Supplementary Information

Substrate and electron donor limitation induce phenotypic heterogeneity in different metabolic activities in a green sulphur bacterium

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This document contains supplementary information used in this study including the description of Material and Methods, a supplementary table, and references.

Material and Methods

Media. The isolation and culture medium for *C. phaeobacteroides* was prepared in a 5 L Widdel-bottle as described previously (Zimmermann *et al.*, 2015). The basal medium contained per litre of distilled water: 0.5 g KH_2PO_4, 0.34 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2$. After autoclaving at 121 °C for 20 min and cooling to 90 °C the headspace was exchanged with 100% N_2 gas for 15 min. After cooling the medium to room temperature under positive N_2 gas pressure, the following anaerobic and sterile solutions were added per litre of basal medium: 30 ml $NaHCO_3$ (1M), 5 ml $Na_2S \cdot 9H_2O$ (0.5M), 0.5 mL Vitamin B_{12} (1M) and 1 mL Trace Element Solution. The Trace Element Solution contained 2.1 g $FeSO_4 \cdot 7H_2O$, 13 mL 25 % HCl, 5.2 g Na_2EDTA, 30 mg H_3BO_3, 100 mg $MnCl_2 \cdot 4H_2O$, 190 mg $CoCl_2 \cdot 6H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, 2 mg $CuCl_2 \cdot 2H_2O$, 144 mg $ZnSO_4 \cdot 7H_2O$ and 36 mg $Na_2MoO_4 \cdot 2H_2O$ per litre of distilled water and was adjusted to pH 6.0 with NaOH before autoclaving. To individually adjust the NH_4^+ content in each incubation, we did not add NH_4Cl at this stage. The medium was adjusted to a pH around 6.8 with concentrated HCl and NaOH and was stored at room temperature in serum bottles that were crimp-sealed with blue butyl rubber stoppers. Sterile and anaerobic NH_4Cl stock solutions (1M, 100mM, 10mM) were prepared to adjust the H_2S/NH_4^+ ratio within the incubations. For $^{13}CO_2$ incubations, the basal medium was prepared with 80 % ^{12}C-$NaHCO_3$ (i.e. 24 mL L^{-1} of 1M). The remaining 20 % were added from concentrated stock solutions as ^{13}C-$NaHCO_3$ upon start of the stable isotope incubation.

The medium for the stable isotope pulse was prepared from the culture medium with 80 % unlabelled $NaHCO_3$. For $^{15}N_2$ incubations, 17 mL $^{15}N_2$ gas per L medium (Sigma, Lot Sigma MBBB0968V) was added as a bubble to $^{14}N_2$-saturated medium in a crimp-sealed bottle. The medium bottles were equilibrated overnight on a rotary shaker, resulting in 50 % $^{15}N_2$ labelling in the pulse medium.

Anaerobic plating was realized in 500 mL Schott bottles sealed with black rubber stoppers. Agar (4.5% Agar-Agar, Bacteriology Grade, Applichem) and growth media were mixed 1:3 in an anaerobic bench (240 mL total volume). After setting the agar,

200 µL of diluted culture (about 100 cells) were evenly distributed over the surface. The colonies can be stored in the dark at 4°C for a few months. We failed in establishing a revivable freeze stock with DMSO as described earlier (Overmann, 2006).

Isolation. *C. phaeobacteroides* was isolated from water samples collected in the chemocline of Lake Cadagno at around 12 m depth with a Niskin bottle in September 2012. A water sample of 2 mL was inoculated into 120 mL NH_4^+-free, anoxic, autotrophic growth media for green sulphur bacteria (described above) and grown at 20 °C under a Radium BioSun Spectralux® fluorescent lamp (NL-T8 36W/965/G13, Radium, Germany) at 20 µmol photons m^{-2} s^{-1}.

We used cells that went through minimal cycles of growth in the laboratory during isolation. Cells grew for 5 to 6 cycles in liquid enrichment culture before the liquid culture was spread on agar. A single colony was picked, dissolved, and spread again on agar. All experiments described in this work were performed with these colonies. For experiments, the bottle was opened in an anaerobic chamber (Coy Laboratories with 2 - 3% H_2 in N_2) and a single colony was picked and dissolved to inoculate an experimental pre-culture from which the experimental cultures were started.

