**Supplemental Information for**

**Enzymatic evidence that the natural product *N*-nitroglycine is degraded by diverse bacteria**

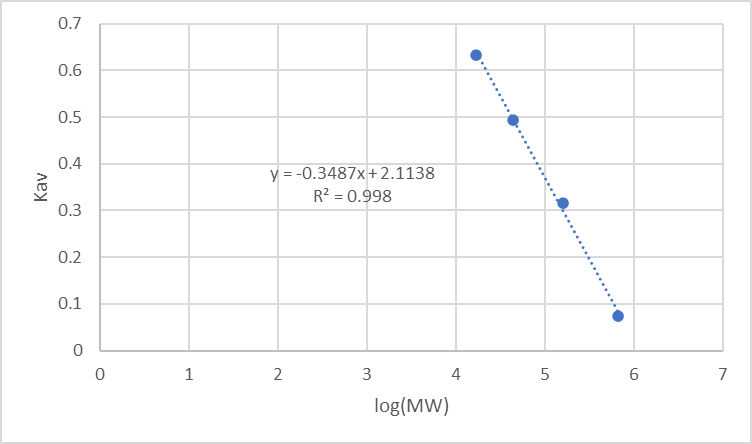
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**Figure S1**. Analytical size exclusion chromatography calibration curve of standards. Flow rate 0.75 mL/min, 100 mM Tricine and 100 mM NaCl buffer pH 7.5.

A graph of a graph showing a number of numbers

Description automatically generated with medium confidence**Figure S2.** Representative LC-MS EICs monitoring molecular anion of 2-NAE (m/z 105.03) in samples containing 2 mM 2-NAE, excess titanium citrate, and either no *Vs* NnlA for the control sample or 20 µM reduced *Vs* NnlA (FeII-NnlA) for the reaction samples. Samples were incubated overnight at room temperature in deoxygenated 23.3 mM tricine buffer, pH 7.5.

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**Figure S3**. Nitrite concentrations observed in overnight cultures of *E. coli* transformed with NnlA homologs or variants grown in the presence of 2-NAE. Cells were incubated overnight in diluted LB containing IPTG and 3 mM NNG and incubated overnight at 37°C.

