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High metabolic versatility and phenotypic heterogeneity in a marine non-cyanobacterial diazotroph

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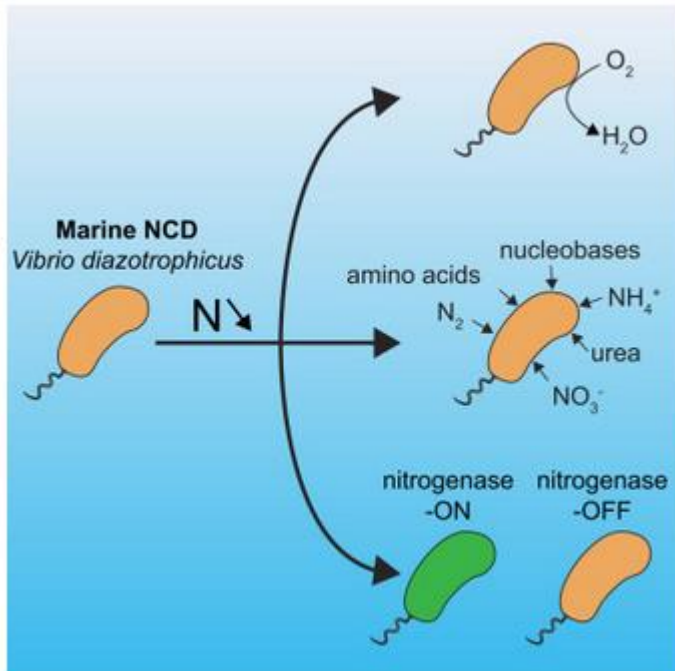
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Abstract :

Marine non-cyanobacterial diazotrophs (NCDs) are widespread in the oceans, but the processes controlling nitrogen fixation in cell populations remain understudied. In this study, we combined high-throughput sequencing, genetic and physiological characterization, and single-cell quantification of nitrogenase expression to investigate the growth strategies of the marine NCD *Vibrio diazotrophicus*. We demonstrate that this marine NCD is highly versatile, capable of utilizing a broad range of organic and inorganic nitrogen sources. Quantitative fluorescence microscopy revealed intense posttranscriptional regulation of nitrogenase expression and that *V. diazotrophicus* regulates both the proportion of cells and their nitrogenase expression levels based on ammonium concentration in an NtrC-dependent manner. We also found that this phenotypic heterogeneity in nitrogenase expression is more widespread among marine NCDs, suggesting it is a conserved trait. These findings help explain their high abundance in the oceans and deepen our understanding of their ecological importance.

Graphical abstract



Highlights

► Intense metabolic rewiring of a marine NCD during diazotrophic growth ► High metabolic versatility to explore alternative nitrogen traces in a marine NCD ► The proportion of nitrogenase-expressing cells is regulated by NH_4^+ and NtrC ► This phenotypic heterogeneity may be a conserved trait among marine NCDs

Keywords : non-cyanobacterial diazotroph, NCD, heterotrophic bacterial diazotroph, HBD, marine bacteria, nitrogen, N_2 fixation, *Vibrio*, phenotypic heterogeneity, single-cell approaches, metabolism, bioreporter

INTRODUCTION

The availability of soluble reactive nitrogen (SRN) —in the form of nitrate and ammonium — is one of the key limiting factors for marine primary production across vast areas of the world's oceans. In these areas, dinitrogen (N_2) fixers, known as diazotrophs, help alleviate N limitation by converting N_2 gas into a more biological accessible form of nitrogen. In doing so, diazotrophs fuel primary production and promote carbon export to the deep ocean ^{1,2}. Among marine diazotrophs, free-living cyanobacteria such as *Trichodesmium* sp., are believed to account for a large proportion of N_2 fixation in the ocean ³. Cyanobacteria such as UCYN-A can also engage in a symbiosis with photoautotrophic micro-eukaryotes eventually turning to an early-stage organelle.

Importantly, most cyanobacteria also perform oxygenic photosynthesis, *i.e.* they produce O_2 when they convert CO_2 into organic matter. Photosynthesis therefore comes into metabolic conflict with diazotrophy with regard to O_2 , since O_2 is known to inhibit nitrogenase ⁴. Strategies have been developed to resolve this metabolic dilemma. Some cyanobacteria, such as the unicellular *Cyanothece* ⁵, can separate photosynthesis during the day from diazotrophy in the night. Other filamentous cyanobacteria, such as *Dolichospermum* (formerly known as *Anabaena*), differentiate into genetically identical but phenotypically distinct subpopulations, one of which expresses the nitrogenase (the heterocysts) and fixes N_2 , while the other subpopulation is dedicated to oxygenic photosynthesis ^{5,6}. The presence of phenotypically distinct subpopulations within an isogenic population, known as phenotypic heterogeneity, is a form of division of labor, with nitrogen and carbon molecules circulating within the cyanobacterial filament. Finally, non-heterocyst diazotrophs can protect their own nitrogenase from O_2 by producing hopanoid lipids ⁷.

Additionally, the key enzymatic complex, called nitrogenase and composed of NifHDK is extremely ATP-demanding, requiring 16 ATP molecules for the conversion of one molecule of N_2 to NH_3 . Due to this energetic burden, known diazotrophs have developed genetic regulatory systems that tightly regulate N_2 -fixation ⁸. Typically, nitrogenase expression is positively regulated by RpoN and NifA, which act as a transcriptional factor and co-factor, respectively. Conversely, this expression is negatively regulated when the concentration of nitrogen, such as ammonium (NH_4^+), is high ⁸.

While the physiological responses to nitrogen starvation have been extensively studied for decades in cyanobacteria and terrestrial diazotrophs, the discovery of marine Non-Cyanobacterial Diazotrophs (NCDs) blurred our understanding of marine diazotrophy and raises key questions regarding their ecophysiology. Indeed, PCR amplicon studies of the *nifH* gene, a recognized hallmark of diazotrophy, have identified bacterial lineages, mainly within the phylum *Proteobacteria*, suggesting the ability of

NCDs to fix N₂ in ocean⁹. Recently, analyses conducted on samples from TARA Ocean expeditions revealed that NCDs were not only widespread but also abundant in the Pacific and north-western Atlantic Oceans^{10,11}. A better understanding of these NCDs is required to reconsider their role in diazotrophy within areas already recognized to be conducive to N₂-fixation. Conversely, NCDs could have a diazotrophic activity in nitrogen-rich environments such as aphotic layer or coastal zones.

Few non-cyanobacterial diazotrophs (NCDs) have been studied using genomic and physiological approaches. The most studied example concerns the strain *Pseudomonas stutzeri* BAL361^{12,13}. This strain can fix N₂ despite the presence of relatively high O₂ concentrations^{12,13} and form aggregates, possibly as a physiological response to the presence of O₂¹². In addition, diazotrophic activity is negatively impacted by NH₄⁺ and nitrate (NO₃⁻)¹².

Deciphering the genetic and physiological responses of marine NCDs during diazotrophy requires the development of genetic and molecular tools for those members, a prerequisite that is not trivial when working with environmental strains. As a consequence, a new strain has emerged as a potential NCD model to study their ecophysiology. This strain belongs to the proteobacterium species *Vibrio diazotrophicus*, with representatives having been isolated from various oceanic habitats, including deep-sea sediments, in association with sea urchins, sea anemones or from phytoplankton exudates¹⁴⁻¹⁷. The type strain, *V. diazotrophicus* NS1 can grow in the absence of SRN, a condition that requires micro-oxic conditions or alternatively production of biofilm likely to restrict O₂ diffusion within the biofilm¹⁸. Moreover, a genetic approach led to the construction of the first marine NCD mutant, with the deletion of the *nifH* gene, rendering this mutant unable to grow under SRN-free conditions¹⁸.

Until now, the genetic and physiological rewiring of marine NCDs to diazotrophic conditions remain underexplored, limiting our global understanding of this polyphyletic group. Using *V. diazotrophicus* as a model, this work aimed at filling this important knowledge gap by coupling an in depth characterization of the genome-wide response of a NCD to diazotrophy with fine-scale determination of the genetic regulation of the process. Moreover, a single-cell based approach using quantitative fluorescence microscopy demonstrated that *V. diazotrophicus* tightly regulates transcriptionally and post-transcriptionally nitrogenase expression depending on ammonium concentration. Importantly, nitrogenase expression was restricted to a subset of the isogenic population of *V. diazotrophicus* NS1 and of another marine NCD, suggesting phenotypic heterogeneity as a conserved NCD trait and a division of labour under SRN-limiting conditions.

RESULTS

***V. diazotrophicus* engages a massive transcriptional rewiring upon nitrogen starvation**

To gain a comprehensive understanding of the genetic response to SRN starvation in a marine NCD, the transcriptomic profile of *V. diazotrophicus* NS1 grown under diazotrophic permissive condition (soft gellan without SRN) was compared to its profile when grown in LB or in softgellan + NH₄⁺ medium. These latter two conditions were shown to inhibit the diazotrophic activity of this strain (**Figure S1**). Overall, *V. diazotrophicus* NS1 engages a massive rewiring of its transcriptomic profile under SRN-free conditions, with 1,258 (when compared with softgellan + NH₄⁺) to 1,880 (when compared with LB) genes being significantly differentially expressed (**Figures 1 and S2**). This accounts for 28.4% and 42.4% of the 4,431 genes composing the genome, respectively (**Figure 1 and Figure S2**). In total, 48.9 to 57.2% of the Differentially Expressed Genes (DEGs) were found upregulated in SRN-free condition as compared to LB and softgellan + NH₄⁺ conditions, respectively (see **Data S1** for a complete list of DEGs).

