A Superfamily of Voltage-Gated Sodium Channels in Bacteria

Ryuta Koishi*, Haoxing Xu*, Dejian Ren$, Betsy Navarro,
Benjamin W. Spiller#, Qing Shi, and David E. Clapham$

Howard Hughes Medical Institute, Department of Cardiovascular Research, Children’s Hospital and Department of Neurobiology Harvard Medical School, 320 Longwood Ave. Boston, Massachusetts 02115, #Children’s Hospital, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 200 Longwood Ave. Boston, Massachusetts 02115

$Present address: 511 Goddard Laboratories, Department of Biology, University of Pennsylvania Philadelphia, PA 19104 USA

Running Title: Novel Prokaryotic Ion Channels

*R. K. and H. X. contributed equally to this work.

$To whom correspondence should be addressed.
E-mail: dclapham@enders.tch.harvard.edu

Fax: 617-731-0787

Phone: 617-355-6163
Summary

NaChBac, a six $\alpha$ helical transmembrane-spanning (6TM) protein cloned from *Bacillus halodurans*, is the first functionally characterized bacterial voltage-gated Na$^+$-selective channel [Ren et al. 2001, *Science* 294, 2372-2375]. As a highly expressing ion channel protein, NaChBac is an ideal candidate for high-resolution structural determination and structure-function studies. The biological role of NaChBac, however, is still unknown.

In this report, another 11 structurally-related bacterial proteins are described. Two of these functionally expressed as voltage-dependent Na$^+$ channels (NavPZ from *Paracoccus zeaxanthinifaciens* and NavSP from *Silicibacter pomeroyi*). NavPZ and NavSP share ~40% amino acid sequence identity with NaChBac. When expressed in mammalian cell lines, both NavPZ and NavSP were Na$^+$-selective and voltage-dependent. However, their kinetics and voltage dependence differ significantly. These single 6TM subunits constitute a widely distributed superfamily (NavBac) of channels in bacteria, implying a fundamental prokaryotic function. The degree of sequence homology (22% to 54%) is optimal for future comparisons of NavBac structure and function of similarity and dissimilarity among NavBac proteins. Thus the NavBac
superfamily is fertile ground for crystallographic, electrophysiological, and microbiological studies.
Introduction

Mammalian voltage-gated sodium (Nav) and calcium (Cav) channels underlie membrane excitability, muscle contraction and hormone secretion (1). In contrast, the function of prokaryotic voltage-gated ion-selective channels is relatively unknown. Na+ channels may drive Na+ -dependent flagellar motors in certain marine and alkaliphilic species (2-6). In marine vibrio, PomAB and MotXY have been proposed to form a functional Na+ channel (5, 6), but the conductance has not been directly measured. In alkaliphilic bacteria, the prokaryotic ion channel responsible has not been identified. A bacterial 6TM channel subunit NaChBac, was expressed in CHO cells as a functional voltage-gated Na+ channel (7), but its role in bacteria is still being elucidated.

The pore-forming subunits (α1) of mammalian Nav and Cav are composed of four similar repeats of 6TM domains (8, 9), probably arising by gene duplication of a single 6TM gene (1, 10). The first bacterial voltage-gated Na+ channel (NaChBac) functionally expressed in mammalian cells was cloned from Bacillus halodurans (7). It contains a single 6TM domain of 274 amino acids, but almost certainly forms a tetramer (7). NaChBac’s voltage-dependent activation and inactivation kinetics are 10-100 times
The Abbreviations used are: Na\textsubscript{V}, Voltage-Gated Sodium Channel; Ca\textsubscript{V}, Voltage-Gated Calcium Channel; TM, transmembrane-spanning; Na\textsubscript{V} Bac, Bacterial Voltage-Gated Sodium Channel; Na\textsubscript{V} PZ, Na\textsubscript{V} Bac from \textit{Paracoccus zeaxanthinifaciens}; Na\textsubscript{V} SP, Na\textsubscript{V} Bac from \textit{Silicibacter pomeroyi}; eGFP, enhanced green fluorescence protein; NMDG, N-methyl-D-glucamine.

slower than that of Na\textsubscript{V} (7). NaChBac inactivation may result from pore inactivation (C-type inactivation) since it does not contain an obvious cytoplasmic inactivation gate (7).

Since single 6TM Na\textsuperscript{+}-selective ion channels do not appear to be present in vertebrates, the 24TM structure of Na\textsubscript{V}s may have arisen under evolutionary selective pressure. It has been proposed that highly Na\textsuperscript{+}-selective Na\textsubscript{V} channels require pore asymmetry, which is achieved by the concatenated 4x6TM structure (11, 12). However, tetramers of identical 6TM NaChBac are equally Na\textsuperscript{+}-selective as Na\textsubscript{V}s (7). Therefore, it seems more likely that the concatenated 4x6TM structure of vertebrate Na\textsubscript{V}s provided an evolutionary advantage by increasing the speed of activation, inactivation, or recovery from inactivation. To begin to address these questions we searched for other bacterial ion
channel subunits that might reveal the range of the gating speeds provided by the simplest tetrameric Na\(^+\)-selective channels. This information will also lay the groundwork for understanding their function in prokaryotes.

**Experimental Procedures**

**Cloning of Na\(\alpha\)PZ.**

NaChBac protein sequence (GenBank accession number NP_242367) was used for a BLASTP search. The nucleotide sequence of Na\(\alpha\)PZ was obtained from the published genomic sequence of CcaA protein of *Paracoccus zeaxanthinifaciens* (CAD24429). We synthesized DNA of Na\(\alpha\)PZ using following oligos:

5’-
TTACCATGGTAATGAGCCTGCGCGCGCCTGCACGCCCTTGTCCACGCCGC
GTCGTGCCCAGGGGGTGTACACCCGGCGTCATCTGTTCTCAA-3’ (1F),
5’-GTCCGCTGATCTGCTGACGGGCGGACCTGCTGCGCAGGGCGTGCGGC
AGATCGCGGCAAGTATCGATCCCGCGGGCCCGCTCTTCT-3’ (2F),
5’-ATGCCGGCGGGCGGCCTTCGTGGCTGTCGCGCGCGCCTGCATCCTGC
TCTGCTGCTGTCTGTGCCGTCACCCCAGGCCTGCAGCCG -3’ (3F),
5’-GCTGATGCTGGCTGATCTTCTACATCTTCTCCTGTCATTCGACAGAAGCTGGTT
CGGCGGCGGGGTCTCCCGGACTGTTGTTCGTCCTGCTTGGCAA -3’ (4F),
5’-GGATCGTGCGTCGGTCATGCAGGAATATCCGCTGGCATGGCTGTTCTTCG
TGCGCTTCATCTCATGACTGCCTTGCGGATGACCT -3’ (5F),
5’-GAAAGCGCCGCCACCCGACGCCCTACAGGTGCTGATGCGCTGCG
CGGATCGGAAGACGGCTGCGAAGGAACCCGGGCGGCTGCG
CCAGCCCCAGCCAGGACGCGGCTGGAACAGGATGACCCGGG -3’ (1R),
5’-AGGCCCTGCCCCGCCGGCATCAGCGCGATGGCCACGACGCTGAAATCGAA
GACGTTCCAGCCGTCGCGGAAGAAGCGCGGGCCGCGCGCGC -3’ (2R),
5’-AGAAGATCACGCCCATCAGCAGGAAGACCGAGGCCATGCCCGGCATCGC
GGCGAACAGCCCCCTGACCACCACAGCGGCGCAGGCGCGGGGTGA -3’ (3R),
5’-CATGACCGGACGCACGATCCCCATCGACCAGCTTTCCAGCGTCATCACCT
GGAACACGGAATAGGCCGACTTGGCAAGCCGAGCGGACCA -3’ (4R),
5’-GCGTCGGTGGCCGCGCTTTTCTCGGCCTGGCGCATCGTCATCAGTTC
ACGATCGACCGACGACAAGGTTCATCACCCGGAAGGTC -3’ (5R), and
5-TTACTCGAGAGAACCGCGTGGCACCAGGACACGCCCAGCCACGAGCCGAACCA -3’ (6R).

After mixing 1µM each of 2F, 3F, 1R and 2R as a template, the first PCR was conducted using 1F and 3R as primers (10µM each). Similarly, 4R, 5F, 5R and 6F were mixed and PCR was conducted using 4F and 6R as primers. These PCR products were excised from agarose gels. Purified fragments were mixed and used as templates for further PCR, using 1F and 6R as primers. The amplified fragment was digested with NcoI and XhoI and purified from an agarose gel. The PCR product was then cloned into pTrcHis2B (Invitrogen). This synthesized DNA contained additional methionine and valine codons prior to the first methionine as required by the cloning strategy. We also intentionally introduced ten silent mutations (45G→T, 321G→T, 330G→T, 564C→G, 606C→G, 624A→C, 775A→C, 777G→T and 783G→T) into the synthetic DNA. Additional silent mutation (495T→C) occurred during the PCR process. Finally, this plasmid DNA was digested with SalI and XhoI and then self-ligated after the linker sequence was deleted.

The resultant expression clone was used to transform E. coli BL-21 (Stratagene).
Na\textsubscript{v}PZ was cloned into a modified pTracer-CMV2 vector (Invitrogen) containing enhanced green fluorescent protein (eGFP) for expression in mammalian cells. Briefly, DNA was amplified by PCR from the \textit{E. coli} expression clone using the following primers: 

5\textsuperscript{'}-AATGGATCCATGAGCCTGCGCGCGC-3\textsuperscript{'} (containing a \textit{BamHI} site) and 5\textsuperscript{'}-ATTGAATTCTCAGACACGCCCACGGCCGCC-3\textsuperscript{'} (containing an \textit{EcoRI} site).

The PCR product was then cloned into the modified pTracer-CMV2 plasmid (Invitrogen) between the single restriction sites for \textit{BamHI} and \textit{EcoRI}. All clones were confirmed by DNA sequencing.

Cloning of Na\textsubscript{v}SP.

The NaChBac DNA sequence (NC\_002570) was used for a TBLASTX search against the Microbial Genomic database at NCBI. A sample of \textit{Silicibacter pomeroyi} was obtained from American Type Culture Collection (#700808). \textit{Silicibacter pomeroyi} genomic DNA was collected by standard procedures (13). The genomic sequence homologous to NaChBac was identified and the preliminary sequence data was obtained from The Institute for Genomic Research (TIGR) website at http://www.tigr.org.

Na\textsubscript{v}SP was cloned into a pTrcHis2A (Invitrogen) for expression in \textit{E. coli}. Briefly, DNA was amplified by PCR from a \textit{Silicibacter pomeroyi} genomic DNA using the
following primers: 5’-AATCCATGGTAATGCAAAGAATGCAGGCCTTT-3’  
(containing an NcoI site) and 5’-  
ATTCTCGAGAGAACCGCGTGGCACCAGGTTTTGGTTTCACCAAG-3’  
(containing a thrombin recognition site and an XhoI site). The PCR product was cloned into the pTrcHis2A plasmid using NcoI and XhoI sites. This cloned DNA contains additional methionine and valine codons prior to the first methionine as required by the cloning strategy. The resulting expression clone was used to transform E. coli BL-21.

NavSP was cloned into a modified pTracer-CMV2 vector containing enhanced green fluorescent protein (eGFP) for expression in mammalian cells. Briefly, DNA was amplified by PCR from a Silicibacter pomeroyi genomic DNA using the following primers:

5’-AATGGATCCATGCAAAGAATGCAGGCCTTT-3’ (containing a BamHI site) and 5’-ATTGAATTCTCATTTCACCTTGGTTTCACCAAG-3’ (containing an EcoRI site). The PCR product was cloned into the modified pTracer-CMV2 plasmid between the single restriction sites for BamHI and EcoRI. All clones were confirmed by DNA sequencing.

*Information related to the cloning of other NaChBac homologs are provided as supplementary data.*
Expression and Purification of Recombinant Proteins.

100-300ml LB medium containing ampicillin (50µg/ml) was inoculated from glycerol stocks and grown overnight at 30°C. 40 ml culture medium was inoculated into 2 liters of Terrific Broth medium (Invitrogen) containing ampicillin and grown at 37°C to A600 = 1.2. Cells were induced with 1mM 1-β-D-thiogalactopyranoside and grown at 37°C for 3h. Cells were then suspended in PBS buffer (pH 8.0) containing protease inhibitors (Protease Inhibitor Cocktail, Sigma) and lysed by sonication. The carboxyl-terminal histidine-tagged protein was extracted by homogenization and solubilization in 15mM n-Undecyl-β-D-Thiomaltopyranoside (Anatrace). Following centrifugation, the supernatant was loaded onto a Talon Co2+ affinity column (CloneTech). Resin was washed with 20mM imidazole and the protein was then eluted in the presence of 400mM imidazole. Purified protein was resolved by 4-12% SDS-PAGE (Invitrogen) and stained with Coomassie Blue. Molecular weight marker was purchased from Invitrogen (BenchMark™ Prestained Protein Ladder).

Mammalian Electrophysiology.

NaγPZ and NaγSP, as well as other NaChBac homologues were subcloned into an enhanced green fluorescence protein (eGFP)-containing pTracer-CMV2 vector.
(Invitrogen) for expression into CHO-K1 and HEK293T cells. CHO-K1 and HEK293T cells were grown in Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum at 37°C under 5% CO2. DNA was transfected using LipofectamineTM2000 (Invitrogen), plated onto coverslips, and recordings were made after 12h (Na\textsubscript{V}PZ) or 48h (Na\textsubscript{V}SP), respectively. Unless otherwise stated, the pipette solution contained; 147mM Cs\textsuperscript{+}, 120mM methane-sulfonate, 8mM NaCl, 10mM EGTA, 2mM Mg-ATP, and 20mM HEPES (pH 7.4). Bath solution contained; 140mM NaCl, 2mM CaCl\textsubscript{2}, 1mM MgCl\textsubscript{2}, 5mM KCl, 20mM HEPES (pH 7.4) and 10mM glucose. All experiments were conducted at 22°C±2°C. Unless otherwise indicated, all chemicals were dissolved in water. Nifedipine (dissolved in DMSO) was purchased from Sigma. As reported previously (7), unknown agents, presumably leached from the perfusion tubing, caused fast inactivation and these perfusion systems were subsequently avoided.

**Results**

Using the whole or partial sequence of NaChBac as the query, we performed standard BLASTP or TBLASTX search on the GenBank databases from various prokaryotic genomic sequencing projects. Several open reading frames (ORF) with significant
sequence homology (22% to 54%) to NaChBac were identified in the following species (Fig. 1A): Vibrio vulnificus (22%), Microbulbifer degradans (two genes, 32% and 33%, respectively), Colwellia psychrerythraea (two genes, 35% and 38%, respectively), Magnetococcus sp (32%), Silicibacter pomeroyi (39%), Paracoccus zeaxanthinifaciens (39%), Hyphomonas neptunium (33%), Thermobifida fusca (30%) and Oceanobacillus iheyensis (54%). Among these species, Vibrio vulnificus (14), Microbulbifer degradans (15), Colwellia psychrerythraea (16), Magnetococcus sp. (17), Silicibacter pomeroyi (18), Paracoccus zeaxanthinifaciens (19) and Hyphomonas neptunium (20) were isolated from sea water or water. Thermobifida fusca (21), a thermophilic gram-positive bacteria was isolated from soil, but grows optimally in alkaliphilic conditions. Oceanobacillus iheyensis (22) and Bacillus halodurans (23) are alkaliphilic gram-positive bacteria isolated from deep sea water. Based on the degree of sequence homology, we consider these proteins to be NaChBac homologs (rather than orthologs). Hydrophobicity analysis of these proteins predicted that all have the 6TM architecture. Importantly, threonine (T), glutamate (E), and tryptophan (W) residues are conserved in the pore region in all proteins (Fig. 1B). These residues have been shown to be critical for the cationic selectivity of NaChBac (24). As is characteristic for voltage-gated channels (25), positively charged amino acids (R) are interspersed every three amino acids in the fourth putative transmembrane region (S4) (Fig. 1C). In Vibrio vulnificus M06, however, the third arginine was not conserved. Based on the sequence homology and the structural
similarity to NaChBac, it is likely that these proteins function as voltage-gated channels.

We cloned all 11 sequences (see Methods) and studied them by expression in mammalian cell lines. As shown below, we were able to measure currents produced by two NaChBac homologs, Na\(\gamma\)PZ (Fig. 2A) from *Paracoccus zeaxanthinifaciens* (a zeaxanthin-producing marine bacteria (19)), and Na\(\gamma\)SP (Fig. 2A) from *Silicibacter pomeroyi* (a dimethylsulfiniopropionate-degrading marine bacteria (18)). Isolation and sequencing of the gene encoding Na\(\gamma\)PZ revealed an open reading frame (ORF) of 262 amino acids with a predicted molecular size of 29kDa. Similarly, the Na\(\gamma\)SP gene encoded an ORF of 258 amino acids with a predicted molecular weight of 29kDa. Na\(\gamma\)PZ and Na\(\gamma\)SP share 39% identity (60% similarity) and 39% identity (59% similarity) with NaChBac, respectively (Fig. 2B). Notably, Na\(\gamma\)PZ is 65% identical (77% similar) to Na\(\gamma\)SP. On electrophoresis, both Na\(\gamma\)PZ and Na\(\gamma\)SP proteins migrated as a single band (~31kDa, Fig. 2C), almost identical to the predicted molecular sizes of the His-tagged constructs.

CHO-K1 or HEK293T cell lines were transfected with NaChBac homologs (in pTracer) and whole-cell currents recorded 12-48h after transfection (see Methods). Among 11
NaChBac homologs, only two (Na\textsubscript{V} PZ and Na\textsubscript{V} SP) produced detectable currents.

Similar current are not present in non-transfected or mock-transfected cells (data not shown). Na\textsubscript{V} PZ-transfected cells exhibited large (up to 10,000pA; 10nA) voltage-activated inward currents (Fig 3A-C). Na\textsubscript{V} PZ-mediated current (\(I_{Na\text{V} PZ}\)) activated with a time constant (\(\tau_{activation}\)) of 21.5 ± 1.3ms at +10mV (n = 19), significantly slower than both mammalian Na\textsubscript{V} channels (\(\tau_{activation}<2\text{ms}\)) and \(I_{NaChBac}\) (\(\tau_{activation}<13\text{ms}\)).

Inactivation of \(I_{Na\text{V} PZ}\) was slow (\(\tau_{inactivation}=102±4.2\text{ms at }+10\text{mV, } n =19\)) compared to the typically fast-inactivating Na\textsubscript{V} currents (\(\tau_{inactivation}<10\text{ms}\)), but faster than \(I_{NaChBac}\) (\(\tau_{inactivation}>160\text{ms}\)).

Cation replacement by NMDG (bath) resulted in complete removal of voltage-dependent \(I_{Na\text{V} PZ}\) inward current (Fig. 3C). Similarly, no significant inward current was seen in isotonic [Ca\textsuperscript{2+}]\textsubscript{o} (monovalent cations replaced with 105mM Ca\textsuperscript{2+}, Fig. 3B, C). \(I_{Na\text{V} PZ}\) reversed at +75mV (Fig 3C), close to the Nernst potential of Na\textsuperscript{+} under our recording conditions (\(E_{Na}=+72\text{mV}\)). These results, together with the large leftward shift of the reversal by external Na\textsuperscript{+} removal (NMDG\textsuperscript{+} and isotonic Ca\textsuperscript{2+} solution substitution), suggested that Na\textsubscript{V} PZ, like NaChBac, is a Na\textsuperscript{+}-selective channel. We assumed that the
outward currents in 0mM [Na\(^+\)]\(_o\) (Fig. 3B) were carried by internal Na\(^+\) (8mM [Na\(^+\)]\(_i\)).

