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bcNOS      1 : -----MSKTKQLIEERSHFTITICKKEL-NK
bsNOS      - : -----
SANOS      1 : -----MLFKERQAFTENMKKEC-HY
deiNOS     1 : -----MSCPARAVLTFDMRALFLRRF
miNOS      77 : QYVRIKNWGSGEILHDTLHHKATSDPTCKSKSCLGSIMNPKSLTRGPRDKPTFLEELLPHRIEFINQYGSFKEA
hnNOS      304 : RFLKVKNWETEVVLTDTLHLKSTLETGTCTEYICMGSIMHFSQHARRPE-DVRTKGQLFPLRAKEFIDQYSSIKRF
beNOS      69 : KFFRVKNWELGSITYDTLCAQSQDGFCTPRCCLGLSLVLFRKLQTRFSPGPPFAEQLLSQRDFINQYSSIKRS

bcNOS      25 : E-QFIEERMKEIRVEIEKTGTVEHTFEELVHGSRMAWRNSNRCTGRLFWSKMHILDRREVNDEEGVYNATIHHTK
bsNOS      1 : ---MKDRLADIKSEIDLTGSVVHTKELEHGARMAWRNSNRCTGRLFWSKMHILDRREVNDEEGVYNATIHHTK
SANOS      20 : ETQILINKRLHDILELEIKETGTVHTTEELIGARMAWRNSNRCTGRLFWSKMHILDRREVNDEEGVYNATIHHTK
deiNOS     21 : HEEMGEPGPARLRAVEEAGLWPTSAELTWGAKWAWRNSTRCVGRLYWEASVRDLRELNTAQAVYEALLQHHD
miNOS      152 : KIEEHLARLEAVTKEIETGTVQLTLDELFATRMAWRNAPRCIGRICWSNLQVFDARNCSTAQEMFQHICRHL
hnNOS      378 : GSKAHMERLEEVNKEIDTSTWQLKDTELIYGARMAWRNASRCVGRICWSKLQVFDARDCTAHGMFNYICNHWK
beNOS      144 : GSQAHEERLQEVAEVASTGTWHLRESELVFGARQAWRNAPRCVGRICWSKLQVFDARDCSSAQEMFTYICNHWK

bcNOS      99 : YATNDGRVKPTITIIFKYQGEENNIRINHOLIRYAGYKTE-MGVIGDSHSAVFTDFCQELGWQGEGTMFDVLPL
bsNOS      72 : TATNGKIRPTIIFFPEEKSEKQVEIWNHOLIRYAGYESD-GERIGDEASCLTAACELGWRGERTDFDLLPL
SANOS      95 : QATNEGKLKPYIIYAPKDGF---KIFNQLIRYAGYDN----CGDPAEKEVTRLANHLGWKGKGTNFDVLPL
deiNOS     96 : DAFCGGHIREVISVFGP---GVRLHNPOLIRYA---DEINADEVDKLRRFGWQERGERFDVLPL
miNOS      227 : YATNGNGIRSAITVFPQRSDEKHDFRLWSQLIRYAGYQMPDGTIRGDAATLEFTQLCIDLGWKFRYGEFDVLPL
hnNOS      453 : YATNKGNLRSAITIFPQRTDGKHDFRVWSQLIRYAGYQPDGSTLGDEANVQFTEICIQQGWKEFRGEFDVLPL
beNOS      219 : YATNRGNLRSAITVFPQRAPERGDFRIWSQLVRYAGYRQDGSVRGDEANVEITELCIQHGWTEGNGEFDVLPL

bcNOS      173 : VFSIDG-KAPIYKEIPKEEVEVPIHPEYP-ISSLGAWYGVPMISDMRLEIGGESYTAAPFNGWYMGTEIGAR
bsNOS      146 : IFRMKGDEQVWYELPRSLVEVPIHPEIEAFSDLELWYGVPISDMKLEVGGEHYNAAPFNGWYMGTEIGAR
SANOS      161 : IYQLPN-ESVKFYEYPTSLIREVPIHNHYPKLRLNLRWYAVPISNMDLEIGGEVYPTAPFNGWYMYTEIGAR
deiNOS     155 : LIEVNG--RAELFSLPQAVQEVAIHPVCLGIGELGLRWHALPVISDMHLDIGGEHLP-CAFSGWYVTEIAAR
miNOS      302 : VLQADG-QDEVFEIPDLVEVTMEHPKYEWFQELGLWYALPAVANMLLEVGGEFFACPFNGWYMGTEIGAR
hnNOS      528 : LLQANG-NDPELFQIPELVEVPIRHPKEFEWFKLDLGLWYGLPAVSNMLLEIGGEFSACPFSGWYMGTEIGAR
beNOS      294 : LLQADD-EAELFVLPELVEVPIRHPELEWFALGLWYALPAVSNMLLEIGGEFSAPFSGWYMYTEIGAR

