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

Running title: Limitation induces phenotypic heterogeneity

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Abstract

Populations of genetically identical cells can display marked variation in phenotypic traits; such variation is termed phenotypic heterogeneity. Here we investigate the effect of substrate and electron donor limitation on phenotypic heterogeneity in N_2 and CO_2 fixation in the green sulphur bacterium *Chlorobium phaeobacteroides*. We grew populations in chemostats and batch cultures and used stable isotope labelling combined with nanometer-scale secondary ion mass spectrometry (NanoSIMS) to quantify phenotypic heterogeneity. Experiments in H_2S (i.e. electron donor) limited chemostats show that varying levels of NH_4^+ limitation induce heterogeneity in N_2 fixation. Comparison of phenotypic heterogeneity between chemostats and batch (unlimited for H_2S) populations indicates that electron donor limitation drives heterogeneity in N_2 and CO_2 fixation. Our results demonstrate that phenotypic heterogeneity in a certain metabolic activity can be driven by different modes of limitation and that heterogeneity can emerge in different metabolic processes upon the same mode of limitation. In conclusion, our data suggest that limitation is a general driver of phenotypic heterogeneity in microbial populations.

Keywords: Phenotypic variation, phenotypic heterogeneity, phenotypic diversity, NanoSIMS, nutrient limitation, Lago di Cadagno, *Chlorobium phaeobacteroides,* dinitrogen fixation, carbon dioxide fixation

Introduction

Phenotypic heterogeneity is a widespread phenomenon manifesting itself in fundamental microbial traits such as antimicrobial persistence (Balaban *et al.*, 2004), competence for DNA uptake (Maamar *et al.*, 2007), chemotaxis (Emonet and Cluzel, 2008), and metabolic activity (Ozbudak *et al.*, 2004; Kiviet *et al.*, 2014; Kotte *et al.*, 2014; New *et al.*, 2014; Solopova *et al.*, 2014; Schreiber *et al.*, 2016). It has been shown that phenotypic heterogeneity is an evolvable microbial trait because it is genetically controlled (Ozbudak *et al.*, 2002). Phenotypic heterogeneity helps microbial populations (Ackermann, 2015) to adapt to fluctuating environmental conditions (Balaban *et al.*, 2004; Kussell and Leibler, 2005; Acar *et al.*, 2008; Beaumont *et al.*, 2009; Ratcliff and Denison, 2010; Arnoldini *et al.*, 2014; Schreiber *et al.*, 2016), aids in the division of labour within isogenic cell populations (Ackermann *et al.*, 2008), and can result from negative frequency-dependent interactions in mixed resource environments (Healey *et al.*, 2016). Multiple studies on phenotypic heterogeneity have been conducted with microbial model strains, while only a few studies have investigated environmental isolates (Ziv *et al.*, 2013; Holland *et al.*, 2014; New *et al.*, 2014; Miot *et al.*, 2015; Guantes *et al.*, 2016) or natural microbial populations (Zimmermann *et al.*, 2015; Sheik *et al.*, 2015; Kopf *et al.*, 2015b). Thus, there remains a knowledge gap as to how phenotypic heterogeneity is controlled in environmental bacteria without long laboratory culture history and in natural microbial populations.

While the molecular mechanisms that generate phenotypic heterogeneity have received considerable attention, it remains unclear how the nutrient environment of

a population affects phenotypic heterogeneity. A recent study showed that heterogeneity in N_2 fixation is induced by the level of NH_4^+ limitation in the heterotrophic model organism *Klebsiella oxytoca* (Schreiber *et al.*, 2016). This study investigated N_2 fixation heterogeneity in glucose-limited and N_2-saturated chemostats with varying degrees of NH_4^+ supply (from depletion to limitation to saturation). It was shown that the closer NH_4^+ limitation approached the transition point between limitation and saturation the higher the heterogeneity in N_2 fixation (Schreiber *et al.*, 2016). However, it remained untested if other types of limitation except those of NH_4^+ can induce heterogeneity in N_2 fixation or if heterogeneity occurs in metabolic activities other than N_2 fixation. Furthermore, it was not tested in the previous study if NH_4^+ limitation also affects heterogeneity in physiologically and phylogenetically distant N_2 fixing bacteria.

Here, we investigated phenotypic heterogeneity in N_2 and CO_2 fixation with stable isotope labelling combined with NanoSIMS imaging in the green sulphur bacterium *C. phaeobacteroides*. NanoSIMS measures the isotopic ratios at single cell resolution (Musat *et al.*, 2012). It allows to quantify the incorporation rate of anabolic substrates on the single-cell level when combined with feeding isotopically labelled substrates for a part of the generation time of the cells. The strain was freshly isolated from the chemocline of the meromictic lake Lago di Cadagno situated in Ticino, Switzerland (Zimmermann *et al.*, 2015) and went through minimal cycles of growth in the laboratory before experimentation. *C. phaeobacteroides* performs anoxygenic photosynthesis under strictly anaerobic conditions with H_2S as electron donor, grows single-celled, but can also form short (approx. 2-5 cells) filaments. It fixes CO_2 as a carbon source and N_2 (if limited and depleted for NH_4^+) as a nitrogen

source. We investigated *C. phaeobacteroides* because previous work indicated that it displays phenotypic heterogeneity in N_2 fixation in its natural habitat (Halm *et al.*, 2009; Zimmermann *et al.*, 2015). In addition, previous work showed that N_2 fixing, phototrophic cyanobacteria living in a microbial mat also display pronounced levels of phenotypic heterogeneity (Woebken *et al.*, 2014). It should be noted that in those two studies phenotypic heterogeneity can also be the result of genetic differences in these natural populations or could be induced by environmental heterogeneity.

Results and Discussion

We grew *C. phaeobacteroides* populations in chemostat and batch culture to disentangle two types of limitation. Chemostats were H_2S (i.e. electron donor) limited and were experimentally varied in the level of NH_4^+ (i.e. substrate) limitation. This was achieved by changing the supply of NH_4^+ in the feed medium from saturated to fully depleted while supplying saturating amounts of N_2 gas (Figure 1a). In the range of NH_4^+ limitation, cells exhaust all the supplied NH_4^+ because NH_4^+ assimilation is preferred over energetically more expensive N_2 fixation. Hence, the amount of N_2 that cells fix depends on the NH_4^+ supply (Supplementary Table 1). In contrast, exponential batch cultures were completely unlimited for both electron donor and substrates. Chemostat and batch populations were pulse-fed with $^{15}N_2$ and $^{13}CO_2$ and analysed for single-cell incorporation with NanoSIMS (Figure 1b). Hence, by comparing cell-to-cell heterogeneity using the coefficient of variation (CV) of chemostat-grown populations with batch-grown populations in the absence of NH_4^+ the effect of electron donor (i.e. H_2S) limitation on two different metabolic activities (N_2 fixation and CO_2 fixation) can be deduced.

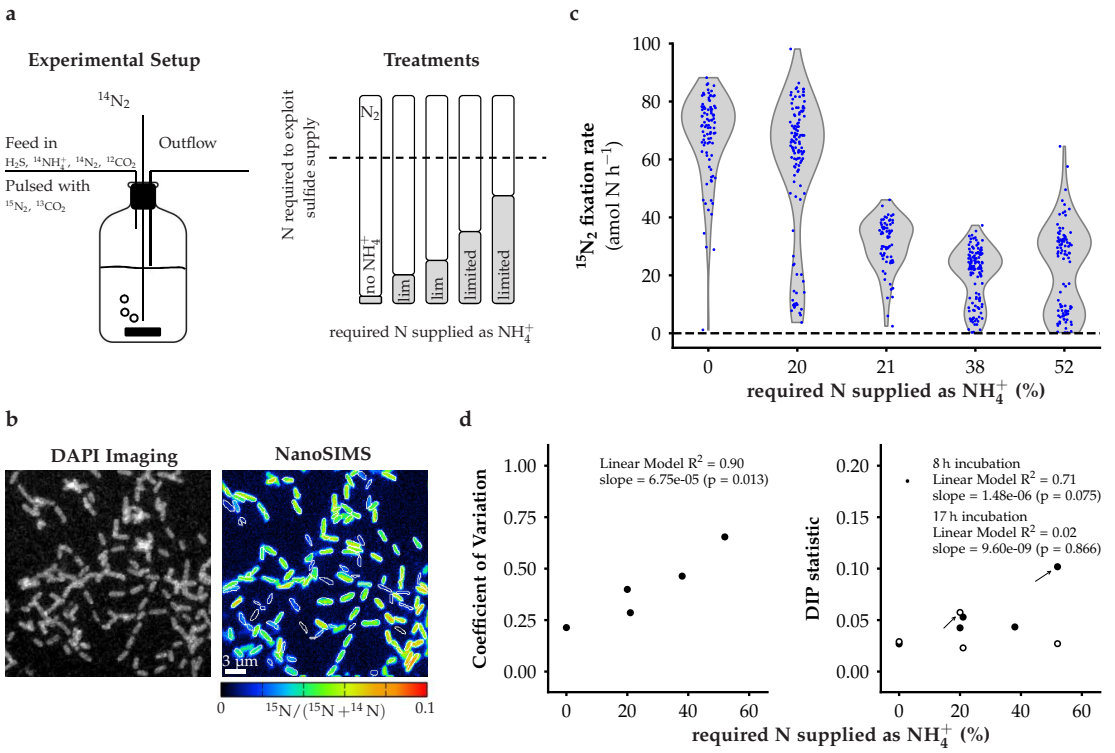

Figure 1. NH_4^+ limitation induces N_2 fixation heterogeneity at the single-cell level in chemostat-grown *C. phaeobacteroides* populations. (**a**) Varying levels of NH_4^+ limitation in the presence of excess N_2 was realized in chemostats. Chemostats were operated under N_2 atmosphere with a constant supply of feed medium with varying H_2S:NH_4^+-ratios (volume = 30 ml; dilution rate = 0.02 h^{-1}). After 10 days of equilibration (five volume exchanges), the chemostats were incubated with a pulse of $^{15}N_2$ and $^{13}CO_2$ for 8 hours (23 % of the generation time) or 17 hours (50 % of the generation time). (**b**) Example of DAPI total fluorescence image and corresponding NanoSIMS measurement for $^{15}N/(^{14}N+^{15}N)$ ratio in single *C. phaeobacteroides* cells. (**c**) $^{15}N_2$ fixation rates of single cells (blue dots) for different levels of NH_4^+ limitation. The kernel probability density is plotted in grey with a constant maximum width. (**d**) Coefficients of variation increase with decreasing NH_4^+ limitation (p = 0.013). Bimodality in N_2 fixation at varying levels of NH_4^+ limitation evaluated by the Hartigan's DIP statistic. Increasing values indicate increasing deviation from unimodality. The significance and the magnitude of the correlation between decreasing NH_4^+ supply and increasing bimodality is stronger after 8 hours of incubation (black circles, p = 0.075) as compared to 17 hours of incubation (open circles, p = 0.866). Approximated p-values < 0.05 for the Hartigan's DIP statistic are indicated with an arrow. See Supplementary table 1 for the number of measured cells and tabulated values for each experimental condition. Note that two of the levels of the required N supplied as NH_4^+ (20 % and 21 %) are close to each other, and that the difference between these two experiments might not be consistently replicated in future experiments.

The experiments showed that NH_4^+ limitation induces heterogeneity, expressed as the coefficient of variation, in N_2 fixation (Figure 1c and d). Heterogeneity increased the closer NH_4^+ limitation approached the transition point between limitation and saturation. These results are consistent with a previous study on the heterotrophic N_2 fixer *K. oxytoca* (Schreiber *et al.*, 2016) and show that phenotypic heterogeneity of

152 the same activity (i.e. N_2 fixation), driven by the same mode of limitation (NH_4^+
153 limitation), is induced in the same way in physiologically and phylogenetically distant
154 bacterial species with different laboratory culture histories.
155
156 Increasing NH_4^+ supply led to a bimodal distribution in N_2 fixation activities in the
157 population as shown by an increasing Hartigan Dip Statistic (i.e. increasing deviation
158 from unimodality) after 8 h of isotopic labelling (Figure 1c and d). The relationship
159 between bimodality and NH_4^+ supply weakened upon 17 h incubation times with
160 stable isotopes (Figure 1d) indicating that cells with high initial rates tended to lower
161 their N_2 fixation rate and cells with low initial activity increased their rate within the
162 duration of the incubation. The generation time (34.7 h) set by the dilution rate was
163 lower than the stable isotope incubation time indicating that cells switch between
164 high and low N_2 fixation rate within their cell cycle.
165
166 Next, we asked if limiting cells experimentally by a different substrate also affects
167 phenotypic heterogeneity. We chose to limit cells with H_2S, the central electron
168 donor for phototrophic growth of *C. phaeobacteroides* in our medium. Populations
169 grown in chemostats are H_2S-limited, while populations grown in batch are
170 unrestricted of any substrate including H_2S. Comparison of phenotypic heterogeneity
171 between NH_4^+-depleted batch- and chemostat-grown populations revealed that H_2S
172 limitation induces heterogeneity in N_2 and in CO_2 fixation (Figure 2). The CV's for
173 N_2 and CO_2 fixation are significantly different between NH_4^+-depleted chemostat
174 populations and NH_4^+-depleted batch populations (Figure 2b, d; two sample t-test,
175 $p(N_2) = 0.0018$, $p(CO_2)=0.0034$). The two sample t-test compares a single
176 measurement from the chemostat experiment with four replicates form batch

experiments under the assumption that the measured chemostat CV is close to the mean value and that the variance is the same as for the batch experiments.

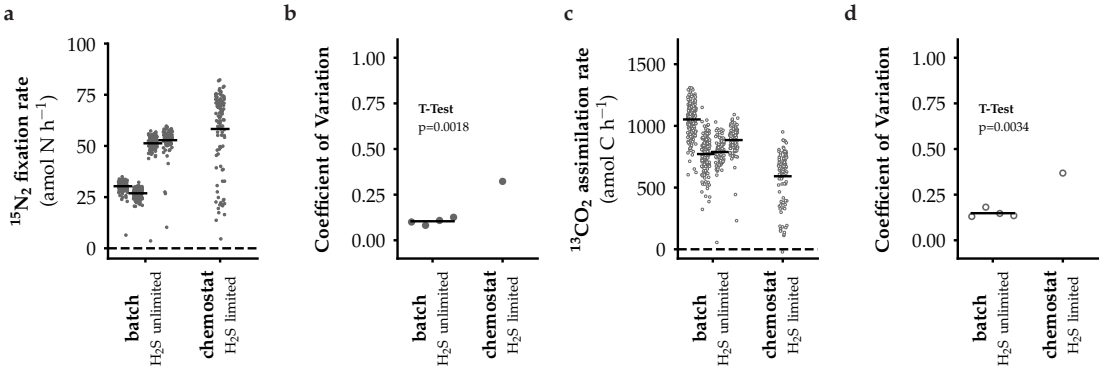

Figure 2. H_2S limitation during chemostat incubations induces heterogeneity in single-cell N_2 and CO_2 fixation activity as compared to unlimited batch-grown *C. phaeobacteroides* populations. Batch and chemostat incubations were both conducted under NH_4^+ depleted conditions. (**a**) $^{15}N_2$ fixation rates of single cells (grey dots) for batch (4 replicates) and chemostat (1 replicate) incubations. Average rates are indicated by a black bar. (**b**) Coefficients of variation (CV) of $^{15}N_2$ fixation rates. The average CV of the four batch incubations is indicated by a black bar. The CV of the chemostat incubation is significantly different from the CV's of the four batch incubations (two sample t-test). (**c**) Calculated $^{13}CO_2$ fixation rates of single cells (grey circles) for batch (4 replicates) and chemostat (1 replicate) incubations. Average rates are indicated by a black bar. (**d**) Coefficients of variation of $^{13}CO_2$ fixation rates. The average CV of the four batch incubations is indicated by a black bar. The CV of the chemostat incubation is significantly different form the CV's of the four batch incubations (two sample t-test).

The results indicate that electron donor limitation can induce phenotypic heterogeneity in different metabolic processes within the same bacterial population. Similarly, pronounced heterogeneity has been observed for ^2H_2O (growth rate) and $^{15}NH_4^+$ assimilation in chemostat-grown, carbon-limited *Staphylococcus aureus* populations (Kopf *et al.*, 2015a). In combination with the NH_4^+ limitation experiment, the results demonstrate that phenotypic heterogeneity in a certain metabolic activity (i.e. N_2 fixation) can be driven by different modes of limitation (here limitation in NH_4^+ and H_2S) in a single microbial population (i.e. *C. phaeobacteroides*). These results might be best understood in terms of a general feedback between growth state and gene expression (Klumpp *et al.*, 2009; Scott *et al.*, 2010; New *et al.*, 2014; Solopova *et al.*, 2014; Kotte *et al.*, 2014; Guantes *et al.*, 2016).

206

207 Taken together, our results and those of previous studies (Kopf *et al.*, 2015a;
208 Schreiber *et al.*, 2016) suggest that limitation might be a general driver of phenotypic
209 heterogeneity in microbial populations regardless of their culture history, the general
210 physiology of the bacterium, the type of limitation, and the considered metabolic
211 activity.

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222

223 **Conflict of Interest**

224 The authors do not declare any conflict of interest.

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