MBW2064617 --------------MAGNGDKRLTELIRLAMECMGVAVTIIDPQGTLLYYNKQAEKILD**R** 46  
MCK4988321 -------------MNENERKTKLGELVNLAMDCLGVAVTIIDTKGTLLYYNQHSAKILD**R** 47  
WP\_189438608 MSQNQHSAFRKQVADRTLDGWELEGCAEWLIDQQGVGVSIIDTEGRLLFYNQWADNKMP**R** 60  
WP\_054784913 -MTDNNNELPEVTDQRILEAWKLSGWADRLLEEAGIGVTIIDKDGKLLYYNKWASENLD**R** 59  
WP\_282531508 ----MDEKLPEVTQQRVLPGWTVSQWAGGLIEHAGVGVTILDREGRVMFYNQWAANRLD**R** 56  
NnlA\_(OUM02170) MNQVNTEELPEVVDQRILAGWRLSEWADRILEYAGVGVTLVDRLGRCVYYNQWAKDHLD**R** 60  
WP\_066989343 --MTTHADLTEVFEHRIVADWALGEWADRLLEQAGLGVTIVDRHGVVMYYNKWAAEHLD**R** 58  
WP\_051342206 ---MTQAILPEVTDARILDGWQLSGWADRLLEQAGVGVTIVDRTGRVLYYNKWADEHLD**R** 57  
WP\_030511367 --MTSQAEPAEAAESRIATDWGLDEWADRLIDQAGVGVTILNRHGTVMYYNKWASEHLD**R** 58  
WP\_191054027 --MSSQVELAEVAESRIATDWGLDQWADRILEQAGFGVTVLDRHGTVMYYNKWASEHLD**R** 58  
WP\_195903080 --MSSQVELAEVAESRIATDWGLDQWADRILEQAGVGVTILDRHGTVMYYNKWASEHLD**R** 58  
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MBW2064617 KPEYIGKDVHS**HHK**RAASNK**K**LDMMLEDFQ-KG**R**TEPFHYQARPYGE-TILVILSPIFED 104  
MCK4988321 KPEYIGTDIHS**HHK**EAAINK**K**VDLMLKEFE-GG**R**KDHFHYEAKPYGK-IIFVTLAPIIKN 105  
WP\_189438608 EPEYLGQKVQE**HHR**KQITNV**R**FEAMLDLFRKEG**R**TEAVKYVAKPYEGLTIIVIVTPIIVE 120  
WP\_054784913 QPRHIGHNVKE**NHR**RSITNP**R**FDAMLQLFR-DG**R**KDPVRYVANPYGTTTILVTVSPIHID 118  
WP\_282531508 KPEYIGKDVRN**HHR**RKITNP**R**FDAMLKLFE-EG**R**TDPVHYVARPYGKITILVTVSPIKVD 115  
NnlA\_(OUM02170) KPGYIGDEIHN**RHR**RAITNP**R**FDAMLKLFE-EG**R**MEPVRYVARPYGKTTILVTVSPIYVE 119  
WP\_066989343 QPGYLGHSVHE**RHH**RKITNP**R**FDAMLKLFV-DG**R**IEPVQYVARPYGKTTILVTVSPIRIG 117  
WP\_051342206 KPEYIGNDVRD**RHR**QPITNP**R**FDAMIALFE-EG**R**VEPVRYVARPYGKTIILVTVSPIWVD 116  
WP\_030511367 RPEYIGNDVRK**RHR**RAVTNP**R**FDAMLKLFE-DG**R**VEPVRYVARPYGKTTILVTVSPIRVD 117  
WP\_191054027 KPEYIGNDVRK**RHR**RAVTNP**R**FDAMLRLFE-EG**R**VEPVRYVARPYGRTTILVTVSPIRVD 117  
WP\_195903080 MPEYIGNDVRK**HHR**RAVTNP**R**FDAMLRLFE-EG**R**VEPVSYVARPYGKITILVTVSPIRVN 117  
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MBW2064617 AKFVGCVQCVRL**K**DDTESR-------------------------------------- 123  
MCK4988321 GEFLGCVQTVRL**K**NTVSANQ------------------------------------- 125  
WP\_189438608 GELVAFCQTVLD**K**DEIQGMCETFDESGNITFQRDILPGSEPG--------------- 162  
WP\_054784913 EELVGFSQFVLL**K**EEVQELCCLFDQHGRDPFEKDMLPNGPPT--------------- 160  
WP\_282531508 GELVGYSQIVLM**K**DEIQELFRRFDESGRESFEKDMLPAWPFSGND------------ 160  
NnlA\_(OUM02170) GELVGYSQIVLL**K**DEVEALCQRFNASGRESFEREMLPDSTPSNDD------------ 164  
WP\_066989343 GELVGLAQLVLL**K**DEVQELFSRFDDSGRESFERDMLPDGYPGA-------------- 160  
WP\_051342206 GELVGFSQIVLL**K**NEVQELCERFDASGRESFEREMLPNGATGY-------LTYKKNT 166  
WP\_030511367 GELVGFSQIVLL**K**DEVQELCARFDESGRESFEREMLPNGPPAT-------------- 160  
WP\_191054027 GELVGFSQVVLL**K**DEIQELCARFDESGRESFEREMLPDTPAVARDPAAGQCSSRRS- 173  
WP\_195903080 GELVGFSQIVLL**K**DEVQELFALFDESGRESFEREMLPNGLPTA-------------- 160  
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**Figure S4.** Amino sequence alignment of NnlA homologs shown in **Figure 6** of main text. Conserved basic residues are colored red.

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| **Table S1.** Elution times for standard by size exclusion chromatography.a | | | |
| Standards | MW | Elution volumes | Gel phase distribution coefficient Kav |
| Thyroglobulin | 670000 | 10.19 | 0.073 |
| γ-globulin | 158000 | 13.82 | 0.317 |
| Ovalbumin | 44000 | 16.45 | 0.493 |
| Myoglobin | 17000 | 18.54 | 0.634 |
| aFlow rate 0.75 mL/min, 100 mM Tricine and 100 mM NaCl buffer pH 7.5. | | | |

