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# Heteromultimerization of Prokaryotic Bacterial Cyclic Nucleotide-Gated (bCNG) Ion Channels, Members of the Mechanosensitive Channel of Small Conductance (MscS) Superfamily

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Supporting Information

**ABSTRACT:** Traditionally, prokaryotic channels are thought to exist as homomultimeric assemblies, while many eukaryotic ion channels form complex heteromultimers. Here we demonstrate that bacterial cyclic nucleotide-gated channels likely form heteromultimers *in vivo*. Heteromultimer formation is indicated through channel modeling, pull-down assays, and real-time polymerase chain reaction analysis. Our observations demonstrate that prokaryotic ion channels can display complex behavior and regulation akin to that of their eukaryotic counterparts.

While the biological roles of proteins are highly conserved across all phylogenetic kingdoms, it is generally true that protein assembly and complexity scale with organismal complexity. For example, eukaryotic and prokaryotic ribosomes conduct very similar functions in protein synthesis; however, the eukaryotic system is composed of 79 proteins and four rRNAs, while the prokaryotic system is composed of only 52 proteins and three rRNAs.[1] From a classical perspective, ion channels exhibit a similar scaling of complexity, with eukaryotic channels often being composed of multiple different protein subunits, which can be further modulated by accessory proteins, while prokaryotic channels are homomeric multisubunit assemblies. This fundamental difference of heteromeric versus homomeric assemblies allows for tissue and cell specific tuning of ion channel function, because different heteromeric assemblies are expressed in tissue specific patterns.[2] Heteromeric modulation can also play a role in development, because, for some channels, the subunit composition changes within a cell or tissue as the organism progresses from an embryonic state to adulthood.[2] Both of these explanations for heteromultiplicity are explicitly tied to the complexity of higher organisms and, thus, are not applicable to prokaryotes. As a result, we were extremely surprised to find two or more copies of different but related bacterial cyclic nucleotide-gated (bCNG) channels within some bacterial genomes. While this suggested to us that bCNG channels might exist as heteromultimeric assemblies, it was also possible that these bacteria contained multiple discrete homomeric bCNG channels or that one of these genes was simply not expressed under normal growth conditions. Although homomeric assemblies of bCNG subunits from some bacteria containing multiple bCNG genes are functional,[3,4] we demonstrate here that some bCNG channels likely exist *in vivo* as heteromultimers. To assess the ability of these channels to form complex assemblies, we have examined the ability of different bCNG subunits to assemble at a molecular level using homology modeling and molecular dynamics (MD) simulations, at the protein level using pull-down assays, and at the organismal level using quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

From a molecular standpoint, bCNG channels are members of the mechanosensitive channel of small conductance (MscS) superfamily with channel domains very similar to *Escherichia coli* MscS (Ec-MscS). Thus, we can use homology modeling to create molecular level structural models of bCNG channels with three transmembrane domains based on the Ec-MscS structure.[5] Previously, we used these models to compare the function of Ec-MscS and a homomeric assembly of bCNG subunits from *Synechosystis* sp. PCC 6803 (Ss-bCNGa).[4] However, *Synechosystis* sp. PCC 6803 (Ss) contains two bCNG genes, which encode two different three-transmembrane domain bCNG subunits that we previously designated Ss-bCNGa and Ss-bCNGb.[3] To evaluate the feasibility of forming heteromultimeric assemblies of bCNG subunits at the molecular level, we produced homology models of homomeric Ss-bCNGb and heteromeric Ss-bCNG in a manner analogous to that used for our homomeric Ss-bCNGa models.[4] These models included four different heteromeric arrangements containing a 1:6, 6:1, or 4:3 Ss-bCNGa:Ss-bCNGb subunit ratio. The DOPE energies, a measure of homology model "quality",[5] for the lowest-energy model of each multimeric state was equivalent to those for our previous models of Ss-bCNGa (Table 1 of the Supporting Information).[4] Moreover, DOPE energies and PROCHECK evaluations (Tables 1 and 2 of the Supporting Information) implied that models for the different homomeric and heteromeric states of Ss-bCNG were equally feasible. Analogous results were observed for Ss-bCNG models produced from three different template structures representing both open and closed states of the channel.

To further evaluate the structural stability of models representing different multimeric states, we compared 50 ns MD simulations of three models (homomeric Ss-bCNGa,

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homomeric Ss-bCNGb, and heteromeric Ss-bCNGa/b 4:3 with alternating subunits) embedded in a lipid membrane.[6] In these simulations, the three Ss-bCNG multimer models maintained a similar level of structural integrity. Each model displayed comparable deviations from its starting structure (Figure 1A

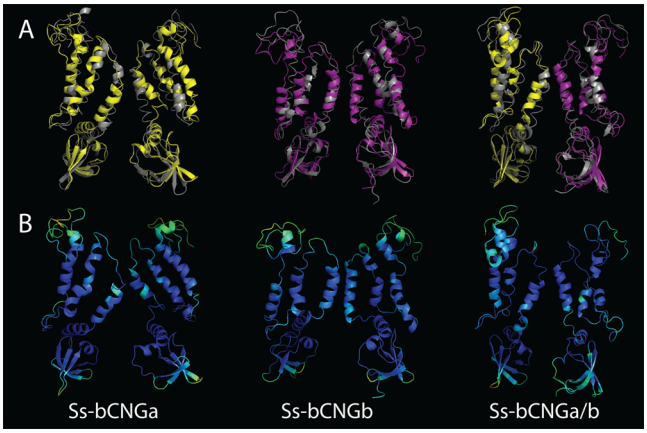


**Figure 1.** (A) Side views of the initial (gray) and final (color) structures after 50 ns MD simulations with Ss-bCNGa subunits (yellow) and Ss-bCNGb subunits (purple). (B) Root-mean-square (rms) C$\alpha$ fluctuation averaged over the last 10 ns of the MD simulations. Greater rms fluctuations are shown as warmer colors (red for $\geq$0.20 nm) and lower rms fluctuations as cooler colors (blue for $\leq$0.05 nm).

and Table 3 of the Supporting Information), maintained consistent regions of secondary structure and overall channel assembly, and showed similar structural stability (Figure 1B). The integrity of all three models was also equivalent to that of Ec-MscS subjected to the same simulation conditions (Table 3 of the Supporting Information). Interestingly, by the end of the simulations, there were more intersubunit hydrogen bonds in the heteromultimeric Ss-bCNGa/b structure than in homomeric Ss-bCNG structures, implying that heteromeric Ss-bCNGa/b interfaces effectively form interactions. Together, our modeling and simulations demonstrate that heteromeric Ss-bCNG channel multimers are possible from a molecular point of view.

To show that heteromeric Ss-bCNGa/b channels could form at the protein level, we used pull-down assays of heterologously co-expressed bCNG channel subunits. For heterologous co-expression, bCNG channel subunits were expressed with a His or HA tag. After induction with IPTG, His-tagged proteins were purified using nickel affinity chromatography and analyzed by Western blotting for both the His tag and the HA tag. We found that His-tagged Ss-bCNGa could pull down HA-tagged Ss-bCNGb, demonstrating that at the protein level heteromeric Ss-bCNG channels were formed when RNA for the two subunits was present (Figure 2A). Controls for these experiments are described in the Supporting Information. An analogous result was observed when using His-tagged Ss-bCNGb and HA-tagged Ss-bCNGa (Figure 2A).

Having observed multimerization of Ss-bCNG subunits, we used pull-down assays to consider whether Ss-bCNG subunits could assemble into heteromers with subunits from different strains. In these experiments, Ss-bCNGa and Ss-bCNGb both formed heteromultimers with bCNG from *Synechococcus elongatus* PCC7942 (Se-bCNG), while bCNG from *Agrobacterium tumefaciens* strain C58 (Rr-bCNG) formed only