COG analysis of the DEGs revealed that 26.0 to 28.8% of those genes were categorized as “Unknown Function” (COG category S) or as “Unclassified” (COG category UC), and were removed from further COG analysis (**Figure S2AB**). We subsequently performed a Fisher’s exact overrepresentation test, to determine which COGs were significantly enriched (in either direction) as compared to their representation in the entire genome. Under diazotrophic condition (softgellan), COG C (Energy production and conversion) and COG T (Signal transduction mechanisms) were significantly differentially enriched in upregulated genes compared to SRN enriched media (LB and softgellan + NH₄⁺) (**Figure 1**). This suggests that growth under SRN limitation is energy-demanding, consistent with the high ATP-requirement of the nitrogenase to convert N₂ into NH₃.

When comparing the transcriptomic profile of NCD cells grown in SRN-free medium (softgellan) to that of NCD cells grown in a medium rich in organic nitrogen in the form of amino acids (LB), genes belonging to COG E (amino acid transport and metabolism) and O (post-translational modification, protein turnover and chaperones) were significantly over-represented among the positively DEGs. In contrast, genes from categories K (transcription) and J (ribosome translation, structure and biogenesis) were downregulated, suggesting a general slowdown of the cellular machinery under SRN-limited conditions (**Figure 1B**).

When comparing the transcriptomic profile of NCD cells grown in softgellan to that of NCD cells grown in a medium rich in SRN source (softgellan + NH₄⁺), categories N (cell motility), G (carbohydrate transport and metabolism) and K were up-represented under diazotrophic conditions, while no COG was shown to be significantly down-represented (**Figure 1A**). These results

demonstrated that the marine NCD completely rewires its metabolism, to commit to diazotrophic conditions.

The entire *nif* cluster is highly overexpressed and required in diazotrophic conditions

The genes found in the *nif* cluster of *V. diazotrophicus* NS1 (**Figure S3A**)¹⁸ were among the most DEGs (**Figure S3A** and **Data S1**). This includes those encoding the key enzymatic nitrogenase complex NifHDK, which were induced by 25 to 551-fold under diazotrophic conditions, as well as genes encoding accessory and/or regulatory proteins such as *nifA*. These findings confirm that the nitrogenase was expressed and active under SRN-free condition, thereby validating both our experimental set-up and RNA sequencing results. Located between the *nifF* and *nifL* genes is an *rnfABCDEG* operon, which is also overexpressed (23- to 70-fold, **Figure S3B**). This operon encodes the Rnf complex, which can reduce ferredoxin or NifF using NADH as an electron donor. The electrons produced are transferred to the nitrogenase, enabling it to reduce N₂ to NH₃¹⁹. Immediately downstream of *nifM*, the molybdenum ModABC transporter was also overexpressed (46- to 233-fold, **Figure S3C**). Molybdenum is crucial for the nitrogenase function, being coupled with iron (MoFe)²⁰ and permitting electron transfer via ATP hydrolysis. Interestingly, *V. diazotrophicus* NS1 seems to bestow an additional operon encoding the molybdenum transporter (BBJY01_440097 to 440099), but this operon is not overexpressed under diazotrophic conditions (**Figure S3C**).

In order to formally demonstrate that this gene cluster is required for growth under diazotrophic conditions, a scarless deletion of the *nifA* gene, encoding a putative transcriptional co-factor required for the expression of *nifHDK* in various diazotrophs was performed. Additionally, since NifA works in combination with the transcriptional factor σ_{54} , we independently also deleted the corresponding *rpoN* gene in the genome of *V. diazotrophicus* NS1. Notably, both mutants share a similar growth shape, similar to that of the wild-type, and achieved a comparable final biomass after 24 hours of growth in LB (**Figure S4AB**). However, when inoculated in softgellan, those mutants were unable to grow while gene complementation restored the wild-type phenotype (**Figure 2AB**). These results demonstrate that both NifA and RpoN are required for the growth in the absence of nitrogen other than N₂. This also suggests that NifA and RpoN act as regulators of *nifHDK* expression in marine NCD. Moreover, this confirms that the experimental set-up, using softgellan, is conducive to commit the growth of *V. diazotrophicus* and its derivative strains under diazotrophic conditions.

The global nitrogen regulator NtrC is not required for growth in SRN-free condition but is implicated in N₂-fixation

NtrC is known to be a major regulator of nitrogen metabolism in many bacterial species. Gene *BBJY01_490003*, putatively encoding the NtrC protein, but also *BBJY01_490004* and *BBJY01_490005*, encoding NtrB and a protein of unknown function, are overexpressed by 4- to 16-fold under SRN-limited conditions (**Data S1**). The *ntrC* gene was deleted in *V. diazotrophicus* NS1, and this deletion did not impair the strain's ability to grow in LB (**Figure S4C**) nor in SRN-free conditions (**Figure 2C**), suggesting an active nitrogenase. To confirm this hypothesis, we next aimed to determine whether the corresponding global regulator plays a role in the N₂-fixation activity. The wild-type strain and the *ntrC* mutant were grown in softgellan and the diazotrophic activity of the population was quantified by an Acetylene Reduction Assay (ARA). Under this condition, *V. diazotrophicus* was able to convert up to 4% of acetylene to ethylene after 3 days (**Figure 2D**). In contrast, the deletion of *ntrC* led to a significant 90% decrease in the population-wide N₂-fixation activity, while complementing this mutant, by the introduction of *ntrC* in the *ntrC* mutant, restored the wild type phenotype (**Figure 2D**). It should be noted that this activity was not completely abolished, explaining the mutant's capability to grow in the absence of nitrogen sources other than N₂ (**Figure 2C**). Therefore, NtrC, while not being strictly required for growth under diazotrophic conditions, plays a role in the N₂-fixation activity of this marine NCD.

***V. diazotrophicus* seeks for trace inorganic sources under SRN-limitation conditions**

NO₃⁻ is the major bioavailable nitrogen source in oceans but is not directly assimilable. *V. diazotrophicus* NS1 is endowed with a genomic region encoding a putative nitrate reductase (*BBJY01_540094*) and nitrite reductase (*BBJY01_540101* and *BBJY01_540102*) flanking nitrate transporters (*BBJY01_540098* to *BBJY01_540100*), which might be involved in the import and reduction (to nitrite and then to NH₄⁺) of NO₃⁻, allowing subsequent ammonium assimilation to produce new biomass. This cluster showed a 7- to 1064-fold expression induction under SRN-free conditions (**Figure S5A**), the NO₃⁻ transporter (*BBJY01_540098*) being the highest differentially expressed gene in softgellan vs LB (**Data S1B**). In order to determine whether this gene cluster is required for growth under diazotrophic conditions, gene *BBJY01_540094* encoding this putative nitrate reductase in *V. diazotrophicus* NS1 was deleted and the mutant complemented. This mutant showed growth in LB comparable to that of the wild-type (**Figure S4D**), and could grow in softgellan, suggesting an active nitrogenase (**Figure 3A**). This diazotrophic activity was confirmed by an ARA experiment, which revealed that deletion of the nitrate reductase did not impact diazotrophy (**Figure 3B**). Therefore, gene *BBJY01_540094* is not required for *V. diazotrophicus* NS1 to grow under SRN-free conditions.

Finally, the growth of the *BBJY01_540094* mutant in the absence or presence of NO_3^- in aerobic liquid MDV was monitored to determine whether this gene cluster was involved in the transport, reduction and assimilation of NO_3^- from the environment. In its absence, the oxygenation of the medium is supposed to inhibit the nitrogenase, forcing it to find another source of nitrogen to thrive. Indeed, under this condition, the wild-type *V. diazotrophicus* NS1 was unable to grow in the absence of NO_3^- , while its presence allowed significant growth, demonstrating the strain's ability to use NO_3^- as an assimilatory nitrogen source (**Figure 3C**). In contrast, the *BBJY01_540094* mutant was unable to grow, either with or without NO_3^- , and gene complementation restored the wild-type phenotype (**Figure 3C**). These results indicated that the nitrate reductase, encoded by *BBJY01_540094*, is essential for nitrogen assimilation, probably via the reduction of nitrate to nitrite.

Urea as a potential organic nitrogen source for *V. diazotrophicus* NS1

In the ocean, the urea concentration varies and is in general between 0.1 and 5 μM ²¹. Urea utilization as a nitrogen (and carbon) source requires the catalytic enzyme urease, a nickel-containing enzyme which releases NH_4^+ from urea, and urea transporters. *V. diazotrophicus* NS1 bestows an operon composed of the entire *ureDABCEFGJ* operon allowing urease production, located immediately upstream of the *urtABCDE* genes required for urea import (**Figure S6B**). Interestingly, the RNA-seq analysis indicates that the transcription of this operon increased 5- to 161-fold in SRN-free conditions compared to softgellan + NH_4^+ and LB conditions, respectively (**Figure S6B**). This overexpression may indicate the NCD ability to metabolize urea either intra- or extracellularly. To test this hypothesis, the *ureA* gene was deleted and the mutant complemented. This mutant reached similar final biomass in LB (**Figure S4E**) and was still able to grow in softgellan without nitrogen other than N_2 , suggesting an active nitrogenase (**Figure 3D**). This diazotrophic activity was confirmed by ARA, which revealed that *ureA* deletion had no impact on diazotrophy (**Figure 3E**). Therefore, the *ureA* gene is not required for *V. diazotrophicus* NS1 under SRN-free conditions or for nitrogenase activity.

We subsequently tested whether urea could serve as a nitrogen source by growing both the wild-type and the ΔureA mutant under diazotrophic-inhibiting conditions, *i.e.* under oxic conditions in MDV. Under these conditions, *V. diazotrophicus* was unable to grow in the absence of urea, but exhibited growth when urea was present, demonstrating its ability to use urea as a nitrogen source. Unlike the wild-type strain, the urease mutant (ΔureA) showed no growth regardless of urea availability. Complementation of the deletion partially restored the wild-type phenotype, since a growth delay of several hours was observed compared to the wild-type strain (**Figure 3F**). This demonstrates that urea is a potential nitrogen source for this marine NCD, due to its ability to degrade urea to NH_4^+ .

Nucleic and amino acids as alternative sources of nitrogen for *V. diazotrophicus* NS1

Other organic nitrogen sources can also be available, like nucleic and amino acids resulting from the decomposition of micro and macro-organisms. The RNA-seq analysis showed that genes involved in nucleic and amino acid (and derivatives) transport (**Figure S6B** and **Data S1**) and degradation were overexpressed under SRN-free conditions, including betaine, choline, arginine, glycine betaine, proline (**Figure S6A** and **Data S1**). In addition, several deaminases and oxidases were also overexpressed, such as nucleoside, ornithine and enamine/enimine deaminases (**Figure S6C** and **Data S1**). Furthermore, the entire pyrimidine (**Figure S6D** and **Data S1**) and purine (**Figure S7**) transport and degradation gene pathway were overexpressed (up to 241-fold). These pathways are known to release NH_4^+ (see **Figure S7** for the purine degradation pathway). To confirm the ability of *V. diazotrophicus* NS1 to use some of these molecules as nitrogen sources, the strain was grown under fully oxic conditions, with or without guanine or arginine as nitrogen sources. Results showed that *V. diazotrophicus* NS1 could only grow when the medium was supplemented with either guanine or arginine (**Table S5**), demonstrating that both guanine and arginine can serve as nitrogen source for this strain.

Transport and assimilation of ammonium by *V. diazotrophicus* NS1

The experiments described above, including RNA-seq analysis combined with genetic mutant construction and physiological characterization, showed that *V. diazotrophicus* NS1 is capable of utilizing a wide variety of organic and inorganic nitrogen sources, all of which are intracellularly converted into NH_4^+ . The major NH_4^+ assimilation pathway in bacteria is the Glutamine Synthetase-Glutamine Oxoglutarate Aminotransferase (GS-GOGAT). In *V. diazotrophicus* NS1, gene *BBJY01_490006* encodes the putative GS, and this gene revealed 43-fold overexpressed in softgellan as comparing with softgellan supplemented with NH_4^+ (**Data S1A**). Interestingly, the GOGAT genes are found in two adjacent copies (*BBJY01_560203-560204* and *BBJY01_560205-560206*) in *V. diazotrophicus* NS1, sharing 37 to 42% amino acid identity. These genes are 5- to 11-fold overexpressed in softgellan vs softgellan with NH_4^+ (**Data S1A**).

Additionally, the gene encoding the NH_4^+ transporter *amt* was found in two copies in *V. diazotrophicus* NS1 (*BBJY01_500114* and *BBJY01_530087*). However, only gene *BBJY01_500114* revealed overexpressed (6- to 121-fold) under diazotrophic conditions, and this gene is located immediately upstream of gene *BBJY01_500115*, encoding the PII-family nitrogen regulator GlnB and also overexpressed (15- to 937-fold, **Data S1**). In contrast, the gene *BBJY01_530087* was not overexpressed and is not located near a cognate *glnB* gene, suggesting that *BBJY01_500114* and

BBJY01_500115 are likely the NH_4^+ transporter and PII regulator involved in nitrogen regulation in *V. diazotrophicus* NS1, respectively.

Reduction of ROS potentially produced by hyper-respiration

The genome of *V. diazotrophicus* NS1 is endowed with both the low- O_2 affinity *bo* cytochrome (*cyoABCDE*, genes *BBJY01_570181* to *570185*) and the high- O_2 affinity *bd* cytochrome (*cydABX*, genes *BBJY01_450042* to *450004*). The latter was overexpressed in softgellan vs softgellan + NH_4^+ and is known to consume large amounts of O_2 during respiration, playing a crucial role in reducing intracellular O_2 levels in a terrestrial diazotroph²². One consequence of (hyper-)respiration is the release of reactive oxygen species (ROS). Interestingly, among the genes that were highly overexpressed under diazotrophic conditions, those related to oxidative stress response were particularly prominent. The *ahpC* gene, encoding the Alkyl hydroperoxide reductase C and reducing hydrogen peroxides and organic hydroperoxides to water and alcohols, showed that *ahpC* transcription increased by a factor of 15 to 284 under SRN-free conditions compared to softgellan + NH_4^+ and LB conditions, respectively (**Figure S8A**). This gene was found immediately upstream of *ahpF* respectively, whose expression also increased by a factor of 4 to 43 (**Figure S8A**). Additionally, *BBJY01_360030*, annotated as *katG* and encoding a catalase/peroxidase, was also overexpressed (8- to 38-fold) under diazotrophic conditions (**Data S1**).

In order to test the role of oxidative stress response during diazotrophy, the *ahpC* gene was deleted and the mutant complemented. Of note, we were unable to obtain this deletion mutant using the standard procedure used for the other genes, as the second recombination always led to the obtention of a wild type revertant. Given that *ahpC* is probably impaired in ROS detoxification, the deletion experiments were repeated under anoxic conditions, which should alleviate O_2 -mediated ROS production and extensive cell burden. Under this condition, an *ahpC* mutant was obtained. This mutant was able to grow in LB under oxic conditions (**Figure S4F**), to grow in softgellan (**Figure S8B**) and to fix N_2 (**Figure S8C**). Therefore, the *ahpC* gene was not required for *V. diazotrophicus* NS1 to grow under SRN-free conditions or to maintain nitrogenase activity.

We subsequently tested the protective role against oxygen of AhpC and showed that the wild-type strain was able to grow under aerobic condition, both with and without H_2O_2 , revealing its resistance to oxidative stress (**Figure S8D**). In the absence of O_2 , the addition of H_2O_2 led to a 10-fold growth decrease for the wild-type, suggesting that genes involved in resistance to H_2O_2 are less expressed under anoxic conditions in *V. diazotrophicus* NS1. In contrast, the *ahpC* mutant strain was severely impaired in growth (x1000 less growth), and this phenotype is exacerbated with the addition of H_2O_2 .

These results demonstrated that *V. diazotrophicus* is able to cope with ROS generated by respiration, and that this adaptation is mediated, at least partially, by AhpC.

***Diazotrophic activity of V. diazotrophicus* NS1 is regulated by NH₄⁺ concentration**

Our results showed that *V. diazotrophicus* NS1 commits to a complete reprogramming of its genetic arsenal when grown in the absence of nitrogen besides N₂. One of the most induced gene clusters was the *nif* cluster (**Figure 2**), which encodes for the production of the nitrogenase complex. In the next set of experiments, the diazotrophic activity of *V. diazotrophicus* NS1 was tested to determine whether this activity was restricted to SRN-free conditions. *V. diazotrophicus* NS1 was grown in softgellan spiked with increasing NH₄⁺ concentrations, and ARA experiments were carried out. Without NH₄⁺, the population-wide N₂-fixation activity, expressed by the ethylene/acetylene ratio, was around 7%, and this ratio decreased with increasing NH₄⁺ concentrations in the medium (**Figure 4A**). Importantly, this analysis also revealed that diazotrophic activity at 0.75 μM NH₄⁺ was low but not null. Above 0.75 μM NH₄⁺, no ethylene was detected, showing that the nitrogenase was not expressed or not active at these concentrations. This demonstrated that the diazotrophic activity of *V. diazotrophicus* NS1 is regulated by NH₄⁺ concentration but it was still active at initial NH₄⁺ up to 0.75 μM.

The nitrogenase is expressed in a subset of the population only

Diazotrophic activity of *V. diazotrophicus* NS1 decreased with increasing NH₄⁺ concentrations. Whether this decrease in activity was due to a decrease *nifHDK* expression remained to be demonstrated. The promoter region of *nifH* of *V. diazotrophicus* NS1 was amplified and cloned upstream of the reporter gene *egfp* in a plasmid and introduced back in *V. diazotrophicus* NS1. This bioreporter allowed single-cell measurement of the promoter activity, with the eGFP intensity being proportional to the activity of the *nifH* promoter. The bioreporters were grown in softgellan and formed a growth disk at a micro-oxic zone. However, as this disk is located at the interface between oxic and anoxic environment, cells located just below the disk are in anoxia. However, the GFP (and all its derivatives) fluorochrome requires O₂ to fluoresce. Bioreporter cells can therefore reveal non-fluorescent because of the absence of oxygen and independently of the (in)activity of the studied promoter. To avoid this issue, we inserted in the same plasmid a second construction containing a constitutive promoter transcriptionally fused to the *mcherry* gene (**Figure 4B**). An absence of mCherry fluorescence indicates cells which were not oxygenated and those were removed from the analysis. Single-cell based *nifH* promoter activity measurement was highest between 0 μM and 0.5 μM initial NH₄⁺ concentration in the medium, revealing that *nifH* is indeed highly expressed at such concentrations. At 0.75 μM NH₄⁺, eGFP fluorescence was higher than the one of the negative controls

(promoterless *egfp* gene, **Figure 4C**). At initial NH_4^+ concentrations above 1 μM , eGFP production is close and not significantly different to the background, highlighting that the nitrogenase was not expressed above 0.75 μM initial concentration.