Due to the very negative E\(_{rev}\) (Fig. 3C), sizable outward currents were observed at most voltages tested (Fig. 3B, C).

We evaluated the voltage-dependent activation of I\(_{Na, PZ}\) by measuring deactivation tail currents (Fig. 4A). A Boltzmann fit of the averaged activation curve yielded a V\(_{1/2}\) of \(-9.5\pm0.8\)mV (n=9) and slope factor (\(\kappa\)) of \(10.7\pm0.7\)mV per e-fold change in current (Fig. 4C). Steady-state inactivation of the channel was determined by sequential depolarization to test voltages followed by voltage clamp to the peak of activation at +10mV (Fig. 4B). Steady-state inactivation was a steep function of voltage, with 50% inactivation at \(-35\pm0.4\)mV (n=10) and slope factor (\(\kappa\)) of \(6.3\pm0.3\)mV/e-fold (Fig. 4C).

We investigated the time course of I\(_{Na, PZ}\) inactivation at \(-30\)mV, where activation was minimal. The degree and speed of inactivation was strongly dependent on the duration of the inactivating pre-pulse (-30mV; \(\tau=2123\pm434\) ms; n=6, Fig. 4D,E). I\(_{Na, PZ}\) recovered slowly with time constant, \(\tau=839\pm90\) ms (n=7, HP = -90mV, Fig. 4F,G).

Na\(\gamma\)SP-transfected cells also yielded voltage-activated inward currents (Fig. 5A), peaking at \(~+30\)mV. The E\(_{rev}\) of Na\(\gamma\)SP-mediated current (I\(_{Na, SP}\)) was +76mV. Ion-
substitution (Ca\(^{2+}\) replacement) experiments confirmed that NavSP, like NavPZ, was
also a Na\(^{+}\)-selective channel (data not shown). \(I_{\text{NavSP}}\) activated and inactivated
significantly faster than \(I_{\text{NavPZ}}\) and \(I_{\text{NaChBac}}\) (\(\tau_{\text{activation}}=3.4\pm0.3\) ms at +30mV, \(n=17\);
\(\tau_{\text{inactivation}}=35\pm1.5\) ms at +30mV, \(n=17\)), but still several-fold slower than Nav currents. The Boltzmann-fit activation curve yielded a \(V_{1/2}\) of +21\pm0.4mV and \(\kappa\) of
11.8\pm0.4mV/e-fold change (\(n=28\); Fig. 5E). Steady state inactivation was strongly
dependent on the voltage (\(\kappa=10.3\pm0.5\) mV/e-fold), with half-inactivation at
−22\pm0.8mV (\(n=11\); Fig. 5E). NavPZ and NavSP were sensitive to high concentrations
of nifedipine (30 \(\mu\)M; data not shown).
Discussion

Expression of bacterial genes in systems where the protein can be studied (mammalian cells for patch clamp) is crucial to interpreting and extending static structural data through structure-function studies. Such functional expression is also important to understanding their native roles in bacteria. However, successful functional expression of bacterial proteins in mammalian cells is rare. Here, we identified 11 putative 6TM Na⁺ prokaryotic channel subunit genes and were able to functionally express 2 of the 11 in mammalian cells, where their electrophysiological properties could be studied. Both channels were Na⁺-selective and activated by voltage. One conclusion based on comparison of bacterial channels and Na\textsubscript{\textsc{v}}s, is that the major evolutionary pressure for gene duplication and concatenation of subunits was to increase the speed of channel gating. Given the high selectivity of presumed homomeric bacterial Na⁺ channels, the case for pore asymmetry as a means to increase Na⁺ selectivity seems a less likely scenario.

Little is known about the molecular determinants that control mammalian Na\textsubscript{\textsc{v}} activation rates. Mammalian Na\textsubscript{\textsc{v}} channels activate and inactivate within a few milliseconds (<10
ms), roughly 10-100 times faster than NaChBac, the only bacterial voltage-gated channel functionally expressed up to now. Interestingly, NavSP activation is ~4-fold faster than NaChBac while NavPZ activation is ~2 times slower than NaChBac, despite 77% sequence homology between NavSP and NavPZ. In NavSP, NavPZ, and NaChBac, the S4 domain and short S3-S4 linker are highly conserved, suggesting that the structural determinants for the kinetics differences are located elsewhere. Notably, there are several NavSP-specific residues in the putative pore forming domains (G143 in S5, I172 in the pore loop, V189 in the linker between the P loop and S6, and M202 in S6). These residues may contribute to the relatively fast activation kinetics of NavSP.

Na+ channel inactivation mechanisms are better understood than those of activation. Interdomain linkers mediate fast inactivation in Navs by “ball and chain” or N type inactivation (26), but these domains are obviously missing in tetramers of 6TM bacterial channels. Additionally, removal of segments within the N and C cytoplasmic domains of NaChBac ((24) and unpublished data) do not substantially alter its inactivation rate. If the cytoplasmic domains do not participate in inactivation, we can then begin to look at other domains. Studies on 6TM HERG K+ channels indicated that the S5-P linker was crucial for its C-type inactivation, probably by providing allosteric coupling between its
outer mouth and the voltage sensor (27). NaChBac inactivates with a time constant similar to Na\(\gamma\)PZ, but the NaChBac S5-P linker (from Q167 to S180) has low homology to Na\(\gamma\)PZ. NaChBac and Na\(\gamma\)PZ inactivates 5-fold more slowly than Na\(\gamma\)SP. In the pore-S6 linker, Na\(\gamma\)SP lacks the negatively-charged glutamate present in both NaChBac and Na\(\gamma\)PZ. Future studies will focus on this and other sequence differences. However, the difficulty of obtaining functional expression of many mutants highlights the need for structural data. By exclusion of alternative mechanisms, we hypothesize that C-type inactivation, in which the Na\(^+\) pore is shut, is the more likely mechanism for Na\(\gamma\)Bac channel inactivation.

Na\(^+\) channels have been proposed to play a central role in Na\(^+\)-dependent flagellar mobility in some prokaryotes. Marine Vibrio species utilize their Na\(^+\)-driven polar flagella for swimming (5, 6), and in the alkaliphilic Bacillus species, [Na\(^+\)]\textsubscript{o} determines the activity of the flagellar motor (4, 28). Under alkaliphilic conditions, the H\(^+\)-motive force is weak due to the high intracellular pH (pH 8-9) of these bacteria (4, 29). Therefore, a Na\(^+\) cycle driven by the Na\(^+\) channel may have evolved to power the flagellar motor (4, 28, 30-32). Interestingly, bacteria whose flagellar motors are powered
by Na\(^+\) (as opposed to H\(^+\)) express NaChBac homologs. In bacteria, the Na\(^+\)/H\(^+\) exchanger prevents cytotoxic Na\(^+\) accumulation and also supports pH homeostasis at elevated pH (4, 31, 33). In low [Na\(^+\)]\(_o\) environments, or in the absence of solutes to support Na\(^+\) uptake through Na\(^+\)-coupled solute transporters, the pH homeostasis function may rely on a Na\(^+\) channel (4, 31, 33). We propose that sustained voltage-gated Na\(^+\) channel opening is primarily responsible for this Na\(^+\) entry (33). It is possible, that some mammalian Na\(^+\) channels play a role in Na\(^+\) or H\(^+\) homeostasis. Interestingly, the mammalian persistent and resurgent Na\(^+\) currents have similar kinetics to Na\(V\)Bac (34, 35). These persistent Na\(^+\) currents may be mediated by subthreshold gating of fast Na\(V\) channels (36), or perhaps by Na\(V\)1.8 and Na\(V\)1.9.

NaChBac selectivity is converted from Na\(^+\) to Ca\(^{2+}\) by replacing an amino acid adjacent to glutamatic acid (E) in the putative pore domain by a negative charged aspartate (D) (from TLESWAS to TLEDWAS or TLDDWAD) (24). Interestingly, two bacterial strains (Colwellia psychrerythraea and Microbulbifer degradans) have a putative pore sequence (TFEDWTD) similar to that of the Ca\(^{2+}\)-selective NaChBac mutant. We have not been able to functionally express these channel subunits in mammalian cells, but one
possibility is that these proteins form heteromeric channels with other related subunits in
the same species.

Acknowledgements

We are grateful for bacteria provided by Drs. Hideto Takami, Arthur. A. Guffanti, Terry.
A. Krulwich (O. iheyensis HTE831), James D. Oliver (V. vulnificus M06) and genomic
DNA from Dr. Barbara Methe (C. psychrerythraea 34H). We also thank the members of
Clapham lab, Nat Blair, and Dr. Terry Krulwich (Mt. Sinai School of Medicine) for
valuable comments and encouragement.
REFERENCE


**Figure Legends**

**Fig. 1. NaChBac homologs in bacteria.**  
**A,** Phylogenic tree of bacterial NaChBac homologs. A multiple alignment of NaChBac homologs was calculated using the ClustalW program based on their conserved sequences. The tree was then generated using the neighbor-joining method (Njplot)\(^{(37)}\). Branch lengths are proportional to the sequence divergence and can be measured relative to the bar shown (top, bar= 0.05 substitution per amino acid site). Genbank accession numbers: *Vibrio vulnificus* M06, AY392139; *Microbulbifer degradans* 2-40, ZP_00067595 and ZP_00065322; *Colwellia psychrerythraea* 34H, AY392140 and AY392141; *Magnetococcus* sp. MC-1, ZP_00043768; Na\(\nu\)PZ (*Paracoccus zeaxanthinifaciens*), CAD24429; Na\(\nu\)SP (*Silicibacter pomeroyi*), AY392137; *Hyphomonas neptunium* ATCC15444, AY392138; *Thermobifida fusca*, ZP_00059295; *Oceanobacillus iheyensis* HTE831, NP_693313, and NaChBac (*Bacillus halodurans*), NP_242367. **B,** Alignment of the putative pore regions of prokaryotic channel subunits. **C,** Alignment of the putative voltage-sensing S4 region of NaChBac homologs. P indicates conserved residues.  

