*, MCPs form a complex with the histidine kinase CheA, which mediates the signalling triggered at the MCP receptor, and with adaptor protein CheW; this complex localizes to the cell poles (Gegner *et al.*, 1992; Liu *et al.*, 1997; Maddock & Shapiro, 1993; Sourjik & Berg, 2000); weaker lateral clusters are also observed (Lybarger & Maddock, 1999; Skidmore *et al.*, 2000). In *Bacillus subtilis*, the core elements that mediate chemotaxis resemble those of the *E. coli*–*Salmonella* systems (Lamanna *et al.*, 2005; Rao *et al.*, 2004; Szurmant & Ordal, 2004; Weis, 2006). As in the enteric bacteria, MCPs are localized to the polar end of other rod-shaped bacteria, including *Bacillus* species (Gestwicki *et al.*, 2000; Kirby *et al.*, 2000; Lamanna *et al.*, 2005). Polar localization and/or clustering of the complexes has been proposed to play a critical role in signal amplification in *E. coli* (Bray *et al.*, 1998; Duke & Bray, 1999; Irieda *et al.*, 2006; Lybarger & Maddock, 2001; Shapiro *et al.*, 2002). If Na_vBP interacts with polar MCPs and/or the kinase/adaptor proteins that are complexed with them in *B. pseudofirmus* OF4, the channels should also localize to the cell poles. On the other hand, if Na_vBP interacts with flagellar motors, a delocalized pattern would be expected, consistent with a peritrichous pattern of flagella in this alkaliphile (Fig. 1) (Fujinami *et al.*, 2007; Sturr *et al.*, 1994). We conducted an immunofluorescence microscopy (IFM) analysis of *B. pseudofirmus* OF4 cells to confirm the polar localization of MCPs and to compare it with the localization pattern of Na_vBP. MCP localization was assessed using mAbs against Na_vBP, and polyclonal antibodies that were raised against *B. subtilis* McpB, a chemoreceptor for Asn, Asp, Gln, Glu and His (Hanlon & Ordal, 1994). The alkaliphile gene whose product cross-reacts with this antibody has not yet been identified; we refer to this putative MCP as McpX. Channel localization was also probed using a plasmid expressing an Na_vBP–cyan fluorescent protein (CFP) fusion protein in *B. pseudofirmus* OF4. We further examined whether disruption of the alkaliphile *cheAW* locus decreases localization of McpX, Na_vBP or both.

The results indicate that Na_vBP co-localizes at cell poles with an alkaliphile protein that is recognized by antibodies raised against *B. subtilis* McpB. They further show that mutational loss of the channel leads to McpX delocalization, while a *cheAW*-disrupted mutant leads to significant delocalization of both the channel and McpX.

METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* and *B. subtilis* strains were grown routinely in LB or LBK medium (Goldberg *et al.*, 1987). Alkaliphilic *B. pseudofirmus* OF4 wild-type (strain 811M) (Clejan *et al.*, 1989) and its derivative strains were grown aerobically in semi-defined malate–yeast extract (MYE) medium (pH 10) (Sturr *et al.*, 1994) at 37 °C. pH 10 medium replaced the pH 10.5 medium used in most of our studies because the SC34 strain that lacked a functional Na_vBP exhibited a significant deficit in mid-exponential growth at the higher pH value; earlier studies of SC34 growth had examined yield at stationary phase and a defect at high pH was not observed (Ito *et al.*, 2004b). Nifedipine-treated wild-type (811M) cells were prepared by adding 50 µM nifedipine to mid-exponential phase cells (OD₆₀₀ 0.6). Growth was continued for 1 h, and the cells were then used for IFM. Antibiotics were added at 5 µg ml^{−1} for chloramphenicol and 0.3 µg ml^{−1} for erythromycin.

Disruption of the *cheAW* genes in *B. pseudofirmus* OF4. The chromosomal DNA fragment that contains the *cheAW* genes (GenBank accession no. DQ150110) was replaced entirely by a chloramphenicol acetyltransferase gene (*cat*) using the gene SOEing method described by others (Horton, 1997). Primers used are shown in Table 2. Two independent PCRs were performed on *B. pseudofirmus* OF4 wild-type chromosomal DNA template with the primer sets del-AW-F1-BH1 and del-AW-R1, and del-AW-F2 and del-AW-R2-ER1. Another PCR was performed on pC194 (Horinouchi & Weisblum, 1982) as DNA template with the primer set del-AW-F-CAT and del-AW-R-CAT to amplify the *cat* gene. The three purified PCR products were used as templates for a second PCR with primer sets del-AW-F1-BH1 and del-AW-R2-ER1. The purified product of this reaction was digested with *Bam*HI and *Eco*RI and cloned into *Bam*HI- and *Eco*RI-digested pG⁺host4, yielding pG4Δ*cheAW*::CAT. The plasmid was transformed into *B. pseudofirmus* OF4-811M protoplasts. The protocol for isolation of single-crossover candidates has been described previously (Ito *et al.*, 1997). From the two kinds of single-crossover candidates, analyses of the *cheAW* disruption mutant indicated that the recombination occurred in the region upstream of the *cheA* gene (region between del-AW-F1-BH1 and del-AW-R-CAT), and this was confirmed by PCR using primer sets OF4*cheA*24 and OF4*cheA*25, OF4*cheA*3 and OF4*cheA*25, and OF4*cheA*24 and OF4*cheA*15. The *cheAW* disruption mutant was designated strain 811M-*cheAW*.