The identity and purity of the strain was confirmed by CARD-FISH with the horseradish peroxidase-labelled oligonucleotide probe Chlp441 (AAATCGGGATATTCTTCCTCCAC; pos. 441–464) targeting *C. phaeobacteroides* (Tonolla *et al.*, 2003; Zimmermann *et al.*, 2015) and by sequencing a 147 bp fragment of the 16S rRNA with a universal eubacterial primer (27F; 5′-AGAGTTTGATCCTGGCTCAG) (as described in Zimmermann *et al.*, 2015: ACTCCTACGGGAGGCAGCAGTGAGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCAACGCCGCGTGGATGATGAAGTTCTTCGGAATGTAAAGTCCTTTTGTGGAGGAAGAATATCCCGATTTATCGGGACTGACGGTACTCCG. The sequence was checked against the NCBI 16S database (28th of October 2016) and was 100 % identical to 16S ribosomal RNA gene of *C. phaeobacteroides* strain DSM 266.

Batch cultivation. All batch growth experiments were conducted at 20 °C under static conditions (no shaking) with a Radium BioSun Spectralux® fluorescent lamp (NL-T8 36W/965/G13, Radium, Germany) constantly (no day-night cycle) emitting 20 µmol photons m^{-2} s^{-1}. A colony was grown to stationary phase at a density of 10^8 cells mL^{-1} in NH_4^+-free medium. The maximum final cell density was stoichiometrically limited by the sulphide concentration because measurements showed that all sulphide was depleted in stationary phase cultures. This pre-culture was used to inoculate the batch incubations in biological duplicates. The incubations were inoculated with 0.75×10^6 cells mL^{-1} to NH_4^+-free medium in half of the incubation volume (18 mL in a 36 mL serum bottle) and were pre-grown for three generations to equilibrate to the culture conditions. We pulsed with pre-prepared pulse medium that contained ^{13}C-$NaHCO_3$ (at 20 % labelling) and that was labelled with 50 % $^{15}N_2$ gas. We pulsed by adding 18 mL pre-prepared pulse medium to the cell suspension leaving no headspace in the serum vial to avoid $^{15}N_2$ outgassing. Thus, the final stable isotope enrichment during the incubation was 10 % ^{13}C-CO_2/HCO_3 and 25 % $^{15}N_2$. The subsequent stable isotope incubation lasted for 18 h, which is equivalent to the generation time. This timing ensured that sulphide did not become limited during the labelling period and that populations remained within the exponential growth phase during the incubation. The final cell concentration at sampling was approx. 6×10^6 cells mL^{-1}, while the maximum cell concentration was approx. 10^8 cells mL^{-1}. The H_2S measurements in the sample at the end of the pulse are shown in Supplementary table 1.

Chemostat cultivation. All chemostat growth experiments were conducted at 20 °C with a Radium BioSun Spectralux® fluorescent lamp (NL-T8 36W/965/G13, Radium, Germany) constantly (no day-night cycle) emitting 20 µmol photons m^{-2} s^{-1}. The chemostat cultures were grown in 60 mL serum flasks containing 30 mL liquid culture and a magnetic stirrer. Feed medium was supplied by a peristaltic pump (IPC-N, Ismatec) with a flow rate of 10 µL min^{-1} ($D = \mu = 0.02$ h^{-1}) and dropped into the chemostat vessel. The dilution rate D determines the specific growth rate µ (and thus the generation time) because in a chemostat D balances the supply of growth limiting nutrients with the wash-out of biomass. We used Tygon tubing (inner diameter of 0.51mm, SC0005, ISMATEC), polypropylene Luer-Lock fittings and

chrome-nickel needles (STERICAN, Huber Lab, Switzerland). The outflow was led over the same peristaltic pump into a waste bottle. Hence, N_2 was only supplied dissolved in the medium and was not purged into the liquid, because preliminary experiments showed that bubbling the culture led to aggregation of the cells. Feed medium and incubations were constantly, gently stirred with a magnetic stirrer placed on a multi-stirrer plate (Variomag).