**Fig. 2. Putative primary structure of Na\(\nu\)PZ and Na\(\nu\)SP.**  
**A,** Deduced membrane topology of Na\(\nu\)PZ (left) and Na\(\nu\)SP (right). **B,** Alignment of the deduced amino acid
sequence of Na\textsubscript{PZ}, Na\textsubscript{SP} and NaChBac. The putative 6TM domains (S1 to S6) and pore region (P) are indicated.  

C, Coomassie-stained SDS-PAGE of Co\textsubscript{2+} affinity chromatography purification of Na\textsubscript{PZ} and Na\textsubscript{SP}, indicating both are \~29kDa as monomers.

**Fig. 3. Functional expression of Na\textsubscript{PZ} in CHO-K1 cells.** 

A, Representative traces (upper) of $I_{Na,PZ}$ activated by the voltage protocol shown. The cell was bathed in 2mM Ca\textsuperscript{2+} Tyrode external solution (140mM Na\textsuperscript{+}, 2mM Ca\textsuperscript{2+}, 5mM K\textsuperscript{+}, 1mM Mg\textsuperscript{2+}, pipette; 147mM Cs\textsuperscript{+}, 8mM Na\textsuperscript{+}; Methods). B, $I_{Na,PZ}$ in isotonic Ca\textsuperscript{2+} (105mM Ca\textsuperscript{2+}/0mM Na\textsuperscript{+}) external solution (+20mV, red; +60mV, blue). C, Averaged (n=24) peak current-voltage (I/V) relation of Na\textsubscript{PZ} in Tyrode’s solution, normalized by cell capacitance (pF). $E_{rev}$=+75mV. Also shown are peak current I-V relations of $I_{Na,PZ}$ from a cell bathed in either isotonic 150mM Ca\textsuperscript{2+} (diamonds) or 150mM NMDG\textsuperscript{+} (0mM Ca\textsuperscript{2+}, 0mM Na\textsuperscript{+}; triangles). $E_{rev}$ \~ -40mV. $V_{HP}$ indicates holding potential, 15s between pulses.

**Fig. 4.** $I_{Na,PZ}$ voltage- and time-dependent activation and inactivation. A, $I_{Na,PZ}$
deactivation tail currents. After pre-pulses of varying depolarization (from −40 to +70mV, increments = +10mV), tail currents were measured upon return to the holding potential (V_Hp, −90mV). B, I_{Na,PZ} steady-state inactivation currents. After a 4s pre-pulse, the currents inactivated to a steady-state level and were re-activated by a second 2s depolarizing pulse. The intersweep interval was 16s. C, Normalized activation curve (n=9; ±SEM) and steady-state inactivation curve (n=10; ±SEM). D, Time dependent-inactivation of I_{Na,PZ} increased slowly at −30mV. V_Hp=−90mV. The currents were elicited by depolarization to +10mV preceded by a pre-pulse (-30mV) with a variable length of time (in ms; 0, black; 500, magenta; 1500, dark yellow; 4500, blue; 9000, red).

E, The average time course of I_{Na,PZ} inactivation at −30mV. The time constant of the development of inactivation development is ~2 s (-30mV). F, Recovery from inactivation. The time interval between the test pulse (+10mV, 1000s) and the inactivation pulse (+10mV, 1000ms) was varied from 0.2s to 9.8s. G, Relative peak I_{Na,PZ} recovered with an approximately exponential time course (τ ~840ms). The ratios between currents elicited by the two pulses (F) were used to construct the recovery curve (n=7; ±SEM).

Fig. 5. Na\textsuperscript{+}SP voltage-dependent inward currents. A, Representative traces (upper) of
$I_{\text{Na}_{\text{v,SP}}}$ in Tyrode’s solution (external) and standard pipette solution. **B**, Averaged peak I/V relation (n=23; ±SEM). $I_{\text{Na}_{\text{v,SP}}}$ activated above -20mV and peaked at +30mV. $E_{\text{rev}} = +76mV$. **C**, $I_{\text{Na}_{\text{v,SP}}}$ activation currents. Tail currents were measured as described in Fig. 4A. **D**, $I_{\text{Na}_{\text{v,SP}}}$ steady-state inactivation currents in response to protocol similar to Fig. 4B. **E**, Normalized activation curve (n=28; ±SEM) and steady-state inactivation curve (n=11; ±SEM).
Online Supplementary Materials

Methods
Cloning of other NaChBac homologs

We identified genomic sequences of NaChBac homologs from the NCBI or TIGR database. GenBank numbers are indicated in the legend of Fig. 1A. Preliminary sequence data from *Colwellia psychrerythraea* 34H and *Hyphomonas neptunium* ATCC15444 were obtained from the TIGR website at http://www.tigr.org. Primer sequences data from *Vibrio vulnificus* M06 was obtained from *Vibrio vulnificus* CMCP6 genomic sequence data (NC_004460).

*Vibrio vulnificus M06*: *Vibrio vulnificus* M06 was kindly supplied by Dr. James D. Oliver. NaChBac homolog was amplified by standard PCR with the following primers: 5’-AATGGATCCATGTTTGTATTATCAAAA-3’, which contains a *Bam*HI site and 5’-ATTGAATTCTTATCCAATCTTTTTGTT-3’, which contains an *Eco*RI site. The PCR product was cloned into the modified pTracer-CMV2 plasmid between the single restriction sites for *Bam*HI and *Eco*RI.

*Colwellia psychrerythraea 34H*: *Colwellia psychrerythraea* 34H genome DNA was kindly supplied by Dr. Barbara Methe. NaChBac homolog (AY392140) was amplified by standard PCR with the following primers:
5'-TGGATCCATGGGGCTTTTGTAGCAAGG-3’, which contains a *BamHI* site and 5’-CGAATTCAAGTAACACAAGCCTATTTTCAC-3’, which contains an *EcoRI* site. Similarly, another NaChBac homolog (AY392141) was amplified by standard PCR with the following primers: 5’-

TGGATCCCATGTCTATATCTGCACACACAGCAAAC-3’, which contains a *BamHI* site and 5’-AGAATTCGGTAATACTTTAATCACATAAAGATAAATCTC-3’, which contains an *EcoRI* site. The PCR products were cloned into the modified pTracer-CMV2 plasmid between the single restriction sites for *BamHI* and *EcoRI*.

**Hyphomonas neptunium ATCC15444**: A sample of *Hyphomonas neptunium* ATCC15444 was obtained from American Type Culture Collection (#15444). NaChBac homolog was amplified by standard PCR with the following primers:

5’-AATGGATCCATGCCGAAAGCAACACTCGAC-3’, which contains a *BamHI* site and 5’-AATGAATTCTCAGCCCTGCCCGATCTGGA-3’, which contains an *EcoRI* site. The PCR product was cloned into the modified pTracer-CMV2 plasmid between the single restriction sites for *BamHI* and *EcoRI*.

**Oceanobacillus iheyensis HTE831**: *Oceanobacillus iheyensis* HTE831 was kindly supplied by Drs. Hideto Takami, Arthur A. Guffanti, and Terry A. Krulwich. NaChBac homolog was amplified by standard PCR with the following primers:

5’-TAAGGATCCATGAGAAACACATACAGAA-3’, which contains a *BamHI* site and
5’-TAAGAATTCTTAATCTTTATGCTGTTGTTT-3’, which contains an EcoRI site.

The PCR product was cloned into the modified pTracer-CMV2 plasmid between the single restriction sites for BamHI and EcoRI.

*Microbulbifer degradans* 2-40, *Magnetococcus sp.* MC-1 and *Thermobifida fusca*.

NaChBac homologs in these species were made by overlapping PCR technology with primers derived from GenBank sequences.
A

AY392139 (Vibrio vulnificus M06)
ZP_00067595 (Microbulbifer degradans 2-40)
AY392141 (Colwellia psychrerythraea 34H)
ZP_00043768 (Magnetococcus sp. MC-1)
NaP (Silicibacter pomeroi)
NaP (Paracoccus zeaxanthinifaciens)
AY392138 (Hyphomonas neptunium ATCC15444)
ZP_00059295 (Thermobifida fusca)
AY392140 (Colwellia psychrerythraea 34H)
ZP_00065322 (Microbulbifer degradans 2-40)
NP_693313 (Oceanobacillus iheyensis HTE831)
NaChBac (Bacillus halodurans)

B

NaChBac (Bacillus halodurans)
NaP (Paracoccus zeaxanthinifaciens)
NaP (Silicibacter pomeroi)
NP_693313 (Oceanobacillus iheyensis HTE831)
ZP_00059295 (Thermobifida fusca)
ZP_00043768 (Magnetococcus sp. MC-1)
AY392138 (Hyphomonas neptunium ATCC15444)
ZP_00065322 (Microbulbifer degradans 2-40)
AY392140 (Colwellia psychrerythraea 34H)
AY392141 (Colwellia psychrerythraea 34H)
AY392139 (Vibrio vulnificus M06)

C

NaChBac (Bacillus halodurans)
NaP (Paracoccus zeaxanthinifaciens)
NaP (Silicibacter pomeroi)
NP_693313 (Oceanobacillus iheyensis HTE831)
ZP_00059295 (Thermobifida fusca)
ZP_00043768 (Magnetococcus sp. MC-1)
AY392138 (Hyphomonas neptunium ATCC15444)
ZP_00065322 (Microbulbifer degradans 2-40)
AY392140 (Colwellia psychrerythraea 34H)
AY392141 (Colwellia psychrerythraea 34H)
AY392139 (Vibrio vulnificus M06)

Koishi et al. Fig. 1
A: 

B: 

C: 

D: 

E: 

F: 

G: 

Koishi et al. Fig. 4