Resource concentration modulates the fate of dissimilated nitrogen in a dual-pathway Actinobacterium

David C. Vuono, Robert W. Read, James Hemp, Benjamin W. Sullivan, John A. Arnone III, Iva Neveux, Robert R. Blank, Evan Loney, David Miceli, Mari Winkler, Romy Chakraborty, David A. Stahl, Joseph J. Grzymski*

* Correspondence: Joseph J. Grzymski: Joe.Grzymski@dri.edu

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SI Materials and Methods

Media preparation: Media preparation was conducted in a 2L Widdel Flask. After autoclaving, the media was immediately put under an anoxic headspace (N2/CO2 80:20 mix) and sterile filtered (0.2µm) trace elements, trace vitamins, and reducing agent were added. The media was cooled under an anoxic headspace and buffered with bicarbonate to maintain a pH of 7.2. Hungate technique was used to dispense media into culture tubes (20 mL) and serum vials (100 mL) pre-flushed with a sterile stream of ultra-high purity (UHP) N2 and sealed with blue 1” butyl rubber stoppers. End-point cultures were grown in Balch tubes (18x150-mm glass tube) sealed with butyl rubber stoppers. Cultures for time-course sampling were grown in 160ml serum vials. All end-point experiments were terminated after 100 hours unless otherwise noted.

Growth Curve/Cell counts/Yield Measurements: Growth curves were measured from scratch-free Balch-tubes grown cultures using an automated optical density reader at OD600 nm (Lumenautix LLC, Reno, NV). End-point cultures were monitored until all replicates reached stationary phase (65-100 hours depending on C:NO3- treatment) (Figure S7).

Cell counts were performed by fixing cells in 4% paraformaldehyde (final concentration) for 20 minutes, filtered onto 0.2µm pore-sized black polycarbonate filters, and washed three times with phosphate buffered saline (PBS, pH 7.2). Filtered cells captured on the black polycarbonate filters
were stained with SYBR® Gold nucleic acid stain (10-minute incubation) (ThermoFisher Scientific) and counted manually with a fluorescence microscope (Olympus BX60, Tokyo, Japan). We collected cells from during lag phase, exponential phase, and stationary phase in order to create a standard curve of cell counts versus optical density (OD$_{600}$). We fit a linear model to cell count versus OD$_{600}$ ($R^2=0.99$) and used the resulting linear equation for cell count enumeration for growth curves during our various treatment conditions.

Biomass concentrations were measured by filtration and drying as per standard protocol (APHA, 2012) for 8mM lactate/12mM nitrate and 0.8mM lactate/1.2mM nitrate treatments and conducted in parallel with growth curve/cell counts as described above. Analysis from triplicate cultures yielded (0.064 ± 0.003) and (0.016 ± 0.001) mg of biomass (dry weight) ml$^{-1}$ for 8mM and 0.8 mM lactate cultures, respectively. Cell counts from stationary phase cultures were (1.5 ± 0.05) x 10$^7$ and (1.16 ± 0.09) x 10$^6$ for 8mM and 0.8 mM lactate cultures, respectively. From these values the dry weight of a single *I. calvum* cell was estimated to be 1.09 x 10$^{-10}$ g. Growth yield ($Y$) (Table S5) was calculated by dividing biomass (g) by lactate mass (g) and moles consumed, as described by (White, 2000).

Lactate measurements are described below.

Thermodynamic calculations for anaerobic lactate oxidation with nitrate and nitrite were carried out using standard Gibbs free-energy values defined by Thauer et al., (Thauer et al., 1977).