Construction and transformation of Na_vBP–CFP fusion or CFP expression plasmid. The large fragment (4.8 kb) of *Xba*I- and *Hind*III-digested shuttle vector pYH30 (Bechhofer & Wang, 1998) was ligated with *Xba*I- and *Hind*III-digested pMW118, yielding pYM1. Plasmid pYM1 is a shuttle vector in *E. coli* and *Bacillus* species that is low copy in both hosts (about six copies per cell) (Ito *et al.*, 2004a). The *ncbA*–*ecfp* fragment that enhanced the CFP gene (*ecfp*) was fused just before the stop codon of the *ncbA* gene using the gene SOEing method (Horton, 1997). A schematic diagram of the *ncbA*–*ecfp* fragment is shown in Fig. 2(a). The primers used for constructions are shown in Table 2. Two independent PCRs were performed on *B. pseudofirmus* OF4 wild-type chromosomal DNA template with the primer sets NC-F1-SC2 and NC-R1-1, and NC-F2-1 and NC-R2-BH1. Another PCR was performed on the pECFP plasmid DNA template with the primer set NC-F-CFP1 and NC-R-CFP1 to amplify the *ecfp* gene. The three purified PCR products were used as templates for a second PCR with the primer set NC-F1-SC2 and NC-R2-BH1. The purified product of this reaction was digested with *Eco*RI and cloned into *Eco*RI-digested pMW118, yielding pMWSC-CFP. pMWSC-CFP was digested with *Bam*HI and *Hind*III, and ligated with the large fragment (5.0 kb) of *Bam*HI- and *Hind*III-digested

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and description	Source or reference
<i>E. coli</i>		
DH5 α MCR	F ⁻ <i>mcrA</i> Δ 1 (<i>mrr-hsd RMS-mcrBC</i>) Φ 80 <i>dlacZ</i> Δ (<i>lacZYAargF</i>) <i>U169 deoR recA1 endA1 supE44 λthi-1 gyr-496 relA1</i>	Stratagene
XL1-Blue MRF'	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1</i> <i>recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI^qZDM15 Tn10</i> (Tetr)]	Gibco-BRL
<i>B. subtilis</i>		
168	<i>trpC2</i>	BGSC*
<i>B. pseudofirmus</i> OF4		
811M	Wild-type, Met ⁻	Clejan <i>et al.</i> (1989)
SC34	811M Δ <i>ncbA</i> ::Spc ^R	Ito <i>et al.</i> (2004b)
SC34-R	SC34, <i>ncbA</i> restored at the native location	Ito <i>et al.</i> (2004b)
811M-cheAW	811M, <i>cheAW</i> disrupted	This study
Plasmids		
pC194	<i>Staphylococcus aureus</i> plasmid Cm ^R	Horinouchi & Weisblum (1982)
pMW118	Cloning vector Amp ^R	Nippon Gene
pG ⁺ host4	Temperature-sensitive vector Erm ^R	Appligene
pG4 <i>ΔcheAW</i> ::CAT	pG ⁺ host4 + Δ <i>cheAW</i> ::CAT	This study
pECFP	ECFP expression vector	Clontech
pMWSC-CFP	pMW118 + <i>ncbA-ecfp</i>	This study
pYH30	pGEM3Zf(+) (Promega) and pBD144 ligated at <i>Pst</i> I sites	Bechhofer & Wang (1998)
pYM1	pYH30 and pMW118 ligated at <i>Xba</i> I– <i>Hind</i> III sites. Low-copy shuttle vector in <i>E. coli</i> and <i>Bacillus</i>	Ito <i>et al.</i> (2004a)
pSC-CFP	pYM1 + <i>ncbA-ecfp</i>	This study
pMWCFP	pMW118 + <i>ecfp</i>	This study
pCFP	pYM1 + <i>ecfp</i>	This study

*BGSC, *Bacillus* Genetic Stock Center.

pYM1, yielding pSC-CFP. In this plasmid, Na_vBP–CFP is expressed under the control of the native *ncbA* promoter.

The *ecfp* fragment (Fig. 2b), pMWCFP and pCFP were constructed by using a parallel method. In pCFP, CFP is expressed under the control of the native *ncbA* promoter. The inserts of pSC-CFP and pCFP were mutation-free. The plasmids were transformed into *B. pseudofirmus* OF4-SC34 protoplasts (Ito *et al.*, 1997).

Antibody preparation. Rabbit polyclonal antibodies against the abundant McpB of *B. subtilis* (Hanlon & Ordal, 1994; Szurmant & Ordal, 2004) were obtained from G. W. Ordal (University of Illinois). An anti-Na_vBP mouse mAb (L30/34; IgG_{2b} isotype) was generated against a recombinant, bacterially expressed full-length Na_vBP protein. Mice were immunized and hybridomas generated and screened by standard methodology (Bekele-Arcuri *et al.*, 1996; Trimmer *et al.*, 1985). L30/34 hybridomas were grown in roller bottles containing Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). Supernatants were collected and mAbs purified with Protein G Agarose (Amersham Pharmacia) following the manufacturer's protocol.

Immunoblot analysis. *B. pseudofirmus* OF4 wild-type and its derivative mutant strains were grown overnight on MYE medium as described above. Overnight culture (1 ml) was inoculated into 100 ml fresh MYE medium, pH 10, and grown to OD₆₀₀ 0.6. Cells were harvested and washed in TSE buffer (50 mM Tris/HCl, pH 8.0, 10% sucrose, 1 mM EDTA). Cells were suspended in the same buffer and a protease inhibitor cocktail (Sigma-Aldrich) and DNase (50 µg ml⁻¹) were added. Cells were disrupted by sonication and unbroken cells were removed by centrifugation at 9100 g for 15 min

at 4 °C, yielding whole-cell suspension. The membrane fraction was harvested by centrifuging at 40 000 r.p.m. for 90 min in a Beckman 70Ti rotor at 4 °C and suspended in TSE buffer. A protease inhibitor cocktail (Sigma-Aldrich) was added. The protein concentrations of the whole-cell suspension and membrane fraction were measured by the Lowry method (Lowry *et al.*, 1951) with BSA as a standard. The same volume of SDS loading buffer was added to each sample, which was boiled for 3 min at 100 °C, after which the proteins were separated by 12% polyacrylamide SDS gels (Schagger & von Jagow, 1987). The gels were then transferred to nitrocellulose filters (Bio-Rad) electrophoretically overnight in Tris/glycine/methanol buffer (25 mM Tris, 192 mM glycine, 20%, v/v, methanol, pH 8.3).

The following antibodies and reagents were used for detection in immunoblot analyses: for McpB (*B. subtilis*) and McpX (*B. pseudofirmus* OF4), 1:2000 rabbit anti-*B. subtilis* McpB antibody and 1:3000 goat anti-rabbit-horseradish peroxidase (HRP) conjugate (Bio-Rad); and for Na_vBP–CFP and CFP, 1:250 (whole-cell analyses) or 1:1000 (membrane fraction analyses) rabbit anti-GFP polyclonal antibody (Clontech), 1:3000 goat anti-rabbit–HRP (Bio-Rad), and Can Get Signal^M Immunoreaction Enhancer Solution (Toyobo). Detection and analysis of chemiluminescence images were conducted using the quantitative imaging system Fluor-S MAX (Bio-Rad) according to the protocol provided in the manufacturer's instructions (Amersham Biosciences). Na_vBP could not be detected in immunoblot analyses of either whole cells or membranes using the anti-Na_vBP mouse L30/34 mAb (data not shown).

IFM. The method described by Hiraga *et al.* (1998) was adapted for *B. pseudofirmus* OF4 strains. Wild-type and mutant strains were grown as described above for immunoblot analyses. Final culture (1 ml) was

Table 2. Primers used in this study

Extra nucleotides that were added to introduce restriction sites are underlined.

Primer	Sequence (5'–3')	Accession number and corresponding sequence (nt)
del-AW-F1-BH1	<u>CGGCATCCT</u> GGTGAGTTTATCTCGATTGC	DQ150110 (180→200)
del-AW-R1	GTCTAAATCAATTTTATTAAAGTTCATCAAGCTCTTGTTCACCTCAATGG	NC002013 [1286→1260 (minus strand)], DQ150110 [1044→1022 (minus strand)]
del-AW-F-CAT	CCATTGAGGTGAACAAGAGCTTGATGAACCTTAATAAAATTGATTTAGAC	DQ150110 (1022→1044), NC002013 (1260→1286)
del-AW-R-CAT	CCTTTGCTCTATGTATTTATTTCTTTCATTATAAAAGCCAGTCATTAGGCC	DQ150110 [3629→3604 (minus strand)], NC002013 [1911→1888 (minus strand)]
del-AW-F2	GGCCTAATGACTGGCTTTTATAATGAAAGAAATAAATACATAGAGCAAAGG	NC002013 (1888→1911), DQ150110 (3604→3629)
del-AW-R2-ER1	CGGAATTCCTAGTCGTATGATCGTCACCAG	DQ150110 [4481→4461 (minus strand)]
OF4cheA3	TGAGAGCCTAGAAGCAGCAT	DQ150110 (1419→1438)
OF4cheA15	TGACGATGCCGCTAATGATTC	DQ150110 [2674→2653 (minus strand)]
OF4cheA24	CGCGATGTGTATCAAGAGTTCTC	DQ150110 (1→23)
OF4cheA25	CCGGTGAGGGTGACCATATTCATT	DQ150110 [4858→4835 (minus strand)]
NC-F1-SC2	TTT <u>CCGCGGGCCCT</u> TAGGAGGAATTCTGT	AY376071 (8→30)
NC-R1-1	GAGCTCCTCGCCCTTGCTCACCATCCCTTTTGTGTTGCTGTTTATG	*[312→289 (minus strand)], AY376071 [1729→1706 (minus strand)]
NC-F-CFP1	CTGAAACAGCAAACAAAAAAGGGATGGTGAGCAAGGGCGAGGAGCTG	AY376071 (1706→1729), *(289→312)
NC-R-CFP1	CTGTCTTTAGTCAGCTCCTTTTGTACTTGTACAGCTCGTCCATGCC	AY376071 [1756→1733 (minus strand)], *[1008→985 (minus strand)]
NC-F2-1	GGCATGGACGAGCTGTACAAGTAACAAAAAGGAGAAGACTAAAGACAG	*(985→1008), AY376071 (1733→1756)
NC-R2-BH1	GAGGATTCCCGGAGTGGAAGCTGACTTATA	AY376071 [2510→2531 (minus strand)]
CN-R1-1	CAGCTCCTCGCCCTTGCTCACCATTACATAATAACTCCTTCTTGCAT	*[312→289 (minus strand)], AY376071 [876→854 (minus strand)]
CN-F-CFP-1	ATGCAAGAAGGAGTTATTATGTAATGGTGAGCAAGGGCGAGGAGCTG	AY376071 (854→876), *(289→312)

*Sequence data from <http://addgene.org/pgvecl?c&cmd=viewvecseq&vectorid=341>.

Fig. 2. Construction strategy for the *ncbA*-*ecfp* fragment and *ecfp* fragment. The schematic diagram of the *ncbA* locus of the wild-type *B. pseudofirmus* OF4 chromosome indicates the primers used in construction of an *ncbA*-*ecfp* fragment (a) and an *ecfp* fragment (b) by PCR. Na_vBP-CFP (a) and CFP (b) are expressed under the control of the *ncbA* promoter. The detailed protocol is described in Methods.

added to 10 ml 70 % ethanol, mixed gently and left for 1 h at room temperature. The fixed cells were harvested by centrifugation (700 g for 15 min at 4 °C) and suspended in 1 ml 70 % ethanol. A glass slide, S-2215 (Matsunami Glass), was covered by 20 µl poly-L-lysine hydrobromide (1 mg ml⁻¹), left for 5 min, washed with distilled water, and air-dried. The ethanol-fixed cell suspension (10 µl) was then dropped on the poly-L-lysine-coated slide and air-dried for 20 min. The slide was covered with 100 µl lysozyme solution (2 mg ml⁻¹ in 25 mM Tris/HCl, pH 8.0, 50 mM glucose, 10 mM EDTA) and incubated for 5 min at room temperature. The slide was washed with 5 ml PBSTE (140 mM NaCl, 2 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.05 % Tween 20, 10 mM EDTA, pH 8) three times and then incubated with PBSTE-BSA (PBSTE containing 2 % BSA) for 15 min at room temperature.

The slide was treated for 1 h at room temperature with the primary antibody solution (1:1000 mouse anti-Na_vBP L30/34 mAb or 1:2000 rabbit anti-*B. subtilis* McpB polyclonal antibody in PBSTE-BSA), and covered with a cover glass in a shading moisture chamber. The slide was washed with 5 ml PBSTE three times (the cover glass was removed by washing). After the slide had been washed with PBSTE, it was again incubated with PBSTE-BSA for 15 min at room temperature. It was then treated for 1 h at room temperature with a secondary antibody solution [1:1000 Alexa Fluor 488 rabbit anti-mouse IgG (Molecular Probes) for the Na_vBP, and Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes) for the McpX in PBSTE-BSA]. The slide was then covered with a cover glass in a shading moisture chamber and again washed with 5 ml PBSTE three times. After washing with PBSTE, it was covered with a cover glass and sealed with enamel. The microscopic images were obtained by an FW4000 Fluorescence Image Analysis Workstation (Leica Geosystems), and processed with Photoshop CS software (Adobe Systems). In each experiment, over 30 cells were assessed with respect to the subcellular localization of Na_vBP and McpX, and the length of the cells. All the results shown are from a total of three independent experiments. The localization of Na_vBP and McpX was classified by two researchers (S. F. and M. I.) as: A, one pole; B, two poles; C, one pole and side(s); D, two poles and side(s); E, side(s); or diffuse (Tables 3 and 4).

Observation of subcellular localization of Na_vBP-CFP and CFP. *B. pseudofirmus* OF4-SC34 cells ($\Delta ncbA$) were transformed with plasmid pSC-CFP (yielding the SC34/pSC-CFP transformant) or pCFP (yielding the SC34/pCFP transformant). The transformants were grown as described above for immunoblot analyses. Small aliquots of the culture were spotted onto glass slides coated with 0.5 % agarose. Microscopic images were obtained on an FW4000 Fluorescence Image Analysis Workstation. Photoshop CS software was used to manipulate images, e.g. to create overlays, without altering the images or patterns of the cells themselves. In each experiment, over 30 cells were classified with respect to the subcellular localization of Na_vBP-CFP and CFP. All the results shown are from a total of three independent experiments.

Migration assays. Migration in soft agar was assayed by the method described previously (Fujinami *et al.*, 2007). *B. pseudofirmus* OF4 wild-type and its derivative mutant strains were grown in MYE medium overnight and then grown to exponential phase on MYE, pH 10, as described above. Migration assays of *B. pseudofirmus* OF4 wild-type and its derivative strains in soft agar were conducted on plates containing MYE medium (pH 10) (Sturr *et al.*, 1994) and solidified by the addition of 0.3 % Noble agar. Liquid culture (1 µl) was spotted onto the centre of the plate. After incubation of the plates at 37 °C for 10 h, the diameter of the colony was measured. All results shown are the means of three independent experiments.

Assays of tumbling bias. *B. pseudofirmus* OF4 wild-type and its derivative strains were grown as described above. Qualitative

assessment of tumbling bias in liquid MYE medium (pH 10) was carried out by the hanging-drop method using a Leica DMLB100 dark-field microscope ($\times 400$), a Leica DC 300F camera, and Leica IM50 version 1.20 software (Leica Geosystems).

RESULTS

Co-localization of McpX and Na_vBP at cell poles in *B. pseudofirmus* OF4 and delocalization of McpX in the *ncbA* deletion strain

The MCPs of *B. pseudofirmus* OF4, and the genes encoding them, have not yet been characterized, because the genome sequence of this micro-organism has not been determined. We anticipated that antibodies raised against a major MCP from *B. subtilis* could be used to study the localization of an alkaliphile MCP, since it has been reported that antibodies raised against an MCP can be used to detect MCPs of different organisms by immunoblotting and IFM (Gestwicki *et al.*, 2000). Before undertaking IFM, confirmation was sought that anti-*B. subtilis* McpB antibody could detect an MCP of *B. pseudofirmus* OF4 by assessing the heterogeneity of any cross-reacting proteins in the alkaliphile on immunoblot analyses (Fig. 3). The bands in expected positions of McpA and McpB were detected in whole cells of *B. subtilis* 168 (Kirby *et al.*, 2000). The location of the band corresponding to McpB is indicated with an arrow in Fig. 3. In whole cells of wild-type *B. pseudofirmus* OF4 (811M), a single band was detected at almost the same location as McpB of *B. subtilis* 168. We designated this MCP of *B. pseudofirmus* OF4 as McpX. Bands corresponding to McpX were also detected in whole

Fig. 3. Immunoblot analyses of *B. subtilis* 168 McpB and *B. pseudofirmus* OF4 McpX in whole cells. Immunoblot analyses were conducted using antibodies against McpB of *B. subtilis* 168. The strain is indicated above each lane. The locations of bands for McpB in *B. subtilis* 168 and McpX in *B. pseudofirmus* OF4 wild-type and its mutant derivatives SC34, SC34-R and 811M-cheAW are indicated with an arrow.

Fig. 4. IFM analyses of the cellular localization of Na_vBP and McpX in *B. pseudofirmus* OF4. The strains are indicated on the left-hand side of the figure and the image analyses are indicated at the top. (a) Wild-type (811M), SC34, SC34-R and 811M-CheAW; (b) nifedipine-treated wild-type 811M. Columns of images: DIC, differential-interference contrast microscopy; Na_vBP, IFM for Na_vBP (Alexa Fluor 488 rabbit anti-mouse IgG in green); McpX, IFM for McpX (Alexa Fluor 546 goat anti-rabbit IgG in red); Overlay, overlay images of the images in columns Na_vBP and McpX. Bar, 5 μm. Underneath the photographs, cartoons are provided to indicate the interpretation of each image that was used to quantify localization.

strain (Fig. 4). Quantification of the co-localization of Na_vBP and McpX is shown in Table 3 (top rows of each section). ‘Total cells’ designates the total number of cells that were analysed. The subcellular localization of Na_vBP was observed in 92 % of wild-type (811M) cells. In 82 % of 811M cells, Na_vBP was localized at a cell pole, and in 90 % of such cells, polar co-localization of Na_vBP and McpX was observed. In the channel mutant SC34, no localized signal was observed with the antibody against the channel, as expected, except for a small signal that reflects the difficulty of setting the parameters ‘counted’ as fluorescence with complete precision. Unexpectedly, the polar localization of McpX was also significantly decreased in the channel mutant, from 78 % in the wild-type to 15 % in SC34 (Fig. 4, Table 3, second row of each section). A channel mutant with a restored *ncbA* gene at the native location (SC34-R) exhibited fluorescence with 76 % polar localization, 89 % of which was co-localized with McpX. McpX fluorescence in the SC34-R strain that was localized at the poles rose from 15 % in SC34 to 71 % in the SC34-R strain and 96 % of that was co-localized with Na_vBP (Fig. 4, Table 3, third row of each section). Measurements of 50 cells of each strain showed that there were no significant differences in the cell length or width of the different strains. The mean cell lengths (μm) of 811M, SC34, SC34-R, 811M-AW, SC34/pSC-CFP and SC34/pCFP were 1.95 ± 0.44 , 1.65 ± 0.36 , 2.43 ± 0.55 , 2.39 ± 0.63 , 2.25 ± 0.45 and 2.13 ± 0.55 , respectively. The mean cell widths (μm) of 811M, SC34, SC34-R, 811M-AW, SC34/pSC-CFP and SC34/pCFP were 0.82 ± 0.05 , 0.82 ± 0.05 , 0.84 ± 0.05 , 0.84 ± 0.06 , 0.84 ± 0.05 and 0.85 ± 0.04 , respectively.

The finding that mutational loss of Na_vBP led to delocalization of McpX raised the possibility that the inverse chemotaxis phenotype observed in the *ΔncbA* strain could result entirely from secondary delocalization of MCPs rather than from a channel function of Na_vBP itself. Evidence against this line of reasoning was the earlier observation that the Na_vBP channel inhibitor nifedipine caused inverse chemotaxis behaviour of wild-type *B. pseudofirmus* OF4 toward the chemoattractant aspartate when the inhibitor was added (at 50 μM) to the chemotaxis assay buffer (Ito *et al.*, 2004b). IFM experiments were conducted to test whether comparable exposure of the wild-type strain to nifedipine caused

cells of the channel mutant SC34, and in a channel mutant strain (SC34-R) to which *ncbA* had been restored to the chromosome (Fig. 3). The expression level of McpX was similar in the three alkaliophile strains.

IFM of Na_vBP and McpX was then conducted in *B. pseudofirmus* OF4 wild-type and its mutant derivatives to assess the localization of these proteins. Both McpX and Na_vBP localized extensively to cell poles of the wild-type

Table 3. Quantification of the co-localization of Na_vBP and McpX

Strain	Subcellular localization of Na _v BP (%)							Total fluorescent cells (%)	Total cells
	A, one pole	B, two poles	C, one pole and side(s)	D, two poles and side(s)	E, side(s)	A + B + C + D polar localization	A + B + C + D + E total

811M (wild-type)	28 (86)*	16	20	12	9.8	82 (90)†	92	100	153
SC34 ($\Delta ncbA$)	1.2 (50)*	0.63	0	0	2.5	1.9 (67)†	4.4	100	158
SC34-R ($\Delta ncbA$, <i>ncbA</i> restored)	52 (89)*	14	7.1	2.1	13	76 (89)†	89	100	141
811M-cheAW (<i>cheAW</i> disrupted)	25 (49)*	2.0	6.7	0	16	33 (50)†	49	100	150

Strain	Subcellular localization of McpX (%)							Total fluorescent cells (%)	Total cells
	A, one pole	B, two poles	C, one pole and side(s)	D, two poles and side(s)	E, side(s)	A + B + C + D polar localization	A + B + C + D + E total

811M (wild-type)	38 (91)‡	17	12	14	15	78 (94)§	93	100	153
SC34 ($\Delta ncbA$)	10 (6.3)‡	3.2	1.9	0	8.9	15 (4.2)§	24	100	158
SC34-R ($\Delta ncbA$, <i>ncbA</i> restored)	49 (96)‡	13	5.0	2.1	13	71 (96)§	84	100	141
811M-cheAW (<i>cheAW</i> disrupted)	23 (54)‡	4.0	5.3	0	17	33 (51)§	49	100	150

*The number in parentheses is the percentage of the cells with Na_vBP localized at one pole that also had McpX localized at one pole.

†The number in parentheses is the percentage of the cells with Na_vBP localized to at least one pole (A + B + C + D) that also had McpX co-localized to at least one pole.

‡The number in parentheses is the percentage of the cells with McpX localized at one pole that also had Na_vBP localized at one pole; this number correlated well with overlay measurements.

§The number in parentheses is the percentage of the cells with McpX localized at at least one pole (A + B + C + D) that also had Na_vBP co-localized to at least one pole.

delocalization of Na_vBP or McpX. These experiments revealed no such delocalization (Fig. 4b).

Na_vBP-CFP expressed from a plasmid localizes at cell poles in the *ncbA* deletion strain

Transformants of *B. pseudofirmus* OF4-SC34 ($\Delta ncbA$) cells with plasmid pSC-CFP (SC34/pSC-CFP), in which CFP is fused to the channel, or control pCFP (SC34/pCFP) were grown in MYE, pH 10, and subjected to immunoblot analyses using antibodies against GFP. As shown in Fig. 5, bands corresponding to the expected location for Na_vBP-CFP fusion were detected in whole-cell and membrane fractions of the SC34/pSC-CFP transformant (white arrows). The band for CFP was detected in whole cells of SC34/pSC-CFP but not in membrane fractions (filled arrows). Anti-Na_vBP mouse mAb was also used for this detection. However, the antibody was not able to detect denatured Na_vBP for immunoblot analyses. This mAb can probably recognize an antigen only when Na_vBP maintains 3D structure.

The subcellular localization of Na_vBP-CFP of SC34/pSC-CFP and CFP of SC34/pCFP was observed by fluorescence

microscopy (Fig. 6). Na_vBP-CFP was localized at cell poles in SC34/pSC-CFP (Fig. 6a), and CFP was not localized in SC34/pCFP (Fig. 6b). Polar localization of Na_vBP-CFP was observed in 88 % of SC34/pSC-CFP cells (Table 4).

Polar localization of both McpX and Na_vBP was decreased upon disruption of *cheAW* in the wild-type strain

The alkaliphile gene encoding the MCP monitored in the IFM experiments has not yet been identified. It was therefore impossible to disrupt this gene and examine whether the disruption caused Na_vBP delocalization. However, it was possible to test whether the delocalization of MCPs that results from mutational loss of CheAW would lead to Na_vBP localization. We therefore introduced a *cheAW* disruption into the wild-type strain, producing strain 811M-cheAW. Immunoblot assays showed that the mutation in 811M-cheAW did not affect the expression level of McpX (Fig. 3). IFM studies of the subcellular localization of McpX and Na_vBP showed a decrease in the polar localization of both McpX and Na_vBP in 811M-cheAW in comparison with the wild-type parent (Fig. 4a, Table 3, fourth rows of both sections). The polar localization

Fig. 5. Immunoblot analyses of Na_vBP-CFP and CFP in whole cells and membrane fractions of *B. pseudofirmus* OF4 wild-type and its mutant derivatives. (a) Whole cells; (b) membrane fractions. Immunoblot analyses were conducted using antibodies against GFP. The strain is indicated above each lane. Bands for the Na_vBP-CFP fusion and CFP are indicated with white arrows and a filled arrow, respectively.

of McpX decreased from 78 % in the wild-type to 33 % in 811M-cheAW, with only 51 % co-localized with Na_vBP in the mutant. The polar localization of Na_vBP decreased from 82 % in the wild-type to 33 % in 811M-cheAW, with only 50 % co-localized with McpX in the mutant.

Migration assays and tumbling bias assessments of *B. pseudofirmus* OF4 strains with different Na_vBP status

Migration assays in soft agar and observations of the tumbling bias in liquid medium were conducted on *B. pseudofirmus* OF4 wild-type and its derivative mutant strains to ascertain whether the effects of *ncbA* status on

Fig. 6. Subcellular localization of Na_vBP-CFP and CFP in the *ncbA* deletion strain. The strains and plasmids are indicated to the left of each panel. (a) SC34/pSC-CFP; (b) SC34/pCFP (control). The imaging analyses are indicated at the top. Left-hand images, differential-interference contrast microscopy (DIC); right-hand images, fluorescence microscopy monitoring Na_vBP-CFP or CFP. Bar, 5 μm. To the right of the photographs are cartoons indicating the interpretation of each image that was used in assessment of localization.

polar localization of McpX correlated with swarming capability in soft agar and tumbliness (Fig. 7, Table 5). As shown in earlier studies of an up-motile alkaliphile variant and its mutant derivative (Ito *et al.*, 2004b), deletion of *ncbA* (in SC34) from the wild-type strain led to a smaller colony diameter and a more tumbling phenotype relative to wild-type. Restoration of *ncbA* (in SC34-R)

Table 4. Quantification of the subcellular localization of Na_vBP-CFP and CFP

Strain	Subcellular localization of Na _v BP-CFP (%)							Total fluorescent cells (%)	Total cells
	A, one pole	B, two poles	C, one pole and side(s)	D, two poles and side(s)	E, side(s)	A+B+C+D polar localization	A+B+C+D+E total		
SC34/pSC-CFP	55	15	16	2.1	7.2	88	95	100	97
Strain	Subcellular localization of CFP (%)							Total fluorescent cells (%)	Total cells
	A, one pole	B, two poles	C, one pole and side(s)	D, two poles and side(s)	E, side(s)	A+B+C+D polar localization	A+B+C+D+E total		
SC34/pCFP	0	0	0	0	0	0	0	100	99

Fig. 7. Migration assay of *B. pseudofirmus* OF4 wild-type and its derivative strains in soft agar. The diameter of each strain was measured after incubation of the plates at 37 °C for 10 h on MYE medium, pH 10, solidified with 0.3% agar. The details are described in Methods.

restored wild-type motility on soft agar and reduced the tumbliness. Transformation of the channel mutant SC34 with a multicopy plasmid encoding *ncbA-ecfp* (SC34/pSC-CFP) led to almost wild-type motility and tumbliness. Control plasmid pCFP, encoding ECFP, did not restore motility or reverse the tumbling phenotype of strain SC34.

DISCUSSION

Na_VBP had previously been shown to have a role in pH homeostasis, motility and chemotaxis in *B. pseudofirmus* OF4 (Ito *et al.*, 2004b). The role in pH homeostasis was presumed to reflect a contribution of Na_VBP to entry of Na^+ in support of the Na^+/H^+ antiport that maintains pH homeostasis during alkalinization of the medium, e.g. during a pH 8.5→10.5 shift of the outside pH. The upward pH change would potentiate opening of the channel (Ito *et al.*, 2004b). The role of Na_VBP observed in the pH-shift experiments was especially notable in the presence of low $[\text{Na}^+]$ and when the medium contained low concentrations of solutes that enter the cell together with Na^+ . No deficit was observed in the growth yield of the channel mutant at pH 10.5 in MYE medium containing optimal $[\text{Na}^+]$ and solutes that are co-transported with Na^+ , so no role for

Na_VBP in pH homeostasis under optimal conditions was indicated in the earlier studies. Here, we found that the channel mutant SC34 exhibited a defect in growth to the mid-exponential phase in the Na^+ -replete, malate-containing medium at pH 10.5. These results indicated that Na_VBP does have a role in alkaline pH homeostasis, even when adequate Na^+ and solutes are present. This raises the possibility that the lag in motility in the channel mutant is a result of suboptimal pH homeostasis, since the pH range for optimal swimming of *B. pseudofirmus* OF4 is below the highest values for optimal non-fermentative growth (Fujinami *et al.*, 2007). However, the role of Na_VBP in pH homeostasis cannot explain the dramatic effect of channel loss on chemotaxis. Most of the original assays of chemotaxis were conducted at pH 8.5, at which the pronounced inverse chemotaxis phenotype was consistently observed in the mutant, although no pH homeostasis problem would exist (Ito *et al.*, 2004b). Rather, we hypothesize that the effect of Na_VBP status on the chemotaxis behaviour of *B. pseudofirmus* OF4 is related to the interaction between Na_VBP and chemotaxis proteins.

