Phenotypic heterogeneity driven by nutrient limitation promotes growth in fluctuating environments

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Abstract

Microbes live in environments where nutrients are limited and fluctuate over time. Cells respond to nutrient fluctuations by sensing and adapting their physiological state. Recent studies suggested phenotypic heterogeneity¹ in isogenic populations as an alternative strategy in fluctuating environments: a subpopulation of cells expresses a function that allows growth under conditions that might arise in the future²⁻⁹. It is unknown how environmental factors such as nutrient limitation shape phenotypic heterogeneity in metabolism and whether this allows cells to respond to nutrient fluctuations. Here we show that substrate limitation increases phenotypic heterogeneity in metabolism, and this heterogeneity allows cells to cope with nutrient fluctuations. We subjected the N₂-fixing bacterium *Klebsiella oxytoca* to different levels of substrate limitation and substrate shifts, and obtained time-resolved single-cell measurements of metabolic activities using nanometer-scale secondary ion mass spectrometry (NanoSIMS). We found that the level of NH₄⁺-limitation shapes phenotypic heterogeneity in N₂ fixation. In turn, the N₂ fixation rate of single cells during NH₄⁺-limitation positively correlates with their growth rate after a shift to NH₄⁺-depletion, experimentally demonstrating the benefit of heterogeneity. The results indicate that phenotypic heterogeneity is a general solution to two important ecological challenges – nutrient limitation and fluctuations - that many microbes face.

Text

Our goal was to investigate whether substrate limitation shapes the level of phenotypic heterogeneity in metabolism, and to analyse whether such heterogeneity would allow microorganisms to grow in fluctuating nutrient environments. We used microbial dinitrogen (N_2) fixation in the heterotrophic N_2 -fixing bacterium *Klebsiella oxytoca* to experimentally study these links. *K. oxytoca* is a facultative anaerobic bacterium that fixes N_2 in the absence of oxygen. N_2 fixation is the biogeochemically important conversion of N_2 to ammonium (NH_4^+) by the enzyme nitrogenase followed by the assimilation into biomass. The conversion of N_2 to NH_4^+ is energetically costly and requires high nitrogenase levels¹⁰. In contrast, NH_4^+ can be assimilated directly from the environment if available. Thus, NH_4^+ is the preferred substrate over N_2 . We reasoned that individual cells engage in mixed-substrate uptake of N_2 and NH_4^+ when NH_4^+ supply is limited, and that the simultaneous uptake of both N-sources shapes heterogeneity in N_2 fixation. Further, a switch to NH_4^+ depletion can cause long lag-times of population growth¹¹, because the cells have to solve the dilemma of needing fixed N to produce the enzyme machinery that allows them to produce fixed N. We therefore hypothesized that phenotypic heterogeneity would help cells to overcome this dilemma: a subpopulation would show high N_2 fixation during mixed-substrate growth in NH_4^+ -limited environments, which would allow these cells to grow faster upon an environmental switch to NH_4^+ depletion. Here we test this hypothesis experimentally.

First, we grew *K. oxytoca* populations with limited NH_4^+ and asked whether we could detect N_2 fixation at the bulk level, which would indicate that at least a fraction of the cells fixed N_2 under these conditions. We grew chemostat cultures into steady state (Fig. 1) and quantified N_2 fixation in the bulk biomass based on the incorporation of ^{15}N -labeled N_2 ($^{15}N_2$) after 1.5 h

incubation time as measured with isotope ratio mass spectrometry. Glucose was the sole carbon source for growth in the chemostats, while NH_4^+ and N_2 were the nitrogen sources. All chemostats were run under glucose limitation with different NH_4^+ supply levels and excess N_2 (Fig. 1a). $^{15}\text{N}_2$ fixation was detected in all treatments in which NH_4^+ was limited and decreased with increasing NH_4^+ supply until the NH_4^+ supply balanced the glucose supply (Fig. 1b). Low (μM -range; Fig. 1c) steady-state NH_4^+ concentrations in the chemostats with limited NH_4^+ supply showed that the cells almost fully consumed the NH_4^+ supplied (mM-range). Together this shows that *K. oxytoca* populations assimilate NH_4^+ and N_2 simultaneously during NH_4^+ limitation to completely consume glucose. The number of cells produced per molecule of glucose (i.e. the yield) increases with increasing NH_4^+ supply (Supplementary Fig. 1). Within this range of NH_4^+ supply, changing the supply of glucose or NH_4^+ alone would affect the yield independently. Thus, the nutrient limitation regime in the chemostat resembles those of independent nutrient co-limitation, which has been suggested to be prevalent in many natural environments^{12,13}.

Figure 1: Mixed-substrate metabolism of N_2 and NH_4^+ by *K. oxytoca* populations under NH_4^+ limitation. (a) Schematic representation of six parallel chemostats that were operated with varying glucose: NH_4^+ -ratios. The height of the bars quantitatively represents the relative pool sizes of supplied carbon (glucose) and nitrogen (NH_4^+ or N_2) for each treatment. The horizontal line denotes the nitrogen pool size required to consume the supplied glucose pool. Chemostats were supplied with no NH_4^+ (black), limiting NH_4^+ (red, green, and blue), sufficient

(brown) and saturated NH_4^+ (grey). At limiting NH_4^+ supply, growth was supported by simultaneous consumption of NH_4^+ or N_2 (yellow) to consume all glucose. The glucose and NH_4^+ concentrations in the feed medium of each treatment are listed below the respective bar. Chemostats were operated under an N_2 atmosphere with a constant supply of feed medium ($V = 20 \text{ mL}$; $D = 0.1 \text{ h}^{-1}$) and were incubated for 1.5 h with $^{15}\text{N}_2$ after they reached steady state. **(b)** N_2 fixation rate of chemostat populations and **(c)** residual NH_4^+ concentrations in the chemostat plotted against the percentage of the required nitrogen supplied as NH_4^+ as calculated from the measured total nitrogen in the biomass and the NH_4^+ concentration in the feed. The horizontal line denotes the point at which the nitrogen in the biomass equals the NH_4^+ supplied with the feed. The symbols share the colour code with the schematic representation in **(b)**.

Next, we established the connection between NH_4^+ limitation and phenotypic heterogeneity in N_2 fixation. We used NanoSIMS to determine N_2 fixation rates of single cells in populations grown with different NH_4^+ supply (Fig. 2a-d). The data shows that phenotypic heterogeneity in N_2 fixation depended on the degree of NH_4^+ limitation (Fig. 2e,f, Supplementary Table 1). We used the coefficient of variation to quantify phenotypic heterogeneity and compare it between different NH_4^+ supply treatments, because it normalizes the standard deviation to the varying levels of average N_2 fixation (Fig. 2e). We observed that the level of heterogeneity depends on the degree of NH_4^+ limitation and increased with increasing NH_4^+ supply (Fig. 2f).