Interestingly, a careful observation of single cells showed that not all cells produced eGFP and therefore express *nifH* (**Figure 4DE**). This intriguing behavior was quantified by QQ plots²³, which revealed the existence of two discrete subpopulations, one not expressing *nifH* (*i.e.* low eGFP fluorescence intensity) and one expressing *nifH* (high eGFP fluorescence intensity (**Figure 4F**). This analysis revealed that the proportion of the subpopulation expressing *nifH* varied from 60% (at 0 and 0.03 μM initial NH_4^+ concentration) to 70-90% at 0.1 to 0.5 μM NH_4^+ (**Figure 4G**). At the highest initial NH_4^+ concentration for which population-wide fluorescence was measured (**Figure 4C**) and showing diazotrophic activity (**Figure 4A**), *i.e.* at 0.75 μM , this subpopulation decreased to 30% of the entire population (**Figure 4G**).

In order to determine whether the P_{nifH} -based eGFP production measured in all cells (**Figure 4C**) reflects the activity of the promoter in the activated cell population, the fluorescence intensity was measured in single-cells of this subpopulation only. This analysis showed that the activity of the P_{nifH} promoter decreased gradually with increasing NH_4^+ concentrations (**Figure 4H**). Thus, our results demonstrated that the diazotrophic activity is restricted to a subpopulation of cells only, and also that NH_4^+ did not only regulate the proportion of expressing cells but also the intensity of this expression in each *nifH*-expressing cells.

Interestingly, the results also highlighted that the population-wide eGFP production from P_{nifH} promoter remained high at 0.5 μM NH_4^+ , while ARA was significantly reduced at this concentration (compare **Figures 4A and 4C**). Therefore, the high expression of the nitrogenase measured at 0.5 μM NH_4^+ did not lead to high nitrogenase activity, suggesting intense post-transcriptional regulation of the nitrogenase.

NtrC regulates the proportion of nitrogenase-expressing cells

In the next set of experiments, we introduced the pPC018 plasmid in the *ntrC* mutant to determine the role NtrC might have in the molecular origin of phenotypic heterogeneity and we quantified the P_{nifH} expression in individual cells when grown in softgellan (**Figure 5A**). We showed that the *ntrC* mutant is severely impacted, since the activated subpopulation decreased to 5% as compared to the 32% found in the wild type (**Figure 5BDE**). In addition, nitrogenase expression level is significantly reduced in the activated subpopulation of the *ntrC* mutant (**Figure 5C**). Therefore, NtrC acts as a global modulator of phenotypic heterogeneity in *V. diazotrophicus* and regulates the activity of the P_{nifH} promoter in the nitrogenase-expressing subpopulation.

Phenotypic heterogeneity may be a conserved physiological trait in marine NCDs

In order to test whether phenotypic heterogeneity in nitrogenase expression is a peculiar trait of *V. diazotrophicus* NS1 or whether this behavior might be shared with other NCDs from other ecological niches, the *nifH* promoter of *V. diazotrophicus* 60.18M, isolated from deep-sea sediments¹⁵ was cloned in pPC018, removing the P_{nifH} promoter of *V. diazotrophicus* NS1. This plasmid was introduced back in *V. diazotrophicus* 60.18M by mating, using the same conditions as for *V. diazotrophicus* NS1.

V. diazotrophicus 60.18M was grown in softgellan, under the same conditions as the one established for *V. diazotrophicus* NS1, demonstrating that *V. diazotrophicus* 60.18M was able to grow in the absence of nitrogen besides N_2 . We repeated the experiment with the bioreporter strain, inoculated into softgellan either spiked or not with $0.75 \mu\text{M NH}_4^+$. The fluorescence values recorded in single-cells were cleaned up as with strain NS1 by removing non-oxygenated cells (those not producing mCherry fluorescence). Measured as a mean of the whole population, the eGFP fluorescence was around 5,000 at both NH_4^+ concentrations (**Figure 6A**), demonstrating high nitrogenase expression at high NH_4^+ concentration. Importantly, fluorescence images also showed the existence of 2 subpopulations (**Figure 6B**), with only 30% of the entire population expressing *nifH* at $0 \mu\text{M NH}_4^+$, a proportion which increased to 70% at $0.75 \mu\text{M NH}_4^+$ (**Figure 6C**). Finally, the fluorescence of the *nifH*-expressing subpopulation decreased when NH_4^+ increase (**Figure 6D**), but still remained at high fluorescence intensity (compare **Figures 6D** with **4H** for *V. diazotrophicus* NS1). The strong fluorescence measured despite an NH_4^+ concentration of $0.75 \mu\text{M}$ (**Figure 6AD**) strongly suggests that strain 60.18M is able to fix nitrogen at this initial NH_4^+ concentration. This hypothesis was confirmed by ARA (**Figure 6E**), even though the ethylene production was lower than for NS1, despite higher *nifH* expression, suggesting stronger nitrogenase post-transcriptional regulation in 60.18M.

DISCUSSION

Diazotrophy is a crucial metabolic process, providing fixed nitrogen to living organisms and sustaining the primary production in ecosystems. While cyanobacteria have been extensively studied for their N_2 -fixing capabilities in marine environments, recent research has highlighted the prevalence of non-cyanobacterial diazotrophs (NCDs). Culture-based efforts led to the isolation of some marine NCDs^{24,25}, and others have been identified as a N_2 -fixing diatom symbiont²⁶. However, knowledge of their lifestyle under- and adaptation to SRN-limitation remains limited because of the low number of NCD strains successfully cultured, and of the even lower number of those having methodological tools (*i.e.*

genetic tools) for detailed characterization. In this study, we combined deep sequencing technologies with genetic engineering, coupled with single-cell quantitative fluorescence microscopy approaches to draw a number of conclusions about the numerous strategies deployed by the model NCD *V. diazotrophicus* during growth under SRN-limitation. Our results unveil the high adaptive potential of marine NCDs and help to understand the ecological success of this understudied polyphyletic prokaryotic group.

Firstly, we demonstrated that *V. diazotrophicus* seeks micro-oxic environments to sustain growth under diazotrophic conditions (**Figure S1**), and that, although high O₂ tension inhibits diazotrophy, respiration is required to fulfil the high ATP requirement for nitrogenase functioning^{20,27}. Under this condition, *V. diazotrophicus* is a powerful diazotroph, able to fulfil its nitrogen requirements exclusively from N₂, *i.e.* without initial amount of SRN (**Figure 4A**). Importantly, carbon-rich but SRN- and O₂-poor environments (oxygen minimum zones OMZ) host a wide diversity of proteobacterial diazotrophs^{28,29}. Other diazotrophy-conducive environments can be encountered in large-size particles in well-oxygenated environments³⁰. These particles offer surface for NCDs to attach and take up nutrients, producing a biofilm to limit O₂ diffusion and create micro-oxic environments³¹. Finally, marine organisms that feed on seaweed such as kelp represent key diazotrophy hotspots because seaweed have a high carbon to nitrogen (C:N) ratio and a low protein content, limiting nitrogen intake from feed^{14,32}. Thus, our finding that both *V. diazotrophicus* NS1, isolated from sea urchin, and strain 60.18M isolated from deep subsurface sea sediments are capable of fixing N₂ under micro-oxic and SRN-depleted conditions, support recent evidence showing the high prevalence of NCDs in oceans and their ability to colonize various ecological niches^{10,33}.

We also demonstrated that *V. diazotrophicus* overexpresses the high-O₂ affinity cytochrome *bd*, encoded by *cydABX* in response to SRN-limitation, which is known to consume large amount of intracellular O₂ during respiration. We hypothesize that this hyper-respiration plays a dual role in *V. diazotrophicus*. On one hand, it lowers intracellular O₂ concentrations, similar to a strategy deployed by the soil diazotroph *A. vinelandii*²². On the other hand, it creates a micro-oxic compartment conducive to diazotrophy, while generating ATP via electron transfer, which is essential for nitrogenase activity³⁴ (**Figure 7**). However, O₂-respiration typically leads to the generation of ROS, requiring detoxification systems. To cope with this additional burden, *V. diazotrophicus* overexpresses genes involved in oxidative stress defence, including *katG* and *ahpCF* (**Figure S8** and **Data S1**). This strategy has been observed in various diazotrophs during diazotrophy, including *Anabaena* sp.³⁵.

Deletion of *ahpC* in *V. diazotrophicus* impairs resistance to oxidative stress associated with respiration (**Figure S8D**), confirming the crucial role of AhpC in protecting cells from oxidative damage.

Furthermore, *V. diazotrophicus* downregulates its nitrogenase activity in response to increasing NH_4^+ concentrations (**Figures 4A** and **6E**), probably to decrease the energetic cost of the nitrogenase when its activity becomes unessential. These results are consistent with a regulation of diazotrophy by NH_4^+ for the marine NCD *P. stutzeri* BAL361¹³. Nevertheless, the molecular machinery involved in the regulation of nitrogenase expression has never been investigated for marine NCDs. As expected, genomic analysis coupled with mutagenesis showed here that both NifA and RpoN are required for growth in SRN-free conditions. This suggests that NifA and RpoN act as regulators of *nifHDK* expression in marine NCDs, similar the regulation in other well-known diazotrophs^{36,37}. In contrast, the role of NtrC, a global nitrogen metabolism regulator, during diazotrophy, is variable among known diazotrophs and has never been investigated in marine NCDs. In some diazotrophs, NtrC is essential to diazotrophy by inducing expression of NifA, the co-factor for *nifHDK* expression^{38,39}. In contrast, in other diazotrophs such as *A. vinelandii*⁴⁰ or *Bradyrhizobium japonicum*⁴¹ NtrC does not seem to be required for *nif* expression, which can be regulated by other regulatory proteins like FixL/FixJ in *Sinorhizobium meliloti*, RegS/RegR in *B. japonicum* or *Candidatus* *Thalassolituus haligoni*^{25,42,43}. Here, we showed the partial influence of NtrC, as this regulatory protein is not strictly required for *V. diazotrophicus* to grow in SRN-free condition (**Figure 2C**), but positively regulates nitrogenase activity (**Figure 2D**). These results, along with the absence of *fixLJ* or *regSR* homologs in the *V. diazotrophicus* NS1 genome, suggest that NtrC plays a role in relieving NifA inhibition by NifL, as already observed in *K. pneumoniae*⁸. However, this inhibition is not fully lifted, since the *ntrC* mutant was still able to grow in SRN-free condition (**Figure 2C**) and conserved a partially active nitrogenase activity (**Figure 2D**), similarly to *P. stutzeri* A1501⁴⁴.

Moreover, we demonstrated that *V. diazotrophicus* engages in intense post-transcriptional regulation of the nitrogenase expression. Indeed, at 0.5 μM or 0.75 μM initial NH_4^+ concentration for the strain NS1 and the strain 60.18M, respectively, nitrogenase expression, revealed by the use of bioreporters, was high (**Figures 4C, 4H** and **6A**). However, the nitrogenase activity remained low under these conditions (**Figures 4A** and **6E**). This post-transcriptional regulation may be an additional strategy to avoid the unnecessary nitrogenase production while still being “ready” to synthesize it, when NH_4^+ concentration decreases. From an ecological point of view, this finding has significant implications in our understanding of the relative importance of the entire diazotroph community (both NCDs and cyanobacteria) *in situ*. Indeed, recent evidences showing that marine NCDs are not only widespread

in oceans but also active largely come from metatranscriptomic approaches focusing on *nifH* gene expression^{10,45,46}. However, we showed here that nitrogenase expression of marine NCDs does not directly reflect nitrogenase activity. As a consequence, caution is needed when interpreting the relative contribution of a given group of diazotrophs to N₂-fixation based only on metatranscriptomic data. Of note, although both strains NS1 and 60.18M post-transcriptionally regulate nitrogenase expression, they display distinct behaviors with respect to diazotrophy, as showed by a nitrogenase expression 10-times higher in strain 60.18M than in strain NS1 at 0.75 μM NH₄⁺ (compare **Figure 4H** with **Figure 6D**), yet with lower nitrogenase activity (**Figure 6E**). This suggests a stronger post-transcriptional regulation of the nitrogenase in the strain 60.18M which was isolated from deep subsurface sediments. Sediments are typically enriched in SRN resulting from the mineralization of accumulated organic matter degradation via ammonification and nitrification, which should prevent unnecessary diazotrophic activity. However, sediments are also the site of the highest rates of nitrogen loss in the ocean by denitrification⁴⁷. The high nitrogenase expression of strain 60.18M despite high initial NH₄⁺ concentrations observed in this study experimentally complement the repeated benthic marine N₂-fixation reported at high SRN concentrations (⁴⁸ and references therein). For benthic diazotrophs, stronger post-transcriptional control of nitrogenase expression may be an additional strategy to be pre-adapted to fluctuating SRN availability driven by the balance between ammonification/nitrification and denitrification processes.

In addition, *V. diazotrophicus* completely rewires its metabolism under SRN-depleted conditions to uptake and assimilate a broad spectrum of nitrogen molecules, ranging from inert N₂ to both nitrogen inorganic and organic forms (**Figure 7**). Indeed, the transcriptional remodelling goes well beyond overexpression of the nitrogenase gene cluster (**Figure S3** and **Data S1**) and of the associated Rnf and molybdenum transporter gene clusters (**Figure S1**), as up to 42.4% of the entire genome was significantly affected (**Figure 1**, **Figure S2** and **Data S1**). This includes genes involved in the transport and assimilation of inorganic nitrogen sources like NO₃⁻ (**Figure 3**) and NH₄⁺ (**Figure 7**), as well as organic nitrogen sources, such as urea (**Figures 3DEF** and **S6B**), purine (**Figure S7**), pyrimidine (**Figure S6D**), amino acids and derivatives (**Figure S6A**), alongside the overexpression of numerous deaminases (**Figure S6C**). The high metabolic versatility of marine Gammaproteobacterial NCDs has been recently inferred from Metagenome-Assembled Genomes (MAGs)⁴⁹, leading to the hypothesis that they are facultative diazotrophs, being able to switch their metabolism to use alternative nitrogen sources when available⁴⁹. In this study, we provide direct experimental evidence supporting this hypothesis, demonstrating that these genes were not only overexpressed, but also required to uptake

inorganic or organic bioassimilable nitrogen forms, as mutants devoid of the corresponding genes revealed unable to grow with the corresponding nitrogen source (**Figure 3**).

Lastly, our single-based approach to monitor nitrogenase gene expression revealed an additional and unexpected adaptive strategy during diazotrophy: phenotypic heterogeneity. Indeed, *V. diazotrophicus* NS1 and 60.18M differentiated into two subpopulations: one actively expressing the nitrogenase (ON-population), and the other one remaining silent (OFF-population (**Figures 4DEF** and **6**). Importantly, *V. diazotrophicus* can modulate both the proportion of ON-subpopulation (**Figures 4G** and **6C**) and the nitrogenase expression level within the ON-subpopulation (**Figures 4H** and **6D**) depending on NH_4^+ concentration. Furthermore, we demonstrated that this proportion is modulated by NtrC, as it decreased in a *ntrC* mutant (**Figure 5**). Therefore, the decreased population-wide nitrogenase activity measured in the *ntrC* mutant (**Figure 2D**) is at least partially due to alteration in the proportion of nitrogenase-expressing cells. To our knowledge, this is the first demonstration of transcriptional-based phenotypic heterogeneity, *i.e.* the existence of two genetically-identical-but-phenotypically-divergent sub-populations, observed in marine NCDs for nitrogenase expression. While such phenotypic heterogeneity was observed at the protein level for *Vibrio natriegens* using immunolabelling⁵⁰, the hypothesis was that this heterogeneity resulted from experimental conditions. Phenotypic heterogeneity has been observed in some filamentous cyanobacterial diazotrophs such as *Anabaena*, for which a subset of cells differentiates into N_2 -fixing cell known as heterocysts⁵¹. This spatial differentiation in filamentous cyanobacteria allows the fixed N_2 to be transferred to neighbouring vegetative cells within the filament, the latter cell subpopulation providing fixed carbon to the heterocysts. However, *V. diazotrophicus* does not grow in filaments and does not perform oxygenic photosynthesis unlike *Anabaena* which can explain this different behavior. Therefore, the ecological reasons underlying phenotypic heterogeneity in nitrogenase-expression for marine NCDs are currently unknown. One hypothesis is that the NCDs adopt a division of labor strategy, with the two subpopulations having distinct roles, the ON-subpopulation providing reactive nitrogen derived of diazotrophy to the OFF-subpopulation, which in turn may perform another task such as biofilm production or hyper-respiration. This strategy requires close contact between cells, such as within biofilms, to prevent loss of signals/molecules between both subpopulations and to optimize energetic advantages⁵². Alternatively, phenotypic heterogeneity might stem from a bet-hedging strategy where the OFF- and ON-subpopulations would coexist to ensure that the population-level genotype is pre-adapted to unpredictable environmental changes such as sudden increase or depletion of SRN sources. Interestingly, a single-cell based approach using nanoSIMS demonstrated that the modulation of N_2 -fixing cells allows the soil diazotroph *Klebsiella oxytoca* to better cope with substrate fluctuations,

demonstrating the importance of phenotypic heterogeneity⁵³. Whether *V. diazotrophicus* is better pre-adapted to environmental changes remains to be demonstrated.

Irrespective of the evolutionary origin of this individual behavior, the demonstration that two different strains of *V. diazotrophicus* (NS1 and 60.18M) differing in their original ecological niche modulate the proportion of nitrogenase-expressing cells (**Figures 4G** and **6C**) suggests that phenotypic heterogeneity might be a conserved trait in marine NCDs.

In conclusion, our results provide an unprecedented view of the cellular and physiological adaptations of a marine NCD during nitrogen limitation, significantly expanding knowledge of this neglected polyphyletic group and contributing to the understanding of their ecological success. Furthermore, the demonstration that nitrogenase expression is restricted to a subpopulation of marine NCDs cells opens a new avenue to decipher the molecular circuit regulating this phenotypic heterogeneity and to unravel the evolutionary origin of this intriguing bacterial cell behavior.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, François Delavat (francois.delavat@univ-nantes.fr).

Materials availability

Strains used and/or generated in this study will be made available on request from the lead contact.

Data and code availability

The RNAseq data generated in this study and associated metadata (biosamples and SRA) are available through NCBI under bioproject accession number PRJNA1133960.

The code used to generate Figures 1 and S2 is available upon request.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization: P.C. and F.D.; Data curation: P.C., N.P., J.V. and F.D.; Formal analysis: P.C., N.P., I.L., J.V., L.M., J-F. M., and F.D.; Funding acquisition: F.D.; Investigation: P.C., L.M., A.J-D., N.P. and E.L.; Methodology: P.C., J-F.M. and F.D.; Project Administration: F.D.; Resources: J-F.M. and F.D.; Supervision: J-F.M and F.D.; Validation: P.C., J-F.M. and F.D; Visualization: P.C. and F.D.; Writing – original draft: P.C. and F.D.; Writing – review & editing: all co-authors;

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURES

Figure 1. COG category distribution of differentially expressed genes, comparing SRN-free softgellan with either softgellan + NH₄⁺ (A) or LB (B). The vertical bars represent the number of upregulated (green) and downregulated (purple) genes in each COG category. The secondary y-axis (left) shows the statistical significance of the Fisher exact test, represented as $-\log_{10}(\text{FDR})$. A threshold of significance ($-\log_{10}(\text{FDR}) \geq 1.30$) is indicated by pink dots, while non-significant values are marked in cyan. COG letters correspond to A: RNA processing and modification. C: Energy production and conversion. D: Cell cycle control, cell division and chromosome distribution. E: Amino acid transport and metabolism. F: Nucleotide transport and metabolism. G: Carbohydrate transport and metabolism. H: Transport and metabolism of coenzymes. I: Lipid transport and metabolism. J: Ribosome translation, structure and biogenesis. K: Transcription. L: Replication, recombination and repair. M: Cell wall, membrane and envelope biogenesis. N: Cell motility. O: Post-translational modification, protein turnover and chaperones. P: Transport and metabolism of inorganic ions. Q: Biosynthesis, transport and catabolism of secondary metabolites. S: Function unknown. T: Signal transduction mechanisms. U: Intracellular traffic, secretion and vesicular transport. UC: Unknown genes. V: Defence mechanisms. See also Figures S1 to S3 and S5 to S8.

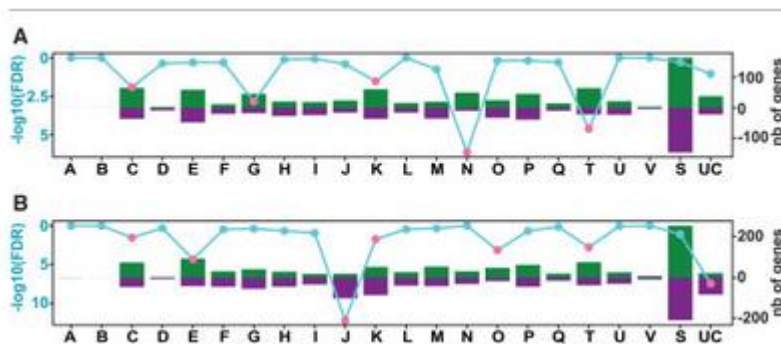


Figure 2. Growth of *V. diazotrophicus* NS1 and derivative mutants after 72 h at 20°C and Acetylene Reduction Assay. *V. diazotrophicus* NS1, (A) $\Delta nifA$, (B) $\Delta rpoN$ and (C) $\Delta ntrC$ contained the pFD085 empty plasmid. *V. diazotrophicus* $\Delta nifA$ compl-*nifA*, $\Delta rpoN$ compl-*rpoN* and $\Delta ntrC$ compl-*ntrC*, correspond to *V. diazotrophicus* NS1 $\Delta nifA$, $\Delta rpoN$ and $\Delta ntrC$ containing pPC005, pFD152 and pPC009, respectively, to complement the corresponding gene deletion. The bacterial disk is indicated by the white arrow. (D) Histogram showing the ethylene to acetylene ratio (%) for *V. diazotrophicus* NS1, the *ntrC* mutant and the complemented strain, done using ARA. A one-way ANOVA with Tukey test was done to compare all strains between them. See also Figure S4.

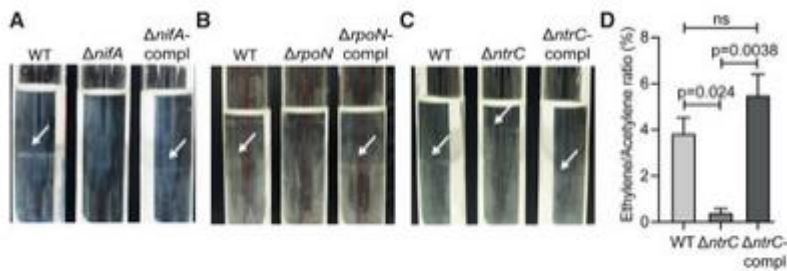


Figure 4. Regulation of nitrogenase expression and activity at population- and single-cell level.

(A) Boxplot showing the population-wide ethylene to acetylene ratio (%) for *V. diazotrophicus* NS1 with increasing initial NH_4^+ concentrations inoculated at identical cell density. Non-parametric t-test was done to compare the ratios obtained at 0.75 μM and 1 μM NH_4^+ . (B) Bioreporter construction used to quantify P_{nifH} expression by quantitative fluorescence microscopy in single cells, with the *mcherry* gene under the control of a constitutive promoter and the *egfp* gene under the control of the P_{nifH} promoter; (C) Mean total eGFP fluorescence of mCherry-positive *V. diazotrophicus* cells carrying pPC018; (D and E) Epifluorescence pictures of the bioreporter constitutively expressing *mcherry* (D) and expressing *egfp* from the P_{nifH} promoter when cultured in softgellan at 0 μM NH_4^+ (E); (F) eGFP QQplot of strain cultured in softgellan at 0 μM NH_4^+ ; (G) Proportion of *nifH*-expressing cells as a function of NH_4^+ concentration. A one-way ANOVA with Tukey test was performed and (H) mean eGFP fluorescence intensity of the *nifH*-expressing subpopulation. A Welch's t-test was performed against the negative control (strain carrying a promoterless *gfp* gene). Experiments done in triplicates. ns=not significant.

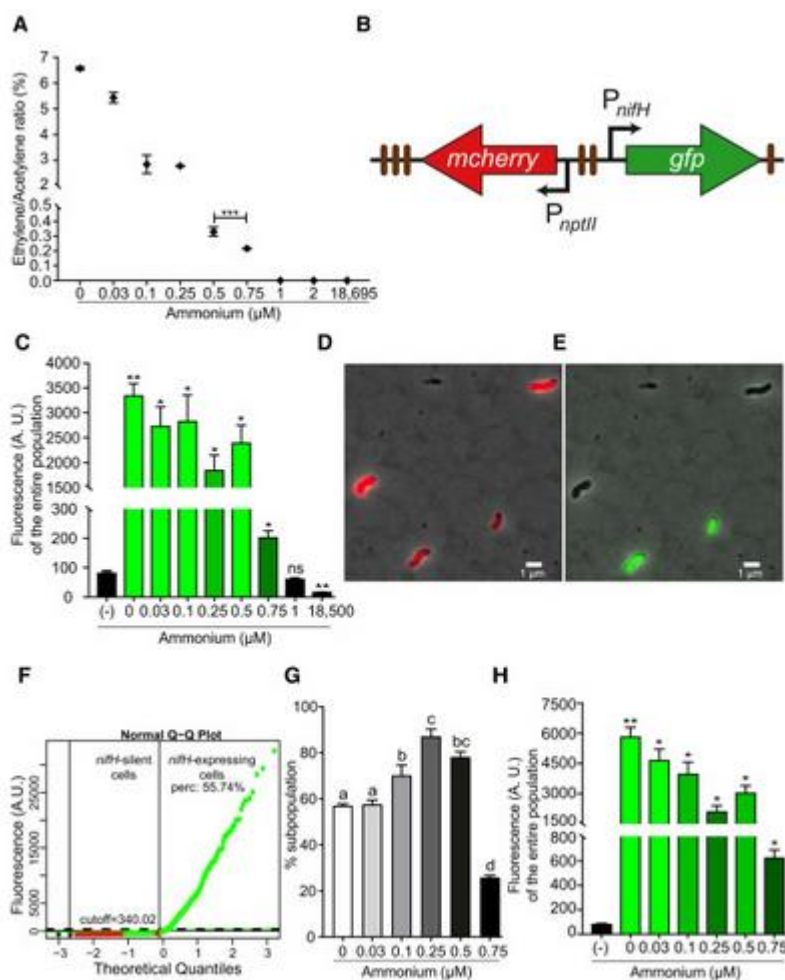


Figure 5. Modulation of the nitrogenase expressing subpopulation by NtrC. (A) Boxplot showing the mean total eGFP fluorescence of mCherry-positive *V. diazotrophicus* NS1 or $\Delta ntrC$ cells carrying pPC018 ; (B) Proportion of *nifH*-expressing cells in both strains ; (C) mean eGFP fluorescence intensity of the *nifH*-expressing subpopulations. (D and E) Phase Contrast and epifluorescence pictures of *V. diazotrophicus* carrying pPC018 (D) or the $\Delta ntrC$ mutant (E) when cultured in softgellan at 0 μM NH_4^+ . Epifluorescence microscopy pictures were adjusted to the same maximal fluorescence intensity, obtained in the wild-type. Unpaired two-tailed t-tests were performed to compare strains. Experiments done in triplicates.

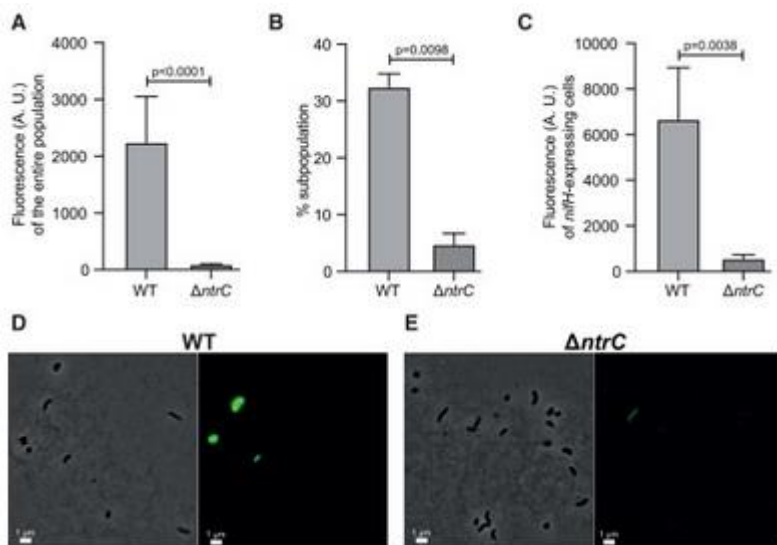


Figure 6. *nifH*-expression and nitrogenase activity *V. diazotrophicus* 60.18M containing pPC019 in MDV softgellan. (A) Mean total eGFP fluorescence regulated by the *nifH* promoter; (B) Epifluorescence picture showing phenotypic heterogeneity; (C) proportion of cells expressing *nifH*; (D) eGFP fluorescence intensity of the *nifH*-expressing subpopulation and (E) ethylene to acetylene ratio of *V. diazotrophicus* NS1 and 60.18M. Welch's t-tests were performed. Experiments done in triplicates. ns=not significant.

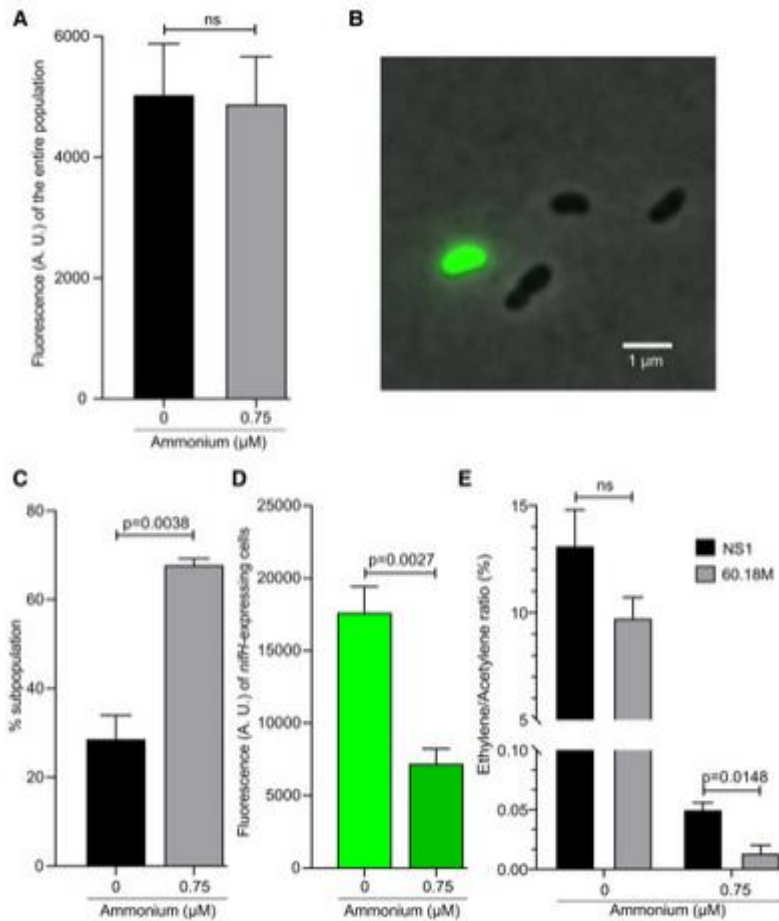
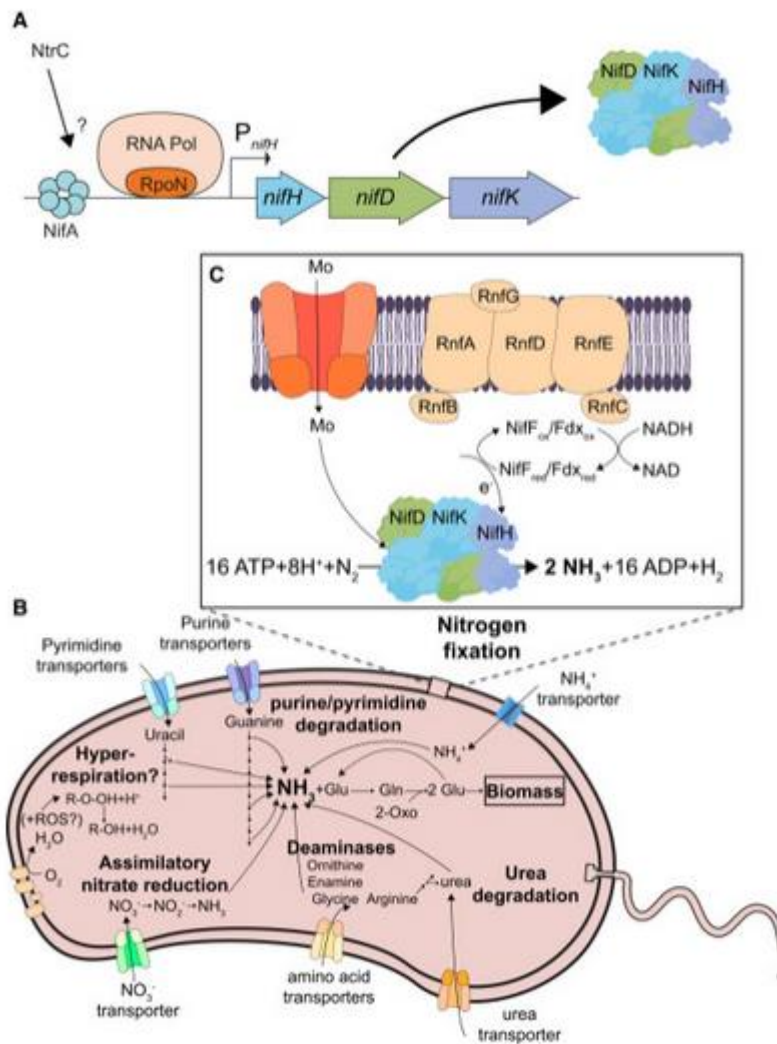


Figure 7. Global response of *V. diazotrophicus* NS1 cultured under SRN-free conditions. (A) NtrC may induce the transcription of *nifHDK* with the involvement of NifA and RpoN. Molybdenum transporters and the Rnf complex are associated with nitrogenase activity. (B) Transport and transformation pathways for alternative nitrogen sources assimilation. Enhanced respiratory activity may reduce O₂ concentrations, facilitating diazotrophy.



STAR METHODS

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Strains and culture conditions

V. diazotrophicus strains and *Escherichia coli* were routinely grown in Lysogeny Broth (LB) at 5 g.l⁻¹ (final concentration NaCl) at 30°C and 37°C with shaking, respectively. The plasmids and strains used in this study are listed in **Table S1** and **Table S2**, respectively.

Physiological tests requiring the absence of SRN were done with shaking in Modified Diazotrophic Vibrio (MDV) medium^{14,18} in which the yeast extract, normally present in the medium, was removed, and with the following modification: Solution 1 was prepared with NaCl (0.3 M), MgSO₄ · 7H₂O (0.0244 M), CaCl₂ · 2H₂O (7.6 mM), KCl (0.01 M), Tris (0.05 M) adjusted to pH 7.5. Three solutions were autoclaved separately: Solution 1, prepared as mentioned above in 333 ml H₂O; Solution 2, prepared in 500 ml H₂O with 0.005 g Na₂MoO₄ · 2H₂O, 40 g glucose, 1 ml of a 3 g.l⁻¹ FeNaEDTA, 3H₂O stock solution. Solution 3 was prepared in 166 ml H₂O with 0.2 g KH₂PO₄ and 0.8 g K₂HPO₄ adjusted to pH 7.2. After sterilization, the three solutions were mixed and 1 ml of a vitamin cocktail (stock solution containing 1 mg.l⁻¹ biotin, 1 mg.l⁻¹ vitamin B12 and 0.2 g.l⁻¹ thiamin-HCl) was subsequently added to 1 l MDV. If necessary, the following compounds were added after autoclaving: trimethoprim (Trim, 10 µg.ml⁻¹); diaminopimelic acid (DAP, 0.3 mM), glucose (0.3 g.l⁻¹), L-arabinose (L-ara, 0,2%), guanine (106.6 µM final concentration from a 5.33 mM stock solution in DMSO), arginine (1 mM final concentration from a 57.4 mM stock solution in H₂O), NaNO₃ (46.67 mg.l⁻¹ from a 46.67 g.l⁻¹ stock solution in water).

Anaerobic growth was promoted as recently developed⁵⁴ by adding sodium thioglycolate to solution 2 (0,152 g.l⁻¹) before autoclaving. When the three solutions were mixed, 0.05% L-cysteine-HCl (from a 5% stock solution dissolved in water) was added. Tubes were sealed with sterile 20 mm crimped caps. Oxygen in the headspace was removed by flushing the headspace for 3 minutes with pure N₂ gas using a 0.2 µM filter and two needles (BD Microlance 3).

METHOD DETAILS

Phenotypic screening using softgellan medium

A softgellan medium was used to screen for the ability of *V. diazotrophicus* and derivative mutants to grow in micro-oxic conditions when cultured in SRN-free conditions. This test is based on a recently

established protocol ¹⁸, with minor modifications. Briefly, *V. diazotrophicus* strains were grown overnight in LB (+Trim if necessary) at 30°C, 200 rpm. The next morning, strains were inoculated in fresh medium. When reaching exponential phase, 1 ml of the preculture was centrifuged at 6,000 rpm for 5 min and pellets were resuspended with 1 ml of nitrogen-free MDV. After three washes, pellets were resuspended with 1 ml of SRN-free MDV. Softgellan medium was prepared as recently published ¹⁸, and tubes were inoculated with 50 µl of this washed preculture at OD_{600nm} 0.5, 20 µl of vitamin solution and 20 µl of Trim (if necessary). Tubes were sealed with a sterile crimped cap. A bacterial growth disk was observed after 48–96 hours at 20°C without shaking. The experiment was performed at least in triplicate.

RNA extraction

LB condition: 100 µl of *V. diazotrophicus* grown overnight in LB was used to inoculate 2 ml of LB in the morning for 8 hours. Subsequently, 200 µl of this culture was inoculated in four 100 ml-Erlenmeyer flasks with 20 ml of LB and incubated at 30°C, 200 rpm, overnight. OD_{600nm} was measured and 5x10⁸ cells were collected and centrifuged at 16,000 g for 1 min. The RNA was extracted using the Direct-zol DNA/RNA miniprep kit.

Softgellan: A LB-grown preculture was washed with SRN-free MDV medium three times, and the pellet was concentrated twice in SRN-free MDV. 100 µl aliquots of the washed preculture were inoculated in 16 tubes with 20 ml of SRN-free MDV softgellan and incubated at 20°C for 48 h. 1 ml of the growth disk was collected with a syringe, and 4 disks were pooled in a falcon tube, before the addition of 6 ml of liquid SRN-free MDV. Samples were centrifuged for 5 min at 4,700 rpm in a swing rotor. The supernatant was removed, and RNA was extracted from the pellet using the RNA Zymo Research kit.

Softgellan + NH₄⁺: The same protocol as for the RNA extraction from softgellan was applied, with the following modifications. The softgellan contained NH₄Cl (18,695 µM final concentration added in solution 3 before autoclaving), and 1 ml of the culture was collected at the same height as the growing disk in the corresponding tubes when grown in softgellan.

Extracted RNA were verified by nanodrop measurements, and 3 µl were deposited in an agarose gel for 1 hour at 50 V. Aliquots were stored at -80°C.

RNA-sequencing

Total RNA samples (4 replicates per condition) were sent to Eurofins (Germany), who did the ribosomal RNA depletion, the DNase treatment, cDNA library preparation and Illumina sequencing

(NovaSeq 6000 S4 PE150 XP). Sequencing was performed on 3 replicates per condition (the replicate with the lowest RIN (RNA Integrity Number) according to Eurofins standards was removed).

Strain constructions

Various genes were deleted in *V. diazotrophicus* NS1 (*nifA*, *ntnC*, *ahpC*, *ureA*, BBJY01_540094) (**Table S2**). All deletions were achieved using a previously established protocol⁵⁵ with some adaptations¹⁸. This protocol employed different suicide plasmids (**Table S1**) in which fragments of approximately 800 bp flanking the to-be-deleted gene were inserted. All deletions were complemented by introducing the deleted gene in a replicative plasmid and introduced back in the mutant.

Gene expression was monitored using bioreporters. Briefly, the *mcherry* gene and a multiple cloning site (**Table S4**) have been inserted in pFD085, a plasmid containing a promoterless *gfp* gene, creating pFD161. Subsequently, the constitutive P_{nptII} promoter was inserted in pFD161, upstream of the *mcherry* gene, creating pFD162. Finally, the *nifH* promoter region of either *V. diazotrophicus* NS1 or 60.18M was inserted in pFD162, upstream of the *gfp* gene, creating pPC018 and pPC019, respectively. These plasmids have been introduced to *V. diazotrophicus* strains by biparental mating¹⁸. The presence of the plasmid was confirmed by PCR.

All constructed strains were stored at -80°C in 20% glycerol and all *in silico* plasmid maps are available upon request.

Fluorescence microscopy – sample preparation and imaging

Strains of *V. diazotrophicus* carrying pPC018 or pPC019 were grown overnight in LB+Trim at 30°C at 200 rpm. 1 ml of precultures was washed 3 times with MDV, and 100 µl were used to inoculate soft gellan as presented above. If necessary, NH_4^+ (from a 187 µM NH_4Cl stock solution) was added to solution 3 before autoclaving. After 24 hours of incubation, the disk was carefully pipetted using a sterile syringe and a needle, and droplets of cultures were deposited on a agarose patch (1% in water) and covered with a coverslip. Pictures were taken using an Eclipse Ni-E microscope (Nikon) equipped with a 100X Apochromat oil objective, a FITC-3540C filter cube (ex 482/35 nm, em 536/40) and a TRITC-B (ex 543/22, em 593/40) filter cube at 100 ms exposure time. The experiment was performed at least in biological triplicate, with at least 3 technical replicates per day and with at least 30 images per technical replicate.

Acetylene reduction Assay

Nitrogenase activity was assessed by measuring its capacity to reduce acetylene to ethylene⁵⁶. Briefly, strains were grown in softgellan as described above (using 100 µl of OD-adjusted washed preculture

as inoculant), and tubes were sealed with rubber stoppers with reversible edge. Fifteen percent of the headspace gas were removed with a HAMILTON Gastight syringe and replaced by acetylene. For aerobic liquid conditions, 100 μ L of preculture was inoculated into 20 ml of LB or 100 μ l of washed preculture was used to inoculate 20 mL liquid MDV in Erlenmeyer flasks, sealed with rubber stoppers with reversible edge. In each Erlenmeyer flask, 15% of air to the headspace were replaced by acetylene. Anaerobic liquid cultures were inoculated with 100 μ l of washed preculture into 20 ml of liquid MDV in 30 ml tubes and 200 μ l of cysteine 5%. Oxygen was replaced by N₂ as previously described. After 3 to 6 days (when grown in softgellan) or overnight (when grown in LB) of inoculation, the quantity of acetylene and ethylene was measured by gas chromatography. Samples were carried out on a Gas Chromatograph Agilent 6890 (*Agilent Technologies*, USA) equipped with a Flame Ionization Detector (GC-FID) and using Hydrogen as carrier gas at a constant flow of 7.7 ml.min⁻¹. The injector temperature was set at 160°C. The injections were carried out in split mode with a split ratio of 10:1. A GS-Alumina capillary column, 50 m \times 0.53 mm \times 0.25 μ m (Agilent, 115-3552) was used. The programmed oven temperature was at 120 °C (isotherm program) for 3 min. The FID detector was set at 160 °C.

The presence of ethylene and its absence in the negative control (culture without bacteria) indicates a nitrogenase activity ²⁴.

QUANTIFICATION AND STATISTICAL ANALYSIS

Transcriptomic analysis

22 million reads were obtained per sample, which were processed using RiboDetector ⁵⁷ to remove reads classified as rRNA. High quality reads were obtained using fastp ⁵⁸. At least 14.24 million “High Quality” reads per sample were obtained. Those reads were aligned to the reference genome of *V. diazotrophicus* (GCA_000740015.1) using STAR ⁵⁹. Genome-wise quantification was achieved using featureCounts ⁶⁰. Differential gene expression between 2 conditions was performed using R/Bioconductor package edgeR ⁶¹. Each gene was annotated according to the Clusters of Orthologous Groups (COG) database from the MicroScope platform (<https://mage.genoscope.cns.fr/microscope/home/index.php>). Genes assigned to multiple COG categories were split and counted individually.

Eurofins initially applied a cutoff of 0.1 for adjusted p-values. We then applied a more stringent threshold of 0.05 to retain only statistically significant genes. Finally, False Discovery Rate (FDR) correction using the Benjamini-Hochberg method was applied to limit false positives due to the large number of genes tested (**Data S1**).

Image analysis

Fluorescence of single cells was quantified for at least 550 cells per condition using a Matlab script⁶². The presence of two populations differing in their fluorescence emission was determined using a R script showing QQ plots, as already published²³.

Statistical analysis and figure production

All statistical analyses were performed with GraphPad Prism v 10.3 and p-value<0.05 were considered significant. Figures were obtained from GraphPad Prism, BioRender and proksee.ca⁶³, and generated on Adobe Illustrator.

SUPPLEMENTAL INFORMATION

Document S1: Figures S1–S8 and Tables S1, S2, S4 and S5

Data S1: Excel file containing all Differentially Expressed Genes (DEGs) too big to fit in a PDF, related to Figure 1. A) DEGs comparing MDV in softgellan vs softgellan+NH₄⁺. B) DEGs comparing MDV in softgellan vs LB.

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