**Ion and Gas Chromatography Measurements:** New glass IC vials were used for every sample in order to ensure no cross contamination of analytes. Ammonium production via respiratory nitrite ammonification was measured as described by (Yoon et al., 2013). Briefly, because the bacterium simultaneously produces (via dissimilation) and consumes (via assimilation) ammonium, ammonium consumption was first measured with O$_2$ and lactate by calculating the difference between starting and ending ammonium concentrations. These ammonium consumption values were then normalized to lactate consumed (0.31µmols NH$_4^+$/lactate) (7.07x10$^{-7}$µmols NH$_4^+$/cell calculated from average cell number of stationary phase biomass; Figure S7). Ammonium production during nitrate reducing conditions was then calculated using the mass balance approach from (Giardina and Ryan, 2002) for Total Belowground Carbon Allocation (TBCA) but adapted for nitrogen flux instead of carbon flux:

$$\Delta\text{NH}_4^+ = (\Delta\text{lactate}_{\text{start-end}} \times 0.31\mu\text{mols NH}_4^+/\text{lactate}) + \Delta\text{NH}_4^+_{\text{end-start}}$$  \hspace{1cm} (1)
Here, $\Delta \text{lactate}_{\text{start-end}}$ (µmols) is multiplied by the ammonium consumed per lactate consumed constant. This value is added to $\Delta \text{NH}_4^+_{\text{end-start}}$ (µmols), denoted as ending minus starting concentration, which defines whether the change in ammonium is positive (more ammonium produced than consumed) or negative (more ammonium consumed than produced).

Headspace gas from Balch tubes and serum vials was sampled with volume appropriate gastight syringes (Hamilton Company, Reno, NV) pre-flushed with UHP N₂. For high and low nutrient treatments, 10µl and 100µl of headspace were sampled and diluted into 12ml exetainers (Labco, Lamptet, Wales, UK) over-pressurized with 15ml UHP N₂, respectively. Similar dilutions were performed for nitrite as e-acceptor experiments, ammonium-free experiments, and time-series experiments. For time-series experiments, an equal volume of headspace gas that was removed at each time-point was replaced with sterile UHP N₂. N₂O and NO were measured by gas chromatography (Shimadzu Greenhouse Gas Analyzer GC-2014) using a 500µl injection volume. The rubber septa on the injection port of the GC was replaced after 100 injections in order to prevent leakage of the sample after the injection needle was lifted out from the injection port. Aqueous concentrations of N₂O were calculated using a Henry’s constant of 1.751 (mM (g)/mM (aq)) corrected for the medium’s ionic strength and temperature. A total of 8-11 replicates per treatment were analyzed for all experiments discussed in this work (Table S2).

Phylogenetic, Genomic, and Transcriptomic Analysis: A set of 34 NrfA amino acid sequences, representing 33 complete genome sequences and 1 octaheme nitrite reductase (ONR) from known respiratory ammonification organisms were downloaded from GenBank (Table S3). A multiple sequence alignment (MSA) was generated from the sequences annotated as cytochrome c nitrite reductase and ONR using MUSCLE (Edgar, 2004). The resulting alignment was visualized within MEGA5 (Tamura et al., 2011) where the alignment was manually screened for the presence of conserved amino acid residues consistent with those found in NrfA (i.e., heme motifs). A maximum likelihood tree was created from the alignment using RAxML (Stamatakis, 2014) with 500 bootstrap iterations. The presence of NapA, NarG, NirK, and Nor modules were manually queried from each NCBI genome in our set and confirmed by MSA, as described above. Metabolic pathway for pool quinone type was queried on BioCyc Pathway/Genome Database (biocyc.org) for each organism in our set. The structure of *I. calvum*'s NirK protein was predicted using the protein structure predicting algorithm Phyre2 (Kelley et al., 2015). Protein atomic composition for C and N was calculated from amino acid sequences as input files, as described by (Baudouin-Cornu et al., 2004; Grzymski and
Dussaq, 2012), using custom python scripts for each element separately (github.com/dvuono/Cost_minimization).