The chemostats were inoculated with pre-cultures originating from one colony that was grown to a density of 10^8 cells mL^{-1} once in NH_4^+-free medium (to inoculate NH_4^+ depleted chemostats) and once in medium with 1000 µM NH_4^+(to inoculate the chemostats that were fed with NH_4^+). The chemostats were inoculated with 4.7×10^7 cells mL^{-1} and equilibrated for 10 days (i.e. five volume turnovers). Next, we pulsed the chemostats with ^{13}C-$NaHCO_3$ and $^{15}N_2$ by exchanging the 30 mL headspace after 20 s of evacuation with 100% $^{15}N_2$ (Sigma, Lot Sigma MBBB0968V). The dissolution of $^{15}N_2$ was supported by vigorous shaking for 1 min. ^{13}C-$NaHCO_3$ was added by exchanging the feed medium supply to medium labelled with 20 % ^{13}C-CO_2/HCO_3. Samples for chemical analysis (NH_4^+, H_2S), cell counting, bulk isotope analysis by isotopic ratio mass spectrometry and NanoSIMS were withdrawn from the chemostats after 8 h and 17 h (0.23x and 0.5x generation time) of incubation.

To calculate the average ^{13}C enrichment during the course of the incubation we described the dilution of the unlabelled HCO_3 after the switch to the pulse medium by the following differential equation:

$$\frac{d}{dt}C_{unlab}(t) = -DC_{unlab}(t) - \frac{B_0\mu_C}{y_C}\frac{C_{unlab}(t)}{C_0} \qquad [1]$$

The first term represent loss due to dilution (with dilution rate *D*) and the second term represent loss due to consumption; the rate of consumption of unlabeled HCO_3 is the rate of consumption of glucose (labeled and unlabeled) $\frac{B_0\mu_C}{y_C}$ times the fraction of unlabeled HCO_3 present in the chemostat $\frac{C_{unlab}(t)}{C_0}$. μ_c is the maximum growth rate (0.047 $hour^{-1}$), C_0 is the standing concentrations of HCO_3 in the chemostat (which is constant through time) (28 mM), B_0 is the equilibrium bacterial

density in the chemostat (3.6×10^6 to 7×10^7 cells ml^{-1}) and y_C is the yield (1.9×10^6 to 3.3×10^7 cells (µmol C)$^{-1}$). The time-averaged ^{13}C enrichment, f_{13CO2}, when pulsing with a feed medium enriched with 20 % ^{13}C-HCO_3 during the incubation time t can then be calculated as:

$$f_{13CO2} = 0.2 \cdot \left(1 - \frac{\int_0^t C_{unlab}(t)dt}{\int_0^t C_0 dt}\right) \quad [2]$$

Total nitrogen in the cell biomass of the chemostat ($N_{biomass}$ in mol) was determined from the elemental analyser data. This parameter was used together with the NH_4^+ concentration in the feed medium (C_{NH4+} in mol L^{-1}) and the chemostat volume ($V_{chemostat}$ in L) to calculate the percentage of required nitrogen supplied as NH_4^+ ($N_{required}$ in %) according to

$$N_{required}\ [\%] = \left(\frac{C_{NH4+} \times V_{chemostat}}{N_{biomass}}\right) \times 100\ . \quad [3]$$

Sample preparation and NanoSIMS analysis. For the single cell analysis, we fixed 2 mL of each incubation in methanol-free 1% PFA in phosphate-buffered saline (Electron microscopy sciences) at 4 °C overnight, filtered 1.57×10^6 cells on 5 mm diameter filter pieces (0.2 µm pore size, GTTP, Millipore, sputter-coated with a ~20nm thick Au film) and rinsed with H_2O and 0.01 M HCL. We used Parafilm and a punch disk to confine the filtration area to a circular area with a diameter of 3mm. Samples were stored dry in a desiccator containing silica-gel at room temperature until NanoSIMS analysis. The filters were coated with 0.1 % low melting agarose pre-warmed to 37°C to avoid cell loss.

The filter pieces were mounted onto glass slides with a mounting solution containing five parts citifluor AF1 (Citifluor Ltd, UK) and one part vectashield (Vectorlabs, UK), and the general DNA stain Hoechst (10 µg mL-1). Areas of interest were marked with laser micro-dissection (PALM micro-dissection, Zeiss 200 M equipped with a 355 nm pulsed UV laser and epifluorescence illumination). For each mark, we gathered images of DNA fluorescence (Dapi filter: ex. 387/11; em. 440/40) that we

could use to overlay with the subsequent NanoSIMS images to improve later cell segmentation.

The marked areas were analysed with a NanoSIMS 50L (CAMECA) at the Center for Advanced Surface Analysis at EPFL/University of Lausanne. The areas were pre-sputtered with a Cs^+ primary ion beam of 4 – 4.2 pA (D1–D2) to remove surface contamination, to implant Cs^+ ions into the sample, and to achieve an approximately stable secondary ion emission rate. A primary Cs^+ ion beam with a beam current between 1 - 1.2 pA (D1–D3) and a beam diameter of around 100 nm was rastered across the cells for analysis with a dwell time of 5 ms per pixel. Secondary ion images for $^{12}C^{12}C^-$, $^{13}C^{12}C^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$, and $^{32}S^-$ were simultaneously recorded from analysis areas of 25 µm × 25 µm or 30 × 30 µm with a resolution of 256 × 256 pixels and 50 µm × 50 µm or 60 × 60 µm with a resolution of 512 × 512 pixels. Five planes from each individual area were measured. Mass resolving power was around 10 000 (Cameca definition), enough to resolve all potential mass interferences from the measured secondary beams. Standards consisting of cells grown in the absence of isotopically labelled substrates were prepared and analysed in the same way and determined independently for each experiment. The background ^{15}N-fraction and ^{13}C-fraction ranged between $X(^{12}C^{15}N)_{background}$ = 0.003581 to 0.0036711 and $X(^{13}C)_{background}$ = 0.01049 to 0.01059.

Analysis of NanoSIMS images was performed with the Matlab-based software Look@NanoSIMS (Polerecky *et al.*, 2012). The images were first corrected for a possible drift of the stage during the measurement and then the counts in each pixel were accumulated over the multiple z-planes measured through the cell. We used the fluorescence image and the $^{12}C^{14}N^-$ ion-image to identify cells and to manually mark these cells as regions of interest (ROI's). The accumulated counts, c, were averaged over the area of a ROI and the atom fractions for ^{15}N-nitrogen, $X(^{12}C^{15}N)_{cell} = c(^{12}C^{15}N)_{cell}/\{c(^{12}C^{14}N)_{cell} + c(^{12}C^{15}N)_{cell}\}$ and ^{13}C-carbon, $X(^{13}C)_{cell} = c(^{13}C)_{cell}/\{c(^{12}C)_{cell} + c(^{13}C)_{cell}\}$ were calculated for each ROI. These fractions are a measure of the N_2 fixation and CO_2 fixation rates, respectively. Calculation of the N_2 fixation and CO_2 fixation rates was done as described earlier (Schreiber *et al.*, 2016). The average nitrogen content per cell and the average carbon content per cell were

determined for each experiment independently from elemental analyser data of associated IRMS measurements and ranged between 3.9 to 11.6 fmol N cell^{-1} and 27.8 to 95.3 fmol C cell^{-1}.

Statistics. The heterogeneity of $^{15}N_2$ and $^{13}CO_2$ assimilation rates within a population was quantified by the coefficient of variation (CV). The correlation between the level of heterogeneity (CV) and the level of NH_4^+ limitation was tested using linear regression. This statistical test assumes that factors that might vary between different experiments show no statistical interaction with NH_4^+ supply in their effect on the CV. A two sample t-test with equal variance was used to compare a single measurement of the phenotypic heterogeneity of a chemostat-grown population to the phenotypic heterogeneity of four batch-grown populations. The assumption that the CV values are normally distributed within each group is legitimate due to the critical limit theorem.

Bimodality was expressed using Hartigan's DIP statistic (Hartigan and Hartigan, 1985). The dip test measures multimodality in a sample by computing the maximum difference between the empirical distribution function and the best fitting unimodal distribution function. The higher the dip statistic the higher the deviation from unimodality. The statistical significance of the dip statistic for each population is inferred with a p-value that is approximated based on tabulated values. We sampled the chemostat incubations 8 and 17 hours after the isotope pulse was introduced and computed the DIP statistic and the corresponding p-values. All statistical analyses were carried out with the scientific computing tools for python (SciPy, version 0.18.1). The Hartigan's dip test for unimodality was performed using a python port of Martin Maechler's R module 'diptest' (available online: https://github.com/alimuldal/diptest).

Contamination Calculation. The $^{15}N_2$ gas lot that we used for these experiments was apparently contaminated with 1900 µmol $^{15}NH_4^+$ per mole $^{15}N_2$ (Dabundo *et al.*, 2014). We measured the contamination in each bottle as described earlier (Schreiber *et al.*, 2016) and found a contamination of 49.3 and 534.9 $^{15}NH_4^+$ per mole $^{15}N_2$ in the two bottles that we used for our experiments. We calculated the fraction

of $^{15}NH_4^+$ molecules that could maximally be fixed into new biomass formed during the incubation per $^{15}N_2$ molecules fixed using (i) the cell counts before and after the incubation for batch experiments, (ii) the dilution rate for chemostat experiments (iii) particulate nitrogen concentrations measured at the end of the incubation with an elemental analyser connected to an isotopic ratio mass spectrometer, (iv) the $^{15}N_2$ labelling%, (v) the quantity of N_2 gas amended to the incubation, (vi) the measured contamination in the bottle, and (vii) by conservatively assuming that all introduced $^{15}NH_4^+$ will be taken up. We excluded all experiments in which $^{15}NH_4^+$ makes up more than 5 % of the new ^{15}N-labeled biomass and thus expect a minor influence on the estimate of phenotypic heterogeneity in N_2 fixation.

Supplementary Table 1. Values for all single-cell measurements of batch and chemostat experiments

Treatment	Relates to Figure	Number of cells measured	Mean single-cell N_2 fixation rate (amol N h^{-1})	CV	Hartigan's DIP/p-value	Mean single-cell CO_2 fixation rate (amol C h^{-1})	CV	H_2S concen-tration (mM)***
Chemostat No NH_4^+ ($^{15}N_2$, ^{13}C-CO_2, 8h)*	1c,d	93	68.5	0.214	0.027/0.940	613.4	0.345	< 0.05
Chemostat 20% NH_4^+ ($^{15}N_2$, ^{13}C-CO_2, 8h)*	1c,d	123	58.6	0.399	0.042/0.106	888.4	0.448	< 0.05
Chemostat 21% NH_4+ ($^{15}N_2$, ^{13}C-CO_2, 8h)*	1c,d	71	30.6	0.286	0.053/0.142	313.4	0.343	< 0.05
Chemostat 38% NH_4^+ ($^{15}N_2$, ^{13}C-CO_2, 8h)*	1c,d	127	20.2	0.464	0.043/0.082	468.2	0.386	< 0.05
Chemostat 52% NH_4^+ ($^{15}N_2$, ^{13}C-CO_2, 8h)*	1c,d	97	22.5	0.654	0.102/>0.000	417.6	0.949	< 0.05
Chemostat No NH_4^+ ($^{15}N_2$, ^{13}C-CO_2, 17h)*	1d,2	108	58.3	0.323	0.029/0.781	591.8	0.368	< 0.05
Chemostat 20% NH_4^+ ($^{15}N_2$, ^{13}C-CO_2, 17h)*	1d	165	57.7	0.378	0.057/>0.000	933.1	0.561	< 0.05
Chemostat 21% NH_4^+ ($^{15}N_2$, ^{13}C-CO_2, 17h)*	1d	100	37.0	0.234	0.023/0.988	411.2	0.313	< 0.05

Chemostat **52% NH_4^+** ($^{15}N_2$, ^{13}C-CO_2, 17h)*	1d	144	29.8	0.561	0.027/0.675	922.0	0.376	< 0.05
Batch **No NH_4^+** **H_2S unlimited** ($^{15}N_2$, 18h)*	2a,2b	124	30.3	0.101	-**	-	-	0.8073
Batch **No NH_4^+** **H_2S unlimited** ($^{15}N_2$, 18h)*	2a,2b	138	26.9	0.082	-	-	-	0.81
Batch **No NH_4^+** **H_2S unlimited** ($^{15}N_2$, ^{13}C-CO_2, 18h)*	2	100	51.3	0.109	-	788.6	0.148	0.9234
Batch **No NH_4^+** **H_2S unlimited** ($^{15}N_2$, ^{13}C-CO_2, 18h)*	2	93	52.8	0.127	-	885.0	0.136	0.9612
Batch **No NH_4^+** **H_2S unlimited** (^{13}C-CO_2, 8h)*	2c,d	168	-	-	-	1052	0.131	1.1394
Batch **No NH_4^+** **H_2S unlimited** (^{13}C-CO_2, 8h)*	2c,d	151	-	-	-	771.8	0.182	1.1502

* type of isotopically labelled substrate and incubation time
** values not computed
*** detection limit < 0.05 mM

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