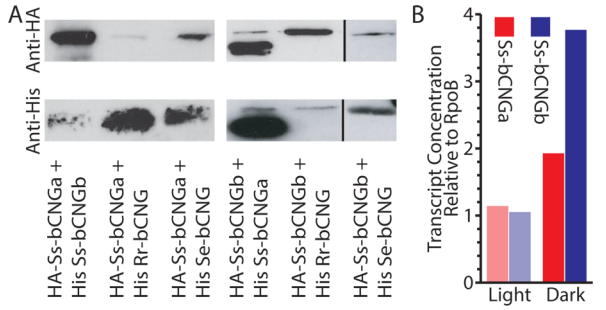


**Figure 2.** (A) Pull-down analysis of heterologously expressed Ss-bCNG channels. (B) Transcript levels for Ss-bCNGa and Ss-bCNGb as determined by qRT-PCR at the midpoint of light and dark portions of the growth cycle relative to RpoB.

heteromers with Ss-bCNGb (Figure 2A). The sequence of Se-bCNG is highly similar with those of both Ss-bCNG subunits and is predicted also to include three transmembrane domains;[3] thus, it is not surprising that heteromultimers form between these channels. However, Rr-bCNG is a much longer sequence predicted to have five transmembrane domains,[3] and interestingly, the subtle molecular interactions between transmembrane domains necessary for assembly occur only with Ss-bCNGb.

Our pull-down data imply that Ss-bCNG channel subunits are able to readily form heteromultimeric assemblies when RNAs for multiple subunits are cotranscribed in *E. coli* using heterologous expression. Evidence of expression of multiple subunits *in vivo* was seen in a shotgun proteomic analysis of *Synechosystis* in which both Ss-bCNGa and Ss-bCNGb proteins were observed.[7] However, we turned to qRT-PCR to further confirm that RNAs for different Ss-bCNG subunits actually are cotranscribed under normal growth conditions. We examined bCNG subunit RNA levels in *Synechosystis* at the midpoints of the light and dark clock cycle (Figure 2B), when the cyanobacteria would undergo photosynthetic and aerobic respiration, respectively. Ss-bCNGa and Ss-bCNGb were cotranscribed under both conditions. Interestingly, the overall RNA level for bCNG subunits changed as a function of respiration state as did the relative ratio of the two subunit RNAs (Figure 2B).

The observation that the bCNG channel population and composition differ under conditions of photosynthetic respiration versus aerobic respiration points to a potential physiological role for Ss-bCNG channel multimerization. Because the plasma membrane of cyanobacteria is the site of ATPase activity in photosynthesis, the resting potential must be different under photosynthetic versus aerobic respiration. In cyanobacteria, these membrane potential changes correlate with changes in cyclic nucleotide monophosphate (cNMP) levels.[8] Thus, Ss-bCNG channels could sense changes in this ligand concentration to serve as a membrane potential governor to stabilize (or possibly control) membrane potential. Altering the channel subunit composition in dark versus light conditions could allow cells to assert an extra level of control on membrane potential in the different respiration states.

To further determine whether there is evidence of bCNG heteromultimer formation in other strains, we considered bCNG channels from *Burkholderia graminis* (Bg). Because Bg-bCNG subunits are predicted to have five transmembrane domains, they were not amenable to our modeling approach. However, pull-down experiments showed evidence of Bg-


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bCNGa/b heteromultimer formation on the protein level, and qRT-PCR measurements confirmed co-expression of both subunits *in vivo* (Figure 3). We expect bCNG heteromultimerization may be even more widespread, because we also observed simultaneous transcription of different bCNG subunits by qRT-PCR in *Acaryochloris marina* MBIC11017 (Am), *Burkholderia xenovorans* LB400 (Bx), *Cyanothece* sp. ATCC 5142 (Cs-2), and *Rhizobium leguminosarum* bv. Viciae (Rl) (Figure 3B).