Due to the high similarity of C5 to 7KIP, reads were aligned to the *Intrasporangium calvum* genomic reference sequence and gtf file (Acc: NC_014830.1) using the STAR RNA-seq aligner (Dobin et al., 2013), with the --limitBAMsortRam parameter set to the recommended value by STAR. Sequence reads were mapped to genomic features to obtain count data using featureCounts (Liao et al., 2014). Systematic changes across experimental conditions were performed on normalized read counts in DESeq2 (Love et al., 2014). The RNA-seq data reported in this study are available within the NCBI BioProject number PRJNA475609.
Supplementary Figure 1. Mean cell concentrations for I. calvum cultures grown over a range of C:NO$_3^-$ ratios (columns) at high nutrient (top row) and low nutrient (bottom row) concentrations of the same ratio. Each growth curve consists of n=6 replicates.
Supplementary Figure 2. Growth curve of I. calvum in a sealed Balch-tube with lactate and O$_2$ as electron donor/acceptor pair and with ammonium as sole nitrogen source.
Supplementary Figure 3. Relationship between growth rate and the fraction of N dissimilated by respiratory ammonification for high and low nutrient concentrations. Treatments under C and NO₃⁻ scarcity, even with low C:NO₃⁻ ratios, disproportionally produce more ammonium and have higher growth rates.
Supplementary Figure 4. Time-series metabolite profiles of a 300-hour incubation for (A) high nutrient and (B) low nutrient concentrations. Shown are the profiles of lactate, nitrate, and nitrite (top pane), production of dissimilated end-products as N₂O-N and net change in NH₄⁺ ammonium production (middle pane), and corresponding growth curves of I. calvum cells (C:NO₃⁻ ratio = 2) (bottom pane).
Supplementary Figure 5. Time-series metabolite profiles of a 72-hour incubation conducted in balch-tubes grown under 8mM lactate 12mM nitrite (C: NO$_2^-$ ratio = 2). Profiles for lactate and nitrite (top pane) and production of dissimilated end-products as N$_2$O-N and net change in NH$_4^+$ ammonium production (bottom pane).
Supplementary Figure 6. The genome-wide transcriptional changes of early exponential, late exponential, and stationary phase I. calvum cells. The first and second outermost rings (dark and light green indicate the open reading frames (ORFs) on the positive and negative strands. The third, fourth, and fifth rings are the relative abundance of transcripts mapped onto the I. calvum genome based on the transcript read counts from early exponential phase, late exponential phase and stationary phase, respectively. The position and locus IDs are marked for the most highly expressed genes and genes involved in the ETC.
Table S1. Literature summary of C: NO₃⁻ ratio controls on N dissimilation.

| Citation                  | C-source     | C:N range | C conc range | NO3 conc range | units             | calc method                  |
|---------------------------|--------------|-----------|--------------|----------------|-------------------|-------------------------------|
| Kraft et al. 2014         | amino acids  | 1.5-3     | 4.4-43.5     | 0.5-14.4       | mmol              | mmol-C/mmol-∑NO₃⁻            |
| Yoon et al. 2015          | lactate      | 1.5-150   | 0.1-10       | 0.2            | mM                | nC*mM-C/nN*mM-N               |
| Van den Berg et al. 2015  | acetate      | 1.8-7.7   | 160-595      | 82-93          | mg/L              | mg-COD/mg-N                   |
| Schmidt et al. 2011       | Soil organic-C | not specified | 2.7-11.4 | 22.4-79.8 | C%,mg-N/kg soil | not specified                 |
| Hardison et al. 2015      | complex      | not specified | C+ - C- | 0.6-5 | μg | not specified |
| Fazzolari et al. 1998     | glucose      | 2.5-10    | 250-1000     | 100            | mg/kg dried soil | mg-C/mg-N                     |
| This study                | lactate      | 0.1-4     | 0.004-16     | 1.2-12         | mM                | nC*mM-C/nN*mM-N               |
Table S2. Summary of all experimental conditions and replicate number in the current study (Figure 2 in main text).

| NO₃⁻ (mM) | Lactate (mM) | Ratio C:NO₃⁻ | experiment type | ammonium-deplete | replicates | Number of samples taken |
|-----------|--------------|---------------|-----------------|-------------------|------------|------------------------|
| 1.2       | 0.04         | 0.1           | end-point       | -                 | 9          | 2                      |
| 1.2       | 0.2          | 0.5           | end-point       | -                 | 9          | 2                      |
| 1.2       | 0.4          | 1.0           | end-point       | -                 | 10         | 2                      |
| 1.2       | 0.6          | 1.5           | end-point       | -                 | 10         | 2                      |
| 1.2       | 0.8          | 2.0           | end-point       | -                 | 9          | 2                      |
| 1.2       | 1.6          | 4.0           | end-point       | -                 | 10         | 2                      |
| 12        | 0.4          | 0.1           | end-point       | -                 | 10         | 2                      |
| 12        | 2            | 0.5           | end-point       | -                 | 8          | 2                      |
| 12        | 4            | 1.0           | end-point       | -                 | 8          | 2                      |
| 12        | 6            | 1.5           | end-point       | -                 | 10         | 2                      |
| 12        | 8            | 2.0           | end-point       | -                 | 10         | 2                      |
| 12        | 16           | 4.0           | end-point       | -                 | 8          | 2                      |
| 12        | 8            | 2.0           | time-series     | -                 | 3          | 17                     |
| 12        | 8            | 2.0           | time-series     | -                 | 3          | 17                     |
| 12*       | 8            | 2.0           | time-series     | -                 | 11         | 4                      |
| 12        | 8            | 2.0           | time-series     | +                 | 10         | 3                      |

* nitrite is used as the electron acceptor
| Organisms                        | Accession #         |
|---------------------------------|---------------------|
| **NrfA**                        |                     |
| Escherichia coli K-12           | NC_000913.3         |
| Salmonella enterica CT18        | NC_003198.1         |
| Yersinia kristensenii           | NZ_CP009997.1       |
| Yersinia frederiksenii         | NZ_CP009364.1       |
| Vibrio fischeri ES114          | NC_006840.2         |
| S._loihica-PV-4                 | NC_009092.1         |
| Shewanella oneidensis_MR-1      | NC_004347.2         |
| Desulfotalea psychrophila_Lsv54 | NC_006138.1         |
| Sulfurospirillum deleyianum     | NC_013512.1         |
| Wolinella succinogenes          | NC_005090.1         |
| Flexibacter tractusus           | NC_014759.1         |
| Porphyromonas gingivalis_W83   | NC_010729.1         |
| Symbiobacterium thermophilum    | NC_006177.1         |
| Carboxythermus hydrogenoformans | NC_007503.1         |
| Desulfovibrio vulgaris_Hildenborough | NC_002937.3     |
| Bacillus vireti                 | NZ_LDN01000003.1    |
| Bacillus bataviensis            | NZ_AJLS01000002.1   |
| Bacillus azotoformans           | NZ_AJLR01000001.1   |
| Bacillus selenitireducens_MLS10 | NC_014219.1         |
| Campylobacter jejuni            | NC_002163.1         |
| Opitutus terreae                | NC_010571.1         |
| Anaeromyxobacter dehalogenans_2_CP-1 | NC_011891.1 |
| Rhodopirellula baltica          | NC_005027.1         |
| Intrasporangium calvum 7KIP     | NC_014830.1         |
| Intrasporangium calvum C5      | This study          |
| Bdellovibrio bacteriovorus     | NC_005363.1         |
| Gimesia maris                   | NZ_ABCE01000001.1   |
| Candidatus Nitrospira inopinata | NZ_LN885086.1       |
| Myxococcus xanthus              | NC_008095.1         |
| Geobacter metallireducens_GS_15| NC_007517.1         |
| Geobacter sulfurreducens_PCA   | NC_002939.5         |
| Thioalkalivibrio nitratireducens | NC_019902.2     |
| Thermodesulfovibrio yellowstonii_THEYE_A0193 | NC_011296.1 |
| **NirK**                        |                     |
| multicopper_oxidase[Intrasporangium_calvum] | WP_013494195.1 |
| nitrite_reductase_copper-containing[Shewanella_loihica] | WP_011867131.1 |
| nitrite_reductase[Candidatus_Nitrospira_inopinata] | WP_062488124.1 |
| nitrite_reductase_copper-containing[Marivirga_tractusuosa] | WP_013454821.1 |
| nitrite_reductase_copper-containing[Symbiobacterium_thermophilum] | WP_070105442.1 |
| nitrite_reductase[Opitutus_terrae] | WP_012373845.1 |
nitrite_reductase,_copper-containing_[Bdellovibrio_bacteriovorus] WP_011165004.1
Nitrite_reductase_OS=Bacillus_azotoformans_GN=nirK ZP_08007035.1
Ochrobactrum_anthropi_ATCC_49188 NC_009668.1
Bradyrhizobium_japonicum_USDA_110 NC_004463.1
Agrobacterium_fabrum_str._C58 NC_003063.2
Sinorhizobium_mellioti_1021 NC_003037.1
Pseudomonas_citronellolis_strain_SJTE-3 NZ_CP015878.1
Rhodanobacter_denitrificans_strain_2APBS1 NC_020541.1
Taylrella_equigenitalis_ATCC_35865 NC_018108.1
Flavobacterium_columnare_ATCC_49512 NC_016510.2
Actinobacillus_suis_ATCC_33415 NZ_CP009159.1
Chromobacterium_violaceum_ATCC_12472 NC_005085.1
Halopiger_xanaduensis_SH-6 NC_015666.1
Halopiger_xanaduensis_SH-6 NC_015666.1
inorhizobium_fredii_HH103 NC_016812.1
Pseudomonas_entomophila_str._L48 NC_008027.1
Pseudomonas_denitrificans_ATCC_13867 NC_020829.1
Flavobacterium_johnsoniae_UW101 NC_009441.1
Rhizobium_etli_CFN_42 NC_007766.1
Ochrobactrum_anthropi_ATCC_49188 NC_009667.1
Caulobacter_segnis_ATCC_21756 NC_014100.1
Rhizobium_giardii_bv._giardinii_H152 NZ_KB902685.1
### Table S4. Concentration and ratio experimental design and produciton values for NH$_4^+$ and N$_2$O-N.