In summary, the major findings on the localization of alkaliphile Na_VBP and an MCP that emerge from this work are as follows. (i) The putative alkaliphile MCP designated McpX localized at cell poles, as anticipated from similar findings in other systems (Kirby *et al.*, 2000). Some McpX appeared to localize at the sides of the cell (Fig. 4, Table 3). MCPs are translocated by the helically distributed Sec machinery (Shiomi *et al.*, 2006). It is possible that the experiments captured McpX that was in transit or that was localized to a central region destined to be a new pole. (ii) Na_VBP also exhibited localization to the cell poles, as shown by both IFM (Fig. 4, Table 3) and fluorescence microscopy on cells expressing CFP fused to the channel (Fig. 6, Table 4). The expression of the fused channel restored wild-type motility properties, as assessed in soft agar plates, and reversed tumbling properties, as assessed qualitatively by microscopic examination (Table 5). (iii) A significant percentage of the polarly localized McpX and Na_VBP exhibited co-localization (Fig. 4, Table 3). On the other hand, the pattern of Na_VBP localization did not correlate well with the pattern of peritrichous flagellum

Table 5. Swarming assay and tumbling bias of *B. pseudofirmus* OF4 strains

Strain	Colony diameter (mm)*	Tumbling†
811M (wild-type)	17.5 ± 2.0	±
SC34 ($\Delta ncbA$)	11.8 ± 1.0	++
SC34/pSC-CFP	15.8 ± 1.9	±
SC34/pCFP	12.0 ± 0.5	++
SC34-R ($\Delta ncbA$, <i>ncbA</i> restored)	17.0 ± 1.8	+

*Measured after 10 h on MYE medium, pH 10, solidified with 0.3% agar. Mean values of three independent experiments with SDs of the mean are shown.

†Qualitative assessment of tumbling bias in liquid MYE medium (pH 10) by the hanging-drop method using a dark-field microscope.

location in wild-type *B. pseudofirmus* OF4. The wild-type strain typically has one to two flagella per cell, and these are located at various locations around the cell body (Fujinami *et al.*, 2007). (iv) The expression level of McpX was not markedly changed in the *ncbA* mutant that lacks Na_vBP, but McpX was significantly delocalized. This effect was reversed appreciably in a strain in which *ncbA* was restored to the chromosome (Fig. 4, Table 3). (v) Significant delocalization of both Na_vBP and McpX was observed when *cheAW* genes of the wild-type were disrupted (Table 3). This delocalization was not as complete as in *E. coli*, in which CheAW has been shown to form a complex with MCPs that is required for polar localization as well as clustering (Irieda *et al.*, 2006; Kentner *et al.*, 2006). Perhaps association of Na_vBP and McpX is sufficient to allow a low level of polar co-localization in *B. pseudofirmus* OF4.

The finding that mutational loss of Na_vBP leads to delocalization of McpX raises the possibility that such delocalization is in part responsible for the altered chemotactic responses, rather than loss of channel function itself being completely responsible. However, the functional capacity of Na_vBP is also essential for normal chemotaxis. Nifedipine addition to chemotaxis assay buffers results in inverse chemotaxis (Ito *et al.*, 2004b), but does not result in delocalization of Na_vBP and McpX. This supports the possibility that Na_vBP-mediated fluctuations in membrane potential play a role in the signalling pathway for chemotaxis of *B. pseudofirmus* OF4, as suggested for not yet identified channels in earlier studies of chemotaxis in *Spirochaeta aurantia* (Goulbourne & Greenberg, 1983) and *E. coli* (Tisa *et al.*, 1993, 2000). The co-localization of Na_vBP and MCPs further raises the possibility that modulation of Na_vBP function occurs via dynamic changes in Na_vBP phosphorylation, as mediated by chemoreceptor activation of the CheA kinase and CheZ phosphatase system, which are presumably also present at the cell poles (Baker *et al.*, 2006). This could lead to changes in Na_vBP gating, as occurs in many eukaryotic voltage-gated ion channels (Levitan, 1999). The cytoplasmic C terminus of Na_vBP contains four aspartate residues that could serve as phosphoacceptors for the CheA kinase; changes in C-terminal phosphorylation of eukaryotic channels can profoundly affect channel gating (Park *et al.*, 2006). Further work will be needed to define the nature of MCP and channel interactions in the alkaliphile, and how such interactions affect channel function and chemotaxis. Finally, these studies raise the question of whether, as inferred by the earlier investigators cited above, voltage-gated ion channels are involved in signalling events or other features of bacterial chemotaxis in bacteria beyond a subset of extremophiles.

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