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| **Table S2.** NnlA Homologs Analytical Size Exclusion Values.a | | | |
| Protein Sample | MW | Elution Volume | Gel phase distribution coefficient Kav |
| *Pd* NnlA | 49,000 | 16.23 | 0.478 |
| *Ps* NnlA | 38,200 | 16.79 | 0.516 |
| *Ms* NnlA | 36,400 | 16.90 | 0.523 |
| *Mr* NnlA | 35,900 | 16.93 | 0.526 |
| Oligomer *Vs* NnlA | 397,000 | 11.51 | 0.162 |
| Dimer *Vs* NnlA | 41,400 | 16.61 | 0.504 |
| aFlow rate 0.75 mL/min, 100 mM Tricine and 100 mM NaCl buffer pH 7.5. | | | |

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| **Table S3.** Nitrogen mass balance resulting from NnlA reaction with NNG | | |
| **NnlAa** | **Reduced NnlA [NO2+]final (µM)** | **As Isolated NnlA [NO2+]final (µM)** |
| *Mr* | 250 ± 10 | 10.6 ± 1.7 |
| *Pd* | 260 ± 10 | 51.3 ± 5.3 |
| *Ps* | 250 ± 10 | 31.0 ± 6.7 |
| *Ms* | 250 ± 20 | 22.2 ± 8.5 |
| aReaction conditions: 5 μM NnlA, 10 µM sodium dithionite for reduced NnlA and no reducing agent for as isolated NnlA, 350 μM NNG in 30 mM tricine buffer at pH 7.5 and room temperature in anaerobic glovebox. | | |

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| **Table S4.** Test of 2-NAE degradation *Vs* NnlA (m/z 105.03).a | | |
| **Sample** | **[NO2+]final (µM)** | **Area of Integration** |
| Control samples | -5.5 ± 1.4 | 2.4 ± 0.4 x 106 |
| Reaction samples | 1.8 ± 11.0 | 2.0 ± 0.5 x 106 |
| aSamples containing 2 mM 2-NAE, excess titanium citrate, and either no *Vs* NnlA for the control samples or 20 µM reduced NnlA (FeII-NnlA) for the reaction samples. Samples were incubated overnight at room temperature in deoxygenated 23.3 mM tricine buffer pH 7.5 | | |

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| **Table S5:** Expected NNG degrading bacteria based on this study. | | | |
| **Species** | **Bacterial Class** | **Location isolated** | **Ref.** |
| *Variovorax sp.* Strain *JS 1663* | betaproteobacteria | USA: activated sludge from  Ammunition Plant | **Ref. 1** |
| *Pseudovibrio denitrificans* JCM 12308 | alphaproteobacteria | Taiwan: seawater | Ref. 2. |
| *Pseudovibrio japonicus* strain KCTC 12861 | alphaproteobacteria | Japan: seawater | Ref. 3. |
| *Pseudonocardia spinosispora* DSM 44797 | actinomycetia | S. Korea: soil | Ref. 4 |
| *Mycobacterium sp.* 1465703.0 | actinomycetia | Mozambique: Host cultures | J. Craig Venter Institute Genome Center for Infectious Diseases.Accession: PRJNA305922 |
| *Microbispora rosea subsp. nonnitritogenes* strain NRRL B-2631 | actinomycetia | Unknown: acidic volcanic ash | Ref. 5 |

1. Mahan, K. M.; Zheng, H.; Fida, T. T.; Parry, R. J.; Graham, D. E.; Spain, J. C., A novel, iron-dependent enzyme that catalyzes the initial step in the biodegradation of N-nitroglycine by Variovorax sp. strain JS1663. *Appl. Environ. Microbiol.* **2017,** *83*, e00457-17.
2. Shieh, W. Y., Lin, Y. T., Jean, W. D. Pseudovibrio denitrificans gen. nov., sp. nov., a marine, facultatively anaerobic, fermentative bacterium capable of denitrification. *International journal of systematic and evolutionary microbiology*, **2004**, *54*(6), 2307-2312
3. Hosoya, S., Yokota, A., Pseudovibrio japonicus sp. nov., isolated from coastal seawater in Japan. *International journal of systematic and evolutionary microbiology*. **2007**, 57(9) 1952-1955.
4. Lee, S. D., Kim, E. S., Kang, S. O., Hah, Y. C. Pseudonocardia spinosispora sp. nov., isolated from Korean soil. *International journal of systematic and evolutionary microbiology*, **2002**, *52*(5), 1603-1608.
5. Nonomura, H. Distribution of actinomycetes in the soil. IV. Isolation and taxonomy of the genus Microbispora. *J. Ferment. Technol.* **1960**, *38*, 401-405.