bcNOS      246 : NLADHDRYNLLPAVAEMMDLDTSRNGTLWKDKALVELNWAVLHSFKKQGVSIVDHETAAQQFQFEKQEAACGRV
bsNOS      221 : NLADEKRYKLKKVASVIGIAADYNTDLWKDQALVELNWAVLHSYKKQGVSIVDHETAASQFKRFEEQEEEAGRK
SANOS      235 : NFIDDYRYNLLENVADAFEFDTLKNSFNKDRALVELNWAVYHSFKKQGVSIVDHLTAAQQELFERNERAQQRGR
deiNOS     227 : DLADVGRYDQLPAVARALGLDTSRERTLWRDRALVELNWAVLHSFDAAGVKLADHETVTAHHVRFEERARAGRE
miNOS      376 : DFCDTQRYNILESVERRMGLEHTLASLWKDRAVEINWAVLHSFQKQNVTIMDHETASESFMKHMQNEYRARGG
hnNOS      602 : DYCDNSRYNILESVAKKMNLDMRKTSSLWKDQALVEINWAVLHSFQSDKVTIVDHHSATESFIKHMENEYRCRGG
beNOS      368 : NLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKALVEINWAVLHSFQLAKVTIVDHEAATVSFMKHLDNEQKARGG

bcNOS      321 : VTGNWWLLPPLSEATHIYHKPYPNEILKNEFHK-----
bsNOS      296 : LTGDWTWLPPTSEAATHIPHRSYDNSIVKPNYFYQDKEY-----
SANOS      310 : VTGKWSWLAPPLSETLTSNYHGYDNTVKPNFFKKKESNANQCPFH-----
deiNOS     302 : VRGKWSWLVPPLSEATTFLWSRRYRAREESRFVRARCEFHTPTVHASTGHAPTG-----
miNOS      451 : CPADWIWLVPPVSGSITEVFHQEMLNYVLSEFYYQIEEWKTHIWQNEKLRFRRR-EIRFRVLVKVVVFASMLMR
hnNOS      677 : CPADWWLVPPMSGSITEVFHQEMLNYLPSEEYQPDFWNTHVWKGTNGTPTKRRAIGFKKLAEAVKFSAKLMG
beNOS      443 : CPADWAWLVPPLSGSLTEVFHQEMVNYLLSAPRYQPDEWKGSATKGAGITRKKT----FKEVANAVKISASLMG

bcNOS      - : -----
bsNOS      - : -----
SANOS      - : -----
deiNOS     - : -----
miNOS      525 : KVMASRVRATVLFATETGKSEALARDL
hnNOS      752 : QAMAKRVKATILYATETGKSQAYAKTL
beNOS      514 : TLMAKRVKATILYASETGRAQSYAQQL

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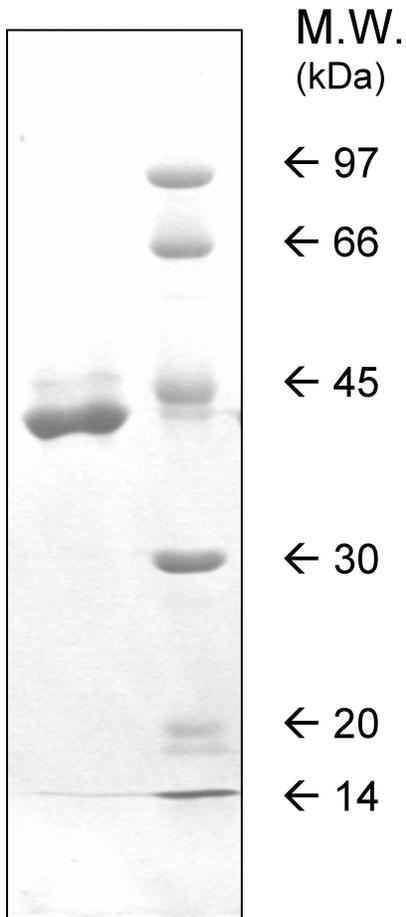
**Supplementary Figure 1. Sequence alignment of the bacterial NOS-like proteins and three mammalian oxygenase domains.** The sequences shown are (Genebank accession numbers between parenthesis): *Bacillus cereus* NOS -bcNOS (ABS24145), *Bacillus subtilis* NOS – bsNOS (CAB12592), *Staphylococcus aureus* NOS – SANOS (BAF78782), *Deinococcus*

*radiodurans* NOS –deiNOS (**ABF44571**), murine macrophage iNOS –miNOS (**M84373**), human brain nNOS –hnNOS (**LO2881**) and bovine eNOS –beNOS (**M89952**). The alignment was performed using Clustal W (30). Degree of shading is indicative of sequence homology.

Sequence alignment of the bcNOS protein with prokaryotic and eukaryotic NOS enzymes showed that the residues involved with heme binding in saNOS, bsNOS and the mammalian NOS proteins are conserved in bcNOS. As well, the highly conserved glutamic acid residue found in mammalian NOS proteins to be important for the binding of L-arginine in the substrate-binding site is also conserved in bcNOS.

*Expression and purification of bcNOS.* Escherichia coli strain BL21(DE3) pLysS transfected with the bcNOS plasmid and grown in Luria-Bertani broth containing 100 µg/mL ampicillin, and induced at an OD (600 nm) of 0.6 with 1 mM IPTG for 4 h. The cells were harvested by centrifugation and stored at –80°C. The temperature was maintained at 4°C throughout the purification. The harvested cells were resuspended by adding 2 mL/g of cells buffer A (50 mM Tris-HCl buffer pH 7.0, 1 mM β-mercaptoethanol, 1.0 M NaCl) containing 0.1 mg/mL DNaseI and 0.55 mg/mL lysozyme, protease inhibitors pepstatin 2 µg/mL, bestatin 10 µg/mL, chymostatin 10 µg/mL, and leupeptin 25 µg/mL and stirred until the frozen pellet was dissolved. The cells were lysed using a homogenizer and the lysate was centrifuged at 48,000 g. The cleared lysate was loaded onto Ni<sup>2+</sup>-agarose resin (Novagen, Madison, WI) equilibrated with tris-imidazole buffer containing 1 M NaCl, and eluted with increasing concentrations of imidazole, always in the presence of 1 M NaCl. Fractions were analyzed by SDS–polyacrylamide electrophoresis and Coomassie blue staining, the fractions containing essentially pure bcNOS protein were pooled, dialyzed against 50 mM Tris-HCl, pH 7.5 and 100 mM NaCl and 10% glycerol, The protein was dialyzed over night at 4°C with the dialysis buffer changed

once after 3 hours. Protein concentration was determined by the Lowry dye binding microassay method using bovine serum albumin as the standard. All assays were performed in quadruplicate.



**Supplementary Figure 2. SDS-PAGE analysis of purified bcNOS expressed in *Escherichia coli*.** Purified bcNOS was electrophoresed on a 10% SDS-PAGE gel (left lane). Comparison with low molecular weight standard in the right lane shows that the approximate molecular weight for bcNOS is 43 kDa.

### *Difference spectroscopy*

Difference spectroscopy was used to measure the binding affinities of imidazole, NOHA, and L-arginine as previously described [see reference 7 in the manuscript]. All experiments were performed at 25 °C. The bcNOS enzyme was diluted to a final concentration of 10 µM with 50 mM Tris-HCl, pH 7.5 and 10 % glycerol before titration with imidazole to a final concentration of 10 mM. The additions of imidazole caused < 2% volume changes to the sample. Spectra were recorded from 350 nm to 500 nm after each addition. The apparent binding constant,  $K_d$ , for imidazole was determined from the x-intercept of a double-reciprocal plot of the difference in the respective peak to trough absorbances versus the imidazole concentration. Linear regression analysis of titration data gave a  $R^2$  value of 0.975. The binding affinities of NOHA and L-arginine were measured after incubation of the bcNOS protein in the presence of 10 mM imidazole for 10 minutes at 25 °C. Titration of either NOHA or L-arginine caused < 6.5% or < 2% volume changes to the sample, respectively. A double reciprocal plot of the NOHA binding data gave an apparent binding constant,  $K_{obs}$ , for NOHA, which was then used to calculate the spectral binding constant,  $K_s$ , for NOHA using equation 1.

$$K_s = K_{obs} / (1 + \{[imidazole]/K_d[imidazole]\}) \quad (1)$$

This analysis was also performed on the spectral data obtained by the binding of L-arginine to bcNOS in order to determine the  $K_s$  for L-arginine.