Figure 2: NH_4^+ limitation affects single-cell heterogeneity in N_2 fixation and *nif* gene expression in chemostat-grown *K. oxytoca* populations. **(a-d)** Single cells were detected with Hoechst general DNA stain shown in red **(a)** and their nitrogen isotopic composition was determined with NanoSIMS **(b-d)**. **(b&c)** The total accumulated ion counts of $^{12}\text{C}^{14}\text{N}^-$ and $^{12}\text{C}^{15}\text{N}^-$ in the analysed cell biomass. **(d)** The calculated atom fraction of $^{12}\text{C}^{15}\text{N}/(^{12}\text{C}^{15}\text{N}+^{12}\text{C}^{14}\text{N})$ for each pixel inside segmented cells in **b & c**. Cells displayed in **(a-d)** were grown at 80% NH_4^+ of the total required N and are representative of 1175 cells from 3 biological replicates. **(e)** Single-cell N_2 fixation rates (circles) calculated from the single-cell NanoSIMS data for a range of different NH_4^+ supplies (see colour code Fig. 1) for replicate chemostat populations (numbers of cells measured are shown in Supplementary Table 1). **(f)** The corresponding coefficients of variation in N_2 fixation activity for each replicate (squares) as shown in **(e)**. **(g)** Transcriptional noise, **(h)** mRNA copy numbers per cell, and **(i)** burst frequency (h^{-1}) of *nifH* (filled circles, black horizontal bar) and *glnK* (open circles, grey horizontal bar) for replicate chemostat populations. The population displayed with a cyan circle was removed from the analysis, since its inflated *nifH* noise and low mean and burst frequency can be explained by the corresponding chemostat being unusually close to the transition point between NH_4^+ limitation and saturation (91% NH_4^+ of the total required N). The x-axes in panels **e-i** are categorical and the horizontal bars display the means. Statistical analysis of the data sets was performed with I-Way-ANOVA: **(f)** $p < 0.0001$; **(g)** $p(\text{nifH}) = 0.0002$, $p(\text{glnK}) = 0.683$; **(h)** $p(\text{nifH}) = 0.028$, $p(\text{glnK}) = 0.575$; **(i)** $p(\text{nifH}) = 0.0069$, $p(\text{glnK}) = 0.627$.

Next, we performed a number of control experiments to evaluate the robustness of this result. First, we asked whether the increase in heterogeneity is specific to N_2 fixation. We analysed heterogeneity in the assimilation of ^{13}C -glucose and $^{15}NH_4^+$. We found that both are low compared to heterogeneity in N_2 fixation (Supplementary Fig. 2a,b) and that glucose assimilation does not strongly vary with NH_4^+ supply. This indicates that metabolic functions were not generally heterogeneous. Second, we asked if the observed heterogeneity in N_2 fixation depends on the incubation time with $^{15}N_2$. We compared populations that were incubated for 1.5 h with $^{15}N_2$ (as in Fig. 1&2) to populations incubated for 7.5 h, and found no significant differences in the coefficient of variation between the two incubation times (Supplementary Fig. 3). This suggests that individual cells did not switch frequently between states of active and inactive N_2 fixation; if they did, the variation in the ^{15}N content between cells – and thus the coefficient of variation – would continuously decrease over time. Third, it has been suggested previously that N_2 fixing cells can leak part of the NH_4^+ produced from N_2 into the medium¹⁴. This leaked NH_4^+ might be assimilated by other cells resulting in the isotopic enrichment of their biomass without concomitant fixation of N_2 . We found that leaked NH_4^+ supports on average only 10 % of the per cell N_2 fixation rate and thus does not have a strong effect on our single-cell N_2 fixation estimates (Supplementary Fig. 4 and Supplementary discussion). Together, these control experiments indicate that phenotypic heterogeneity in N_2 fixation during NH_4^+ limitation is a robust pattern.

Our results raise the question about the molecular mechanism that generates heterogeneity in N_2 fixation. To address this question, we used single-molecule mRNA fluorescence in situ hybridization (smFISH) to quantify the single-cell expression of two genes that play a central

role in N_2 fixation: the structural gene *nifH* and the regulatory gene *glnK*, which encodes a P-II regulatory protein that senses the nitrogen status of the cell and post-translationally activates NifA, the master regulator for the expression of nitrogen fixation structural genes (*nif* genes) including *nifH*¹⁰. We determined the mean mRNA number per cell, the transcriptional noise, and the symmetry of the distribution of mRNA numbers per cell in populations with varying NH_4^+ supply (Fig. 2g-i; Supplementary Fig. 5). The symmetry of the distribution is characterized by the fitting parameter r of a negative binominal distribution and is used to estimate the burst frequency of gene promoters¹⁵. First, we found that transcriptional noise in *nifH* increases with increasing supply of NH_4^+ and increased heterogeneity in N_2 fixation activity (Fig. 2e-g). This indicates that noise in gene expression transmits to variability in N_2 fixation activity. Second, we found that noise in *glnK*, as opposed to noise in *nifH*, remains unchanged between different NH_4^+ supply regimes (Fig. 2g). This suggests that noise in *nifH* expression emerges below the level of *glnK* expression. Third, we found that the mean *nifH* mRNA number and the burst frequency of *nifH* decrease with increasing NH_4^+ supply (Fig. 2h,i). This indicates that inducer levels for *nif* gene expression decrease as NH_4^+ supply increases. Similarly, previous work on the *lac* promoter in *E. coli* showed that the burst frequency decreases as inducer levels decrease¹⁵ and that intermediate inducer concentrations can shape the response of the *lac* regulatory circuit from a graded to a bistable response¹⁶. Taken together, the smFISH data suggest that intermediate inducer concentrations lead to variation in *nif* gene expression (including *nifH*) that emerges below the level of the regulator *glnK*, and that this variation in *nif* gene expression ultimately transmits to variation in activity.

Figure 3: Consequences of heterogeneity in N₂ fixation for growth during a shift from limited NH₄⁺ supply to NH₄⁺ depletion. We performed incubations in which cells were switched between the same environmental conditions, but with two different sequences of isotope labelling. The cells were grown in chemostats with limited NH₄⁺ equal to 87% NH₄⁺ as required N during the pulse-phase and for 3 h in batch mode without NH₄⁺ during the chase-phase after which samples for NanoSIMS analysis were taken. **(a)** In the first incubation a $^{15}\text{N}_2$ pulse serves to capture heterogeneity in NH₄⁺-limited chemostats before the environment switches. The cells are then subjected to the chase/pulse-phase by switching them from NH₄⁺ limitation to NH₄⁺ depletion with concomitant removal of $^{15}\text{N}_2$ and addition of ^{13}C -glucose to capture the single-cell growth rate after the transition. **(b)** A second incubation with $^{15}\text{N}_2$ and ^{13}C -glucose both added after the switch was performed to establish the link between N₂ fixation and growth during the chase-phase. Blue and cyan colours distinguish cells from two independent biological replicates. Spearman correlation coefficients for the combined data of the two replicates (r), their corresponding Gaussian approximated (GA) p-values, and confidence intervals (CI; 95%) are displayed.

Next, we asked whether cells that fix N₂ in the NH₄⁺-limited environment could more rapidly resume growth after an environmental switch to full NH₄⁺ depletion. To answer this question it is necessary to obtain single-cell N₂ fixation and growth rate measurements before and after the environmental transition. NanoSIMS is a destructive method and thus cannot directly provide dynamic measurements on substrate assimilation in the same cell. However, the isotopic composition of a cell grown with an isotopically-labelled substrate is a representation of its

metabolic activity in the past. We used this fact to devise a pulse-chase/pulse approach to extract quantitative and time-resolved information about the metabolism and growth of individual cells during an environmental transition from a non-dynamic, single NanoSIMS measurement (Fig. 3). The pulse-chase/pulse approach assumes that glucose assimilation is a good proxy for growth and that cells do not transiently accumulate carbon storage compounds without concomitant N_2 fixation. These assumptions have been confirmed separately for cells grown in steady-state chemostats and in batch after the switch (Supplementary discussion).

The pulse-chase/pulse experiment showed that individuals that invested more in N_2 fixation during NH_4^+ limitation had a growth benefit after a switch to NH_4^+ depletion. This conclusion is based on a strong positive relationship between $^{15}N_2$ fixation rates of individual cells grown under NH_4^+ limitation and their ability to assimilate ^{13}C -glucose after a switch to NH_4^+ depletion (Fig. 3a). Importantly, a second experiment with $^{15}N_2$ and ^{13}C -glucose both added only in the chase-phase showed that the ability to grow after the switch is ultimately linked to the ability to fix N_2 (Fig. 3b). This positive correlation is still evident 3 h after the switch which indicates that the heterogeneity in activity is reflected in the available enzyme pool that individual cells established before the switch and not solely due to differences in the energy charge (e.g. ATP and NADPH levels), which would dissipate rapidly after the switch. Recent studies on phenotypic heterogeneity in microbial metabolism have reported the emergence of phenotypic heterogeneity in the expression of metabolic genes during diauxic shifts in carbon sources; i.e. the switch between two different modes of unrestricted growth^{4,17,18}. In contrast, our data show that phenotypic heterogeneity can emerge during restricted (i.e. limited) growth. The connection between these different scenarios could be that diauxic shifts also represent

transient nutrient limitation, which can result in phenotypic heterogeneity due to intermediate inducer concentrations.

A simple, stochastic growth model showed that the patterns observed at the single-cell level translate into a growth benefit for populations with increasing heterogeneity (Fig. 4). We simulated populations of individuals with varying coefficients of variation at a fixed, experimentally derived mean activity ($103 \text{ amol N h}^{-1}$). The growth of these populations after the transition to NH_4^+ depletion was simulated using the experimentally derived growth benefit of N_2 fixation during NH_4^+ limitation from the slope in Fig. 3a ($0.000279 \text{ [amol N]}^{-1}$). The model demonstrates that increased heterogeneity allows populations to increase their biomass faster after a switch to substrate depletion.

Figure 4. The effect of heterogeneity in N_2 fixing populations on initial population growth after a switch simulated with a stochastic growth model. The x-axis shows the coefficient of variation in N_2 fixation before a switch to NH_4^+ depletion. The y-axis shows the mean increase of the population biomass (M_t) relative to the initial population biomass (M_0) after individual cells increase their mass (M_{cell}) at $M_{\text{cell}}(3h) = M_{\text{cell}}(0h) \cdot e^{\mu t}$ for a time (t) of 3 h at a specific growth rate μ (h^{-1}). Populations of 10060 individuals were randomly generated with varying standard deviation and a fixed, experimentally determined mean N_2 fixation activity. The specific growth rate μ of each individual cell drawn from the resulting distributions was predicted based on its N_2 fixation activity before the switch from the slope in Fig. 3a, showing the relation between N_2 fixation rate before the switch (amol N h^{-1}) and the carbon-based growth rate μ (h^{-1}). The error bars show the standard deviation between 5 replicated simulations.

Our results show that substrate limitation increases phenotypic heterogeneity in a metabolic pathway, and that this heterogeneity can be advantageous in dynamic nutrient environments. The single-cell mRNA data further suggest that phenotypic heterogeneity emerges when limited substrate supply to the cells translates into intermediate intracellular inducer concentrations. Multiple microbial metabolic activities are controlled by internal metabolite concentrations^{19,20}. Thus, biologically relevant phenotypic heterogeneity can be potentially generated in many other metabolic pathways that translate nutrient-limited, flux-controlled growth into intermediate inducer concentrations. In turn, this heterogeneity allows populations to deal with nutrient fluctuations that are expected to be pervasive under limiting conditions. Therefore, our work predicts that phenotypic heterogeneity in microbial metabolism is stronger in nutrient-limited, dynamic habitats as compared to nutrient-saturated, stable habitats. Furthermore, we observe that nutrient co-limitation - a prevalent limitation regime in many natural environments^{12,13} - provides the basis for high phenotypic heterogeneity, because it forces cells to perform mixed-substrate uptake. This connection allows establishing testable hypotheses for phenotypic heterogeneity assessments across eco-systems based on bulk nutrient addition experiments.

Phenotypic heterogeneity in metabolic activities has repeatedly been observed in natural microbial communities²¹⁻²³ and chemostats²⁴, but its origin and functional consequences in natural communities remained unresolved. The pulse-chase/pulse approach coupled to NanoSIMS provides an experimental approach to study the functional consequences of phenotypic heterogeneity in the metabolism of non-model microorganisms and directly in natural microbial communities²⁵, because it is independent from pure cultures of genetically tractable bacteria modified with fluorescent-based reporter proteins. We expect that studying the function of phenotypic heterogeneity in natural environments will add a new dimension to

253 biodiversity-ecosystem-functioning research in microbial ecology: phenotypic diversity is – along
254 with genetic diversity - potentially an important component of biodiversity sustaining ecosystem
255 functioning in ever changing environments.

Methods

Bacterial strains and media. All experiments were done with *Klebsiella oxytoca* M5A1 (DSM 7342) obtained from the German Collection of Microorganisms and Cell cultures (DSMZ) at a growth temperature of 30°C. The chemostat medium was modified from Hill²⁶ and consisted of a basal medium to which supplements were added after cooling. The basal medium consisted of 25 mM KH_2PO_4 , 69 mM K_2HPO_4 , 0.88 mM sodium thioglycolate as reducing agent, and 0.002 mM Resazurin as O_2 indicator dye. This medium was autoclaved and cooled under N_2 atmosphere to achieve anoxia in the medium. Supplements were added anoxically to the basal medium to give final concentrations of 0.177 mM CaCl_2 , 0.25 mM MgCl_2 , 0.0315 mM Na_2MoO_4 , 0.002 mM Mn(II)Cl_2 , 0.25 mM Na_2SO_4 , 0.147 mM Fe(II)Cl_2 . This medium was filled anoxically into serum bottles and supplemented with glucose and NH_4Cl according to the experimental treatments: No NH_4^+ treatments contained 30 mM glucose and no NH_4Cl , limiting NH_4^+ treatments contained 20 mM glucose and 0.5 mM, 1.25 mM, or 2 mM NH_4Cl , the sufficient NH_4^+ treatment contained 20 mM glucose and 3.5 mM NH_4Cl , and the saturated NH_4^+ treatment contained 20 mM glucose and 8 mM NH_4Cl . The treatment without added NH_4^+ contained more glucose than the other treatments to account for the lower biomass yield under purely N_2 -fixing conditions and to establish similar biomass across all treatments. Serum vials containing the medium were sealed with blue butyl-rubber stoppers (Ochs) and were equipped with a magnetic stir bar.

Chemostat cultivation and pulse-chase/pulse experiments. Freeze stocks were prepared as follows: the cell lyophilisate obtained from the DSMZ was dissolved in LB broth and then streaked on LB agar. A single colony was inoculated into LB broth. Cells were grown to mid-exponential phase, diluted at 1:1 ratio with 30% glycerol and frozen at -80°C. These freeze

stocks were used to inoculate all reported experiments. Pre-cultures were inoculated with 10 μ L cell suspension from freeze stocks into 2 mL LB broth and grew for 6-8 h on a shaker at 220 rpm. These cells were inoculated at 1:100 ratio into 60 mL serum vials containing 20 mL anoxic chemostat medium with the glucose and NH_4Cl concentrations respective to the experimental treatment. The cells were grown overnight in batch mode on a multi-position stirrer plate (Variomag with Telemodule 40S) and connected to continuous medium flow on the following morning. The stirrer speed was set to 300 rpm and the force was set to 60%, which heated to culture liquid to 30°C. Starting the supply of medium on the next day started the chemostats after batch overnight growth. All connections were made with sterile needles, polypropylene Luer-Lock adaptors and O_2 -tight fluran tubing (Ismatec), which was sterilized with 70% ethanol.

The chemostats were operated at a dilution rate of 0.1 h^{-1} corresponding to a generation time (g) of 6.9 h, which is below the maximum growth rate (μ) of *K. oxytoca* under N_2 -fixing conditions ($\mu = 0.13 \text{ h}^{-1}$; g = 5.33 h). The medium was constantly dropped into the chemostat at a pump rate of 2 mL h^{-1} into a volume of 20 mL with one medium turnover being achieved in 10 h. The outflow needle was adjusted to the liquid surface and the outflow pump was operated at a rate of 93 mL h^{-1} . The withdrawn excess gas volume was replaced with N_2 gas purging through the cell suspension. This resulted in a constant liquid volume in the chemostat and in the constant supply of N_2 gas to the culture. The chemostats were operated for 5-7 volume changes before further analysis (glucose concentration, NH_4^+ concentration, smFISH) and experimentation (pulse or pulse-chase/pulse with bulk isotope and NanoSIMS analysis).

Pulse-labelling of chemostats cultures for the limitation experiment shown in Figures 1 and 2 was performed by replacing the headspace volume of the chemostat vessels with $^{15}\text{N}_2$ (Sigma,

Lot MBBB0968V) at a ^{15}N fraction, $X(^{15}\text{N})_{\text{N}_2}$, of 0.49. The original headspace volume was evacuated for 1 min and the established vacuum in the bottle was used to transport $^{15}\text{N}_2$ from a gastight syringe into the chemostat vessel. Thereafter, the bottle was shaken thoroughly for 1 min. The constant supply of N_2 gas was stopped during pulse-labelling. A constant volume during the pulse-labelling phase was maintained by setting the pump rate of the outflow equal to the inflow. ^{13}C -glucose labelling was done by switching the unlabelled feed medium to a feed medium labelled with ^{13}C -glucose (Sigma) at a fraction, $X(^{13}\text{C})_{\text{glucose}}$, of 0.5. Pulse labelling was performed for 1.5 h (0.22x generation time) or 7.5 h (for control experiments). We assured that the degree of $^{15}\text{NH}_3$ contamination in the $^{15}\text{N}_2$ gas stocks obtained from Sigma did not affect the experimental results (see Supplementary discussion)²⁷.

The pulse-chase/pulse experiments shown in Figure 3 consisted of two phases; (i) the pulse-phase in which cells were labelled with $^{15}\text{N}_2$ in the chemostats and (ii) the chase/pulse-phase in which cells were switched to batch growth in NH_4^+ -free medium amended with ^{13}C -glucose. *Pulse-labelling* with $^{15}\text{N}_2$ was performed as described above, but labelled at $X(^{15}\text{N})_{\text{N}_2}$ of 0.98 with $^{15}\text{N}_2$ supplied by Cambridge Isotopes (Lot I-15312) instead of Sigma and omitting ^{13}C -labelling. The *chase/pulse-phase* was initiated by two times washing the pulse-labelled cells in an anaerobic chamber (Coy Laboratory Products). Washing consisted of centrifugation at 5000 rpm for 5 min and re-suspending the cells in medium without NH_4^+ . After washing, cells were inoculated to a final OD of around 0.05 in fresh NH_4^+ -free medium containing $^{14}\text{N}_2$ and 50 mM glucose with $X(^{13}\text{C})_{\text{glucose}} = 0.1$ or 0.5. Control pulse-chase/pulse incubations for NH_4^+ -limited chemostats were incubated with unlabelled $^{14}\text{N}_2$ and ^{12}C -glucose during the pulse-phase and with $^{15}\text{N}_2$ at $X(^{15}\text{N})_{\text{N}_2} = 0.98$ and ^{13}C -glucose at $X(^{13}\text{C})_{\text{glucose}} = 0.1$ or 0.5 during the chase/pulse-phase.

Total nitrogen, NH_4^+ and bulk isotopic analysis. Samples for bulk isotope measurements were filtered on 0.7 μm pore-sized glass microfiber filters (GF/F, Whatman) which were pre-combusted at 400°C for 3 h. The filters were immediately frozen at -20°C, dried for at least 3 h at 65°C and decalcified in an atmosphere generated by 37% hydrochloric acid. The filters were stored dry in a desiccator at room temperature until further processing. Abundances of ^{15}N and ^{13}C in the biomass on the filters were measured as N_2 and CO_2 released by flash combustion in excess oxygen in an automated Thermo Flash EA 1112 elemental analyser coupled to an isotopic ratio mass spectrometer (Thermo Delta Plus XP, Thermo Fisher Scientific). Unlabelled standards were prepared from cells grown in the absence of $^{15}\text{N}_2$ and from caffeine.

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 ($C_{\text{NH}_4^+}$ in mol L^{-1}) and the chemostat volume ($V_{\text{chemostat}}$ in L) to calculate the percentage of required nitrogen supplied as NH_4^+ (N_{required} in %) according to

$$N_{\text{required}} [\%] = \left(\frac{C_{\text{NH}_4^+} \times V_{\text{chemostat}}}{N_{\text{biomass}}} \right) \times 100 . \quad [1]$$

The steady-state NH_4^+ concentration in the chemostats was measured with the orthophthaldialdehyde assay²⁸.

Measuring metabolic activity of single cells with NanoSIMS. Chemostat samples for NanoSIMS analysis were mixed with ice-cold PBS (phosphate-buffered saline), centrifuged for 5 min at 4°C and 5000 rpm, and re-suspended in methanol-free 3.7% paraformaldehyde (PFA, Electron Microscopy Sciences) in PBS and fixed for ca. 1-4 h at room temperature. Thereafter,

cells were filtered on 0.22 μm pore-sized polycarbonate filters (GTTP, Millipore) with a diameter of 5 mm that were sputter-coated with a conductive gold-palladium layer. Filtration of cells was immediately followed by filtration of 200 μL double-deionized water for washing the filters. The filters were stored dry at room temperature until NanoSIMS measurements.

The filtered cells were stained with the general DNA stain Hoechst ($10\text{ }\mu\text{g mL}^{-1}$). Areas of interest were marked with laser micro-dissection (PALM micro-dissection, Zeiss 200M equipped with a 355 nm pulsed UV laser and epifluorescence illumination) and imaged for Hoechst fluorescence. The marked areas were analysed with a NanoSIMS 50L (CAMECA, Gennevilliers Cedex-France) at the Max Planck Institute for Marine Microbiology in Bremen (pulse-chase/pulse experiment as shown in Figure 3) and at the Center for Advanced Surface Analysis at EPFL/University of Lausanne (limitation experiment as shown in Figure 2). The areas were pre-sputtered with a Cs^+ primary ion beam of 300 pA for approximately 1 ms per pixel to remove surface contamination, to implant Cs^+ ions on the sample, and to achieve an approximately stable ion emission rate. A primary Cs^+ ion beam with a beam current between 1 and 1.2 pA and a beam diameter of around 60-120 nm was rastered across the cells for analysis with a dwell time of 1 ms or 5 ms per pixel. Secondary ion images for $^{12}\text{C}^-$ or $^{12}\text{C}^{12}\text{C}^-$, $^{13}\text{C}^-$ or $^{12}\text{C}^{13}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{12}\text{C}^{15}\text{N}^-$ and $^{32}\text{S}^-$ were simultaneously recorded from analysis areas of $20 \times 20\text{ }\mu\text{m}$ to $40 \times 40\text{ }\mu\text{m}$ with a resolution of 256×256 or 512×512 pixels. We measured 1 to 2 individual areas per biological sample and acquired between 30 and 60 planes from each area for 1 ms dwell times and 5 planes for 5 ms dwell time.

Analysis of NanoSIMS images was performed with the Matlab-based software Look@NanoSIMS²⁹. The images were first corrected for a possible drift of the sample 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 $^{12}\text{C}^{14}\text{N}^-$ ion-image to identify cells and to mark these cells as regions of interest (ROI). The accumulated counts, c , were averaged over the area of a ROI and the atom fractions for nitrogen, $X(^{15}\text{N})_{\text{cell}} = c(^{15}\text{N}^{12}\text{C})_{\text{cell}} / \{c(^{14}\text{N}^{12}\text{C})_{\text{cell}} + c(^{15}\text{N}^{12}\text{C})_{\text{cell}}\}$ and carbon, $X(^{13}\text{C})_{\text{cell}} = c(^{13}\text{C})_{\text{cell}} / \{c(^{12}\text{C})_{\text{cell}} + c(^{13}\text{C})_{\text{cell}}\}$ were calculated for each ROI. Together with the incubation time, these atom fractions are a measure of the rates at which N_2 and glucose were assimilated into biomass, respectively.

Calculations. The single-cell N_2 fixation rate, r_{N_2} (amol h^{-1}), was calculated according to

$$r_{\text{N}_2} = \frac{X^E(^{15}\text{N})_{\text{cell/reference}} \cdot b}{X(^{15}\text{N})_{\text{N}_2} \cdot t}, \quad [2]$$

Whereby t denotes the incubation time (h), b denotes the average nitrogen content per cell measured from elemental analyser data during IRMS measurements of NH_4^+ -limited cells grown in the medium described above ($8.5 \pm 0.6 \text{ fmol N cell}^{-1}$; mean $\pm$ SD, $N=3$), and $X(^{15}\text{N})_{\text{N}_2}$ - the isotopic enrichment of $^{15}\text{N}_2$ in the incubation medium. $X^E(^{15}\text{N})_{\text{cell/reference}}$ is the excess atom fraction of ^{15}N in the cell against the average background isotopic composition of cells grown in the absence of isotopic label measured independently with NanoSIMS ($X(^{15}\text{N})_{\text{background}} = 0.0036797$) calculated according to

$$X^E(^{15}\text{N})_{\text{cell/reference}} = X(^{15}\text{N})_{\text{cell}} - X(^{15}\text{N})_{\text{background}}. \quad [3]$$

The specific carbon-based growth rate (μ) was calculated from the doublings, n , that a cells needs to perform with ^{13}C -glucose as growth substrate to reach a certain excess atom fraction, $X^E(^{13}\text{C})_{\text{cell/reference}}$, of ^{13}C in its biomass relative to the isotopic enrichment of ^{13}C -glucose in the incubation medium, $X(^{13}\text{C})_{\text{glucose}}$, according to

$$n = \log_2 \left(1 - \frac{X^E(^{13}\text{C})_{\text{cell/reference}}}{X(^{13}\text{C})_{\text{glucose}}} \right)^{-1}, \quad [4]$$

whereby $X(^{13}\text{C})_{\text{glucose}}$ denotes the isotopic enrichment of ^{13}C -glucose in the incubation medium. $X^E(^{13}\text{C})_{\text{cell/reference}}$ is the excess atom fraction of ^{13}C in the cell against the average background isotopic composition of cells grown in the absence of isotopic label measured independently with NanoSIMS ($X(^{13}\text{C})_{\text{background}} = 0.010634$) calculated according to

$$X^E(^{13}\text{C})_{\text{cell/reference}} = X(^{13}\text{C})_{\text{cell}} - X(^{13}\text{C})_{\text{background}}. \quad [5]$$

Using equation 3, a previously unlabelled cell reaches $X^E(^{13}\text{C})_{\text{cell/reference}} = 0.5$ if it divides once grown in ^{13}C -glucose with $X(^{13}\text{C})_{\text{glucose}} = 1$; or $X^E(^{13}\text{C})_{\text{cell/reference}} = 0.75$ if it divides twice. Subsequently, the specific carbon-based growth rate, μ , during the incubation time, t , was calculated from the doublings, n , according to

$$\mu = \frac{n \cdot \ln 2}{t}. \quad [6]$$

The projected excess atom fraction of ^{15}N in a cell before the switch, $X^E(^{15}\text{N})_{\text{cell-before/reference}}$, was calculated from its dilution to the measured ^{15}N excess atom fraction after the switch, $X^E(^{15}\text{N})_{\text{cell-after/reference}}$, and from the carbon-based doublings, n , according to

$$X^E(^{15}\text{N})_{\text{cell-before/reference}} = X^E(^{15}\text{N})_{\text{cell-after/reference}} \cdot 2^n \quad [7]$$

and

$$X^E(^{15}\text{N})_{\text{cell-after/reference}} = X(^{15}\text{N})_{\text{cell-after}} - X(^{15}\text{N})_{\text{background}} \quad [8]$$

Using equation 6, a cell measured with NanoSIMS after the pulse-chase/pulse experiment with a $X^E(^{15}\text{N})_{\text{cell-after/reference}} = 0.5$ and carbon-based doubling $n = 1$ will have a calculated $X^E(^{15}\text{N})_{\text{cell-before/reference}} = 1$.

Statistical analysis. One-Way Analysis of Variance, unpaired t-tests, Deming regression, and Spearman rank correlation coefficients were calculated using the Prism 5 (GraphPad Software, Inc., CA). We always calculated two-tailed p-values. The sample size was chosen to balance analytical costs for NanoSIMS analysis with the possibility for statistical comparison of the results. We confirmed that all data sets that were statistically evaluated by unpaired t-tests and One-Way Analysis of Variance had equal variances ($p > 0.05$) as tested by an F-test and the Browne-Forsythe test, respectively. We used parametric statistical tests to compare between biological replicates and assume – without testing – that the individual values within groups follow a normal distribution, because common normality tests need higher sample size to be sensitive. We calculated a non-parametric Spearman correlation coefficient for the pulse-

chase/pulse experiment since the single-cell growth rates do not follow a normal distribution according to D'Agostino-Pearson test ($p < 0.002$). During Spearman correlation Prism computes a p-value by a Gaussian approximation and reports an approximated 95% confidence interval. The slopes of carbon-based growth rate (or glucose assimilation) vs. N_2 fixation rates before or after the switch were determined by fitting a linear model with Deming (Model II) regression. A runs test confirmed that 3 of the 4 populations are best fitted with a linear model.

***NifH*-targeted smFISH.** Single-molecule fluorescence in situ hybridization (smFISH) of *nifH* and *glnK* was performed as described by Skinner et al.³⁰. Probes were manufactured by Biosearch Technologies (Petaluma, CA) and designed using their web-based Stellaris Probe Designer. The probes were designed using the *nifH* and *glnK* sequence of *K. oxytoca* reported by Arnold et al.³¹ and consisted of 40 or 17 DNA oligonucleotides, respectively. Each probe had an average length of ~20 nucleotides. Probes for *nifH* were labelled with the fluorescent dye CalFluorRed 590 (max. ex. 569 nm and max. em. at 591 nm) and probes for *glnK* were labelled with Quasar 670 (max. ex. 647 nm and max. em. at 670 nm). The probe sequences are reported in the Supplementary Information. Phase contrast and fluorescent images were acquired with an inverted epifluorescence microscope (Leica DMI6000B, Leica Microsystems, Germany) equipped with Cy3 (Ex. 555/25 nm BP, dichromatic mirror 575nm, Em. 605/52 nm BP) and Cy5 (Ex. 620/60 nm BP, dichromatic mirror 660nm, Em. 700/75 nm BP) filter cubes, and a 100x 1.47NA Oil HCX PlanApo objective. Images were acquired using the 1.6x lens in front of the camera resulting in a resolution of 71.4 nm per pixel. Z-stacks with 6-9 slices at 200 nm step size were recorded for each field-of-view. Images were analysed with Matlab-based scripts (The MathWorks, Inc.); segmentation was done from phase contrast images using Schnitzcells³² and fluorescent spot recognition was done with Spätzcells³⁰. We analysed approximately 500 cells in

each of the 3 biological replicates. Summed maximum intensities of spots contained in a cell were used to calculate the mRNA numbers in each fluorescent spot. Strain UNFI07rpsL1Δ107(gnd-his-nif-deletion) kindly provided by Ray Dixon³³ was used as a negative control. Calibration of spot intensities corresponding to a single *nifH* mRNA was done with cells from an NH₄⁺-grown batch culture that was switched to growth without NH₄⁺ for 30 min. Calibration of spot intensities corresponding to a single *glnK* mRNA was done with cells from a chemostat culture grown with 86% NH₄⁺ of the total required N, because *glnK* was highly abundant in a NH₄⁺-grown batch culture that was switched to growth without NH₄⁺ for 30 min. The spot intensities obtained for single mRNA molecules were used to calculate mRNA numbers in each cell based on the fluorescence intensities in each spot (peak height) and a linear calibration. The Matlab curve fitting toolbox was used to fit negative binominal distributions to the measured mRNA distributions according to

$$P(n) = \binom{n+r-1}{n} p^r (1-p)^n \quad . \quad [9]$$

The fits were used to estimate the mean expression level (m) and the variance (σ^2) as well as the fitting parameter r , which was further used to calculate the burst frequency (f). The burst frequency was estimated by $f=r/\tau_{\text{RNA}}$ (REF 33), where τ_{RNA} is the mRNA life-time, which has been determined earlier for *nifH* to be 18 min under N₂ fixing conditions³⁴. This value was used to also calculate the burst frequency of *glnK*. As the life-time of *glnK* mRNA might be different, values of the *glnK* burst frequency should be considered only relative to each other. The transcriptional noise (η^2) was estimated by $\eta^2=\sigma^2/m^2$.

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End notes

Additional Information Supplementary Information is available online. Correspondence and requests for materials should be addressed to FS or MA.

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Author contributions FS, MMMK, and MA designed research. FS performed the experiments. SL, SE, and FS did the NanoSIMS measurements. SL, GL, SE, AM, and MMMK contributed analytical tools. FS and MA analyzed data. FS and MA wrote the paper with input from all coauthors.

Competing interests The authors declare no competing financial interest.

Phenotypic heterogeneity driven by nutrient limitation promotes growth in fluctuating environments - Supplementary Information

Frank Schreiber, Sten Littmann, Gaute Lavik, Stéphane Escrig, Anders Meibom, Marcel M.M. Kuypers, Martin Ackermann

Sequence information for probes used for smFISH

nifH-targeted smFISH probes:

GCGCATTGACGCATGGTCAT
GATACCGCCTTTACCGTAAA
TCTGCGTGGTGGTGGATTTA
ATCTCCGCCAGCGCGCGAC
GACGATCATCACTTTCTTAC
AGTCCGCCTTCGGATCGCAG
GCGTGCAGAATCAGACGGGT
CATAATGGTGTTCTGTGCTT
AGCCGACTTCCGCGGCCATC
TCGAGTTCGAGGTCTCGAC
GTAGCCAATTTGCAGCACGT
ATTCCGCGCAGCGCACATCG
ACGCCTGGCTCCGGGCGGCC
CACGCCGCGTCCCGCGCAGC
CAAGAAAGTTGATCGCCGTG
TCCTCGTAGGCGCCTTCTTC
ATAGAACACGAAATCGAGAT
AGACCACGTCGCCGAGCACG
ATCGGCATGGCGAAGCCGCC
CTCCTGGGCTTTGTTTTCGC
CGCCGGAGCAGACGATGTAG
GCCGCGTACATCGCCATCAT
GATCCCTTTGGAGATTGT
TGCCGGATTTGGCGTATTTA
ATCAGGCCGCCGAGGCGCAC
GTCGGTCTGACGTGAGTTAC
CAATAATCAGTTCGTCTTCA
GTACCGAGCTTTCCGCCAG
GGGCACAAAGTGGATCATCT
CGCGCTGCACGATGTTGTCG
ACCGTCATGCGGCGGATCTC
ACAGGCGGGGTCTGACTCGA
TGCGGTATTGTTGGCCTGT
TTGACGATCTTCTGCGCCAG
CGGCACCACTTTCATGGTGT
GCTCATCCATGGTGCAGGGC
AACTCCATCAGCAGCGATTTC
GTCTTCCTCTTCCATGATGC
CGGTTTTGCCAATGATGCTG
CAGGCCGCGTTTTCTTCGGC

glnK-targeted smFISH probes:

TAATGGCCGCAGCTAACACG
GTCCCCTCCAGTCAGAATTC
ACCACGGTAACCAGCTTCAT
TTCCAGTTTGAATGGTTTGA
AAGATAATCGTTCACGCACA
GTCAGGCCCTGAATACCCAT
GAACCCTTTAACTTCAGTGA
CCGCGTGGCCTTTCTGACGG
TATTCGGCGCCGCGATAGAG
TTTCGGTAGAAAATTGACGC
CAATCGCGACATCAATCTTC
ACTTCGTCCAGCTGATCGTC
CGCTTTACTGATGACATCGA
CGCCAATCTTTCGGGTGTAC
CAGCTCGGCGACAAAGATTT
CGGTACGATGCGAATCACG
AGTGCGGCTTCGTCAGATTTC

Supplementary Figure I. Biomass yield expressed in cells per mmol glucose in chemostats with increasing NH_4^+ supply levels as shown in Figure I. The symbols share the colour code with the schematic representation in Figure IA.

Supplementary Figure 2. Phenotypic heterogeneity in glucose and NH_4^+ assimilation. **(a)** The coefficient of variation in glucose assimilation at different NH_4^+ supply levels comparable to Figure 2F. Statistical analysis was performed with a I-Way-ANOVA ($p=0.029$). **(b)** The coefficient of variation in NH_4^+ assimilation (cyan diamond) as compared to N_2 fixation (blue square, taken from Figure 2F) at NH_4^+ supply with 80% of the total required N. Statistical analysis was performed with an unpaired t-test ($p=0.0002$). Data originates from replicated chemostat populations.

Supplementary Figure 3. Phenotypic heterogeneity in N_2 fixation at different NH_4^+ supply levels and different incubation times with $^{15}N_2$. NH_4^+ was supplied at 0% (black and grey) and 80% (blue and cyan) of the total required N. Incubations were run for 1.5 h (black and blue, taken from Figure 2F) and 7.5 h (grey and cyan). **(a)** Single-cell N_2 fixation rates (circles) and the average (horizontal bars) calculated from the single-cell NanoSIMS data for replicate chemostat populations (replicates and number of cells measured are shown in Supplementary Table 1). **(b)** The corresponding coefficients of variation in N_2 fixation activity for each replicate chemostat population (circles) as shown in **(a)**. The horizontal bar displays the mean. Statistical analysis between groups with different incubation times at the same NH_4^+ supply regime were performed with an unpaired t-test ($p_{0\%(1.5h:7.5h)}=0.523$ & $p_{80\%(1.5h:7.5h)}=0.892$).

Supplementary Figure 4. Single-cell N-uptake rates of N₂ fixing wild-type *K. oxytoca* cells (black circles) and cells of the non N₂-fixing strain *K. oxytoca* UNF107::rpsL1Δ107Δgnd-his-nif(pUA66::P_{rpsM}::gfp::km) (open circles) incubated in a mixed chemostat in the presence of ¹⁵N₂ and without supply of external NH₄⁺. N₂-fixing wild-type cells apparently leak ¹⁵NH₄⁺ produced from ¹⁵N₂ that is being taken up by *K. oxytoca* UNF107. The uptake rate of NH₄⁺ produced from N₂ per cell makes up about 10% of the uptake rate of N₂ directly to wild-type cells.

Supplementary Figure 5. Micrographs of *nifH* and *glnK* mRNA expression measured with smFISH in single *K. oxytoca* cells grown in chemostats supplied with 0% (a), 29% (b), 60% (c), and 86% (d) NH_4^+ of the total required N and the corresponding histograms for *nifH* (e-h) and *glnK* (i-l) expression. We analysed approximately 500 cells from 3 biological replicates per experimental condition. The experimental data from the 3 replicated chemostat populations (filled circles) and the average fit to a negative binomial distribution (grey line) are shown in histograms. Non-expressing cells in (e) and (f) were excluded from the fit. The population displayed with cyan circles in panels h & i was removed from the analysis for reasons described in the caption of Figure 2. The scale bar in (a-d) corresponds to 4 μm . A threshold was applied to the green (41 of 255) and the red (10 of 255) channel to remove background signal.

Supplementary Table I

NanoSIMS measurements: data values and number of analysed cells

	Relates to Figure	Number of analysed cells	Mean single-cell N ₂ fixation rate amol N h ⁻¹	Coefficient of variation N ₂ fixation
No NH₄⁺	2e; SF** 2a; SF 3	270	617	0.115
(¹⁵ N ₂ , ¹³ C-glucose, 1.5 h)*		317	682	0.144
		414	529	0.187
No NH₄⁺	SF 3	244	339	0.162
(¹⁵ N ₂ , 7.5 h)*		223	499	0.138
		323	572	0.084
20% NH₄⁺	2e; SF 2a	504	493	0.129
(¹⁵ N ₂ , ¹³ C-glucose, 1.5 h)*		375	472	0.157
		414	398	0.143
57% NH₄⁺	2e; SF 2a	265	275	0.225
(¹⁵ N ₂ , ¹³ C-glucose, 1.5 h)*		461	269	0.266
		513	186	0.277
80% NH₄⁺	2e; SF 2a&b; SF 3	337	119	0.507
(¹⁵ N ₂ , ¹³ C-glucose, 1.5 h)*		443	77	0.548
		395	112	0.509
80% NH₄⁺	SF 3	169	109	0.442
(¹⁵ N ₂ , 7.5 h)*		316	54	0.450
		423	61	0.643
80% NH₄⁺	SF 2b	262	579***	0.199****
(¹⁵ NH ₄ ⁺ , 1.5 h)*		256	466***	0.208****
		316	542***	0.131****

*type of isotopically labelled substrate and incubation time

**SF (Supplementary Figure)

***NH₄⁺ assimilation rate

**** coefficient of variation in NH₄⁺ assimilation

Supplementary discussion

Supplementary discussion on the relevance of NH_4^+ leakage and re-uptake

We performed an experiment to test how much of fixed NH_4^+ leaks from cells and is then being taken up by cells that do not fix N_2 . We constructed a reporter strain (*K. oxytoca* UNF107::rpsL Δ 107 Δ gnd-his-nif[pUA66::P_{rpsM}::gfp::km], denoted in short UNF107) based on UNF107rpsL Δ 107(gnd-his-nif-deletion)¹ by transforming the strain with plasmid pUA66::P_{rpsM}::gfp::km² (gfp under the control of the *E. coli* rpsM promoter and kanamycin resistance). We grew *K. oxytoca* wild-type cells in a chemostat without NH_4^+ supply as described in the Methods section. Further, we grew *K. oxytoca* UNF107 in a parallel chemostat under NH_4^+ limitation (20 mM Glucose and 2 mM NH_4Cl in feed medium). The feed medium for UNF107 contained in addition: 40 mg L⁻¹ histidine (UNF107 is his-auxotroph) and 50 mg L⁻¹ Kanamycin (to stabilize the plasmid). After both chemostats reached steady state, we pulsed the chemostat that contained the wild-type cells with citrate buffer-washed ¹⁵N₂ (50%; Sigma Lot MBB0968V, see Supplementary discussion) and incubated the chemostat for 1.75 h. Simultaneously, we washed and concentrated *K. oxytoca* UNF107 in a anaerobic glove box as described in the Methods section.

The concentrated cell suspension of *K. oxytoca* UNF107 was added to the chemostat with the wild-type cells to reach a relative abundance of 5 % after the wild-type cells were incubated 1.75 h with ¹⁵N₂. The pre-incubation was done to allow for the removal of any residual ¹⁵NH₃ supplied with the ¹⁵N₂ gas and to facilitate the equilibration of the external NH_4^+ pool with ¹⁵NH₄⁺ that leaked from ¹⁵N₂ fixing cells. The mixed cell suspension was incubated for additional 1.75 h in the presence of ¹⁵N₂ before cells were exposed for 5 min to the air (to allow for the maturation of Gfp in UNF107) followed by washing in PBS and fixation in 3.7% paraformaldehyde as described in the Methods section. Cells were filtered on polycarbonate membrane filters, imaged and marked with a laser micro-dissection microscope, and subjected to NanoSIMS analysis as described in the Methods section. UNF107 cells were distinguished from wild-type cells based on their Gfp-fluorescence. The experiment was performed in duplicate. We analysed 500 and 521 wild-type cells and 25 and 45 UNF107 cells per repeat, respectively.

The results show that the wild-type cells fixed $973 \text{ amol N h}^{-1}$ ($\pm 152 \text{ amol N h}^{-1}$; SD, N=2) into their biomass, while UNF107 incorporated 96 amol N h^{-1} ($\pm 5 \text{ amol N h}^{-1}$; SD, N=2) into their biomass (Supplementary Fig. 4). The data demonstrates that the uptake of leaked NH_4^+ per cell makes up about 10 % of the total fixed N under N_2 fixing conditions. This indicates that leakage and re-uptake does not strongly affect our estimates of heterogeneity in N_2 fixation.

Supplementary discussion that ^{13}C -glucose assimilation is a proxy for growth

The pulse-chase/pulse experiment is based on the assumption that glucose assimilation is a good proxy for growth. To support this assumption, we concomitantly labelled chemostats with ^{13}C -glucose and determined single-cell, ^{13}C -carbon-based specific growth rates (calculated with equations 4-6) of chemostat-grown cells. We find that the ^{13}C -carbon-based specific growth rates averaged across all NH_4^+ supply levels ($0.095 \pm 0.01 \text{ h}^{-1}$; mean $\pm$ SEM, $N=13$) are similar to the set dilution rate of the chemostat (i.e. 0.1 h^{-1}).

Further, the data of the pulse-chase/pulse experiment indicates that ^{13}C -carbon assimilation is a good proxy to assess growth also after a switch to NH_4^+ -depletion, because the single-cell, carbon-based growth rates averaged across all 4 experimental runs ($0.093 \pm 0.008 \text{ h}^{-1}$; mean $\pm$ SEM; $N=4$ as calculated from data in Fig. 3a,b) are in the same range as the population growth rates ($0.08 \pm 0.01 \text{ h}^{-1}$; mean $\pm$ SEM; $N=4$) determined from OD measurements during the experiment. Populations do not have a considerable lag-time under the conditions of the experiment, because they already contain the enzymes for N_2 fixation that have been produced during NH_4^+ -limited growth before the switch (Fig. 3b). This experiment also indicates that most carbon-assimilation after the switch is linked to growth and not to production of carbon storage compounds, because nitrogen and carbon are assimilated at a ratio (Fig 3b; Deming regression slope = $2.9 \pm 0.15 \text{ amol C per amol N}$; mean $\pm$ SEM, $N=2$) that is not above the C:N ratio of the biomass in NH_4^+ -free chemostats (4.7 ± 0.1 ; mean $\pm$ SEM, $N=2$; assessed from elemental analyser data).

Supplementary discussion on the purity of $^{15}\text{N}_2$ gas stocks

It has recently been reported that commercially available $^{15}\text{N}_2$ gas stocks could be contaminated with $^{15}\text{NH}_3$ ³. We calculated the potential effect of the contamination by calculating the ratio of $^{15}\text{NH}_3/^{15}\text{N-N}_2$ in the new biomass based on the new biomass formed during the incubation time, the $^{14}\text{NH}_4^+$ supplied from the feed during the incubation, the amount of $^{15}\text{NH}_3$ supplied during the pulse, the isotopic labelling% of $^{15}\text{N}_2$, and by assuming that cells would consume $^{15}\text{NH}_3$ and $^{14}\text{NH}_4^+$ before engaging in N_2 fixation. The calculation showed that a contamination of 6 $\mu\text{mol NH}_3$ per mole N_2 would result in a $^{15}\text{NH}_3/^{15}\text{N-N}_2$ ratio ≤ 0.01 across different NH_4^+ supply treatments. Dabundo et al. (2014) reports that gas provided by Cambridge Isotopes is relatively pure with values ranging between 0.014-0.052 $\mu\text{mol NH}_3$ per mole N_2 across different Lots. We conclude that the pulse-chase/pulse experiments conducted with gas provided by Cambridge Isotopes are not affected by the contamination.

In contrast, Dabundo et al. (2014) reported that gas provided by Sigma (Lot MBB0968V, as used by us) is contaminated with 1900 $\mu\text{mol NH}_3$ per mole N_2 . This contamination could theoretically affect the N_2 fixation measurements. Therefore, we removed NH_3 from gas provided by Sigma by incubating 72 mL gas at 0.8 bar with 20 mL anoxic citrate buffer (0.1M, pH3) in a 60 mL sealed serum flask for 2 h on a shaker (200 rpm) at room temperature. 20 mL of the purified gas was withdrawn from the serum flask and was used in the pulse labelling experiments. We confirmed the purity of the gas incubated with citrate buffer by equilibrating it with 10 mL 0.1x phosphate buffered saline (pH 7.4; citrate buffer cannot be used since it interferes with the NH_4^+ assay) in a 36 mL closed serum flask on a shaker (200 rpm) at room temperature. Subsequently, we measured the NH_4^+ concentration in the liquid phase using the sensitive orthophthaldialdehyde assay⁴. We found that the remaining contamination is about 1.7 ± 1.5 (N=4) $\mu\text{mol NH}_3$ per mole N_2 . We conclude that our cleaning procedure reduces the $^{15}\text{NH}_3$ contamination in the gas provided by Sigma to levels that will not affect our results.

Supplementary references

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