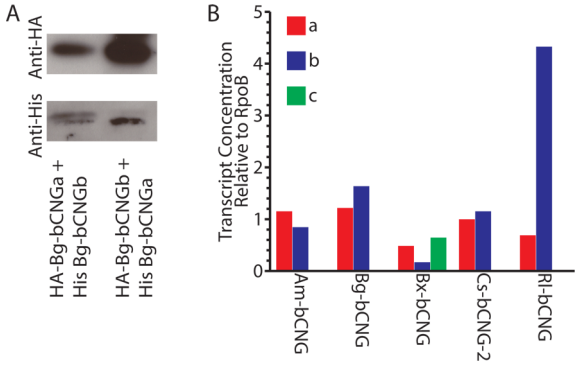


**Figure 3.** (A) Pull-down analysis of heterologously expressed Bg-bCNG channels. (B) Transcript levels as determined by qRT-PCR for bCNG channels relative to RpoB controls.

Our results suggest that like eukaryotic ion channels, some prokaryotic ion channels can exist as heteromultimeric assemblies. One possible role for heteromultimeric assemblies of prokaryotic ion channels is to allow for dynamic regulation of ion channel function based on environmental stresses, as we have suggested for Ss-bCNG. Formation of heteromultimers in bacterial channels previously believed to be homomeric leads to significantly more molecular diversity than what was previously appreciated and may explain the difference between *in vivo* and *in vitro* physiology. For example, six MscS superfamily homologues are known to be expressed in *E. coli*,[9] and heteromultimer formation might explain physiological discrepancies that have been observed for MscS channel function between early work using native channel levels and more modern studies, which have used overexpression.[10,11] Considering heteromultimeric assembly of bacterial channels is critical to developing an understanding of *in vivo* bacterial physiology and highlights the similarity between prokaryotic and eukaryotic ion channels.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Supporting details, figures, and tables, including detailed materials and methods and supplemental discussion of molecular models and pull-down asssays. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ■ REFERENCES

(1) Klinge, S., Voigts-Hoffmann, F., Leibundgut, M., and Ban, N. (2012) Atomic structures of the eukaryotic ribosome. *Trends Biochem. Sci. 37*, 189−198.

(2) Hille, B. (2001) *Ion channels of excitable membranes*, 3rd ed., Sinauer, Sunderland, MA.

(3) Caldwell, D. B., Malcolm, H. R., Elmore, D. E., and Maurer, J. A. (2010) Identification and experimental verification of a novel family of bacterial cyclic nucleotide-gated (bCNG) ion channels. *Biochim. Biophys. Acta 1798*, 1750−1756.

(4) Malcolm, H. R., Heo, Y. Y., Caldwell, D. B., McConnell, J. K., Hawkins, J. F., Guayasamin, R. C., Elmore, D. E., and Maurer, J. A. (2012) Ss-bCNGa: A unique member of the bacterial cyclic nucleotide gated (bCNG) channel family that gates in response to mechanical tension. *Eur. Biophys. J. 41*, 1003−1013.

(5) Shen, M. Y., and Sali, A. (2006) Statistical potential for assessment and prediction of protein structures. *Protein Sci. 15*, 2507−2524.

(6) Lindahl, E., Hess, B., and van der Spoel, D. (2001) GROMACS 3.0: A package for molecular simulation and trajectory analysis. *J. Mol. Model. 7*, 306−317.

(7) Gan, C. S., Reardon, K. F., and Wright, P. C. (2005) Comparison of protein and peptide prefractionation methods for the shotgun proteomic analysis of *Synechocystis* sp. PCC 6803. *Proteomics 5*, 2468−2478.

(8) Ohmori, M. (1989) Camp in Anabaena-Cylindrica: Rapid Changes in Cellular-Levels in Response to Changes in Extracellular Environments. *Plant Cell Physiol. 30*, 911−914.

(9) Booth, I. R. (2014) Bacterial mechanosensitive channels: Progress towards an understanding of their roles in cell physiology. *Curr. Opin. Microbiol. 18*, 16−22.

(10) Martinac, B., Buechner, M., Delcour, A. H., Adler, J., and Kung, C. (1987) Pressure-sensitive ion channel in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 84*, 2297−2301.

(11) Sukharev, S. (2002) Purification of the small mechanosensitive channel of *Escherichia coli* (MscS): The subunit structure, conduction, and gating characteristics in liposomes. *Biophys. J. 83*, 290−298.