| [C] (mM) | [NO3] (mM) | C:NO3- ratio | NH$_4$ produced (µmoles) | NH$_4$ produced (µmoles) | N$_2$O produced (µmoles) | % Recovery of Dissimilated N |
|----------|------------|--------------|--------------------------|--------------------------|--------------------------|-----------------------------|
| 16       | 12         | 4            | 1.94 ± 1.31              | 7.79 ± 3.3               | 27.4 ± 7.5               | 91.64 ± 12.9                |
| 8        | 12         | 2            | 4.91 ± 1.07              | 10.8 ± 4.1               | 18.1 ± 6.7               | 72.87 ± 9.3                 |
| 6        | 12         | 1.5          | 3.07 ± 4.50              | 10.2 ± 3.8               | 19.1 ± 6.2               | 72.77 ± 4.1                 |
| 4        | 12         | 1            | 8.06 ± 2.19              | 14.5 ± 4.2               | 18.1 ± 6.8               | 61.79 ± 5.1                 |
| 2        | 12         | 0.5          | 3.82 ± 1.92              | 8.76 ± 2.9               | 10.2 ± 3.7               | 64.21 ± 9.8                 |
| 0.4      | 12         | 0.1          | 2.05 ± 0.50              | 1.55 ± 0.2               | 0.48 ± 0.1               | 24.44 ± 7.5                 |
| 1.6      | 1.2        | 4            | 1.12 ± 0.99              | 2.39 ± 0.7               | 1.77 ± 0.2               | 70.47 ± 10.4                |
| 0.8      | 1.2        | 2            | 1.50 ± 0.57              | 2.32 ± 0.5               | 3.72 ± 0.4               | 90.71 ± 8.2                 |
| 0.6      | 1.2        | 1.5          | 0.90 ± 0.53              | 2.27 ± 0.5               | 4.53 ± 0.7               | 88.31 ± 8.6                 |
| 0.4      | 1.2        | 1            | 1.18 ± 1.17              | 2.45 ± 0.3               | 0.88 ± 0.3               | 50.20 ± 9.4                 |
| 0.2      | 1.2        | 0.5          | 1.91 ± 0.33              | 1.13 ± 0.2               | 0.18 ± 0.0               | 43.34 ± 20.0                |
| 0.04     | 1.2        | 0.1          | 0.03 ± 0.95              | 0.28 ± 0.2               | 0.06 ± 0.0               | 18.10 ± 10.9                |
Table S5. Growth rate and growth yield values for concentration and ratio experiment.

| [C] (mM) | [NO3] (mM) | C:NO3- ratio | Specific Growth Rate (µ) | Doubling Time (hours/generation) | Growth Yield cells (g)/Lac(g) | Molar Growth Yield cells (g)/moles Lac | Growth Yield cells (g)/NO2(g) | Molar Growth Yield cells (g)/moles NO2 | Growth Yield cells (g)/NO3(g) | Molar Growth Yield cells (g)/moles NO3 |
|----------|------------|--------------|--------------------------|-------------------------------|-------------------------------|----------------------------------------|-------------------------------|----------------------------------------|-------------------------------|----------------------------------------|
| 16       | 12         | 4            | 0.143 ± 0.02             | 4.96 ± 0.73                   | 0.25 ± 0.05                   | 22.3 ± 4.6                              | 0.10 ± 0.02                   | 6.43 ± 1.23                           | 0.63 ± 0.19                   | 28.8 ± 8.6                             |
| 8        | 12         | 2            | 0.144 ± 0.02             | 4.86 ± 0.55                   | 0.29 ± 0.02                   | 26.4 ± 1.4                              | 0.12 ± 0.03                   | 7.61 ± 1.77                           | 0.66 ± 0.21                   | 30.1 ± 9.6                             |
| 6        | 12         | 1.5          | 0.150 ± 0.03             | 4.77 ± 0.94                   | n.a.                         | n.a.                                   | 0.11 ± 0.02                   | 6.84 ± 1.45                           | 0.62 ± 0.21                   | 28.6 ± 9.5                             |
| 4        | 12         | 1            | 0.150 ± 0.01             | 4.65 ± 0.34                   | 0.26 ± 0.11                   | 23.5 ± 9.6                              | 0.12 ± 0.05                   | 7.46 ± 2.79                           | 0.62 ± 0.25                   | 28.5 ± 11.3                            |
| 2        | 12         | 0.5          | 0.150 ± 0.03             | 4.82 ± 1.12                   | 0.27 ± 0.02                   | 24.3 ± 2.2                              | 0.10 ± 0.03                   | 6.38 ± 1.88                           | 0.58 ± 0.18                   | 26.7 ± 8.1                             |
| 0.4      | 12         | 0.1          | 0.290 ± 0.05             | 2.46 ± 0.51                   | 0.46 ± 0.003                  | 41.1 ± 0.27                            | 0.15 ± 0.01                   | 9.41 ± 0.66                           | 0.92 ± 0.20                   | 42.5 ± 9.4                             |
| 1.6      | 1.2        | 4            | 0.241 ± 0.05             | 2.98 ± 0.69                   | 0.26 ± 0.03                   | 23.5 ± 3.1                              | 0.11 ± 0.01                   | 6.86 ± 0.72                           | 0.83 ± 0.05                   | 38.32 ± 2.48                           |
| 0.8      | 1.2        | 2            | 0.146 ± 0.05             | 5.31 ± 1.96                   | 0.29 ± 0.02                   | 25.7 ± 2.2                              | 0.11 ± 0.00                   | 6.99 ± 0.01                           | 0.78 ± 0.06                   | 36.07 ± 2.93                           |
| 0.6      | 1.2        | 1.5          | 0.164 ± 0.03             | 4.39 ± 0.97                   | 0.23 ± 0.02                   | 20.8 ± 2.3                              | 0.10 ± 0.00                   | 6.47 ± 0.02                           | 0.63 ± 0.09                   | 29.06 ± 4.20                           |
| 0.4      | 1.2        | 1            | 0.284 ± 0.02             | 2.45 ± 0.17                   | 0.27 ± 0.01                   | 23.9 ± 0.5                              | 0.10 ± 0.00                   | 6.51 ± 0.03                           | 0.73 ± 0.09                   | 33.72 ± 4.16                           |
| 0.2      | 1.2        | 0.5          | 0.214 ± 0.04             | 3.33 ± 0.67                   | 0.57 ± 0.01                   | 51.4 ± 0.6                              | 0.12 ± 0.00                   | 7.67 ± 0.19                           | n.a.                         | n.a.                                   |
| 0.04     | 1.2        | 0.1          | 0.344 ± 0.10             | 2.16 ± 0.64                   | n.a.                         | n.a.                                   | n.a.                         | n.a.                                   | n.a.                         | n.a.                                   |