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RESEARCH ARTICLE

Genetic and physiological insights into the diazotrophic activity of a non-cyanobacterial marine diazotroph

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Abstract

Nitrogen (N₂) fixation, or diazotrophy, supports a large part of primary production in oceans. Culture-independent approaches highlighted the presence in abundance of marine non-cyanobacterial diazotrophs (NCD), but their ecophysiology remains elusive, mostly because of the low number of isolated NCD and because of the lack of available genetic tools for these isolates. Here, a dual genetic and functional approach allowed unveiling the ecophysiology of a marine NCD affiliated to the species *Vibrio diazotrophicus*. Physiological characterization of the first marine NCD mutant obtained so far was performed using a soft-gellan assay, demonstrating that a $\Delta nifH$ mutant is not able to grow in nitrogen-free media. Furthermore, we demonstrated that *V. diazotrophicus* produces a thick biofilm under diazotrophic conditions, suggesting biofilm production as an adaptive response of this NCD to cope with the inhibition of nitrogen fixation by molecular oxygen. Finally, the genomic signature of *V. diazotrophicus* is essentially absent from metagenomic data of *Tara Ocean* expeditions, despite having been isolated from various marine environments. We think that the genetically tractable *V. diazotrophicus* strain used in this study may serve as an ideal model to study the ecophysiology of these overlooked prokaryotic group.

INTRODUCTION

Nitrogen is a macro-element essential for life, as it is found in many biological molecules (i.e. nucleic acids, proteins). The atmosphere is composed by 78% of dinitrogen (N₂), but this form cannot be assimilated by the vast majority of the organisms. The process of N₂ fixation or diazotrophy is pivotal in oceanic ecosystems, since it makes the newly fixed nitrogen accessible to the entire plankton community, fuelling primary production (Tang et al., 2019). Diazotrophy is considered a key player in the biogeochemical cycles of nitrogen, but also of carbon, since it stimulates the biological pump, a mechanism absorbing a significant portion of the

carbon dioxide emitted into the atmosphere and exporting it to the ocean seafloor. Indeed, diazotrophy can fuel oceanic primary production in some tropical and subtropical area, where dissolved inorganic nitrogen (DIN) concentration is limited (Capone et al., 2005). In addition, recent in situ quantification of N₂ fixation demonstrated high diazotrophic activity in DIN-replete marine environments (Mulholland et al., 2019; Tang et al., 2019), showing the overall importance of diazotrophy in oceans.

Diazotrophy is an anabolic process restricted to some prokaryotic species (found both in Bacteria and Archaea). Cyanobacteria like the free-living *Trichodesmium* are known since decades to be important

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oceanic nitrogen fixers (Carpenter & Romans, 1991), accounting for at least 1.6 Tmol N in the tropical North Atlantic alone (Capone et al., 2005). Other Cyanobacteria, typically living in symbiotic association with photosynthetic microalgae also contribute significantly. These associations include diatom-diazotroph associations (DDA), where filamentous Cyanobacteria like *Richelia intracellularis* and *Calothrix rhizosoleniae* can be located either intra- or extracellularly to fix N₂ and transfer the newly fixed nitrogen to sustain diatom growth (Nieves-Morion et al., 2020), receiving organic carbon from its host in return (Foster et al., 2022). DDA associations can account for 0.19 and 0.62 Tmol N per year in Atlantic and Pacific oceans, respectively (Foster et al., 2011). Another example includes the unicellular cyanobacterial lineage UCYN-A, which associate with haptophytes and fixes N₂ even under nitrogen-replete conditions (Mills et al., 2020) and which significantly contribute to N₂ fixation in oceans (Martinez-Perez et al., 2016).

Despite the tremendous data related to cyanobacterial N₂-fixation in oceans, the global nitrogen (N) budget is frequently considered unbalanced (Marconi et al., 2017), as the N loss by denitrification and anammox largely exceeds N input (Landolfi et al., 2018). This suggests that other factors are missing to equilibrate the N budget. More and more evidence show the presence in all oceans of previously overlooked nitrogen-fixers, not affiliated to the phylum Cyanobacteria. Indeed, culture-independent approaches based on the amplification or immunodetection of the *nifH* gene, a gene encoding a subunit of the nitrogenase, conserved among diazotrophs and considered as a biomarker for diazotrophy, have demonstrated the presence of marine non-cyanobacterial diazotrophs (NCDs) (Moisander et al., 2017; Zehr et al., 1998) in the water column attached to particles (undefined aggregates, faecal pellets) (Farnelid et al., 2019; Geisler et al., 2019) and in deep sea sediments (Kapili et al., 2020). Moreover, *Tara Ocean* expeditions provide metagenomic data from diverse oceanic locations, depths and size fractions, coupled with physical and chemical information (Karsenti et al., 2011; Planes et al., 2019). Analyses of the metagenomes allowed the reconstruction of metagenome-assembled genomes (MAGs) from diverse diazotrophs, including many NCDs, found in abundance in diverse oceans (Delmont et al., 2018, 2022). Metatranscriptomic analysis from *Tara Ocean* data showed that many NCDs express their *nifH* gene and are therefore active (Delmont et al., 2022).

From a biochemical point of view, diazotrophic activity faces a strong metabolic constraint under oxic conditions (Gallon, 1992). Indeed, O₂ acts at different levels to block diazotrophy: it can (i) inhibit the expression of *nif* genes including *nifH* (Dixon & Kahn, 2004;

Hubner et al., 1991), (ii) lead to irreversible damages of the nitrogenase (Gallon, 1992), and (iii) reversibly inhibit the nitrogenase activity (Goldberg et al., 1987). This O₂-based inhibition of diazotrophy poses a metabolic conundrum, since many diazotrophs are also aerobes and therefore use O₂ as a terminal electron acceptor for aerobic respiration. To solve this dilemma, terrestrial diazotrophs committed to symbiotic interactions with legume roots (and sometimes on stems; Bonaldi et al., 2011) are engulfed within a plant organ called nodule. This nodule serves as a physical barrier, limiting O₂ diffusion and creating micro-aerobic environments where both nitrogen fixation and respiration co-occur. Other terrestrial diazotrophs, like the free-living diazotroph *Azotobacter vinelandii* produce a cytochrome *bd* terminal oxidase, that consumes a high volume of O₂, which leads to a rapid decrease in intracellular O₂-tension, allowing nitrogen fixation in overall aerobic environments (Kelly et al., 1990; Poole & Hill, 1997). Additionally, *A. vinelandii* secretes an alginate capsule, whose thickness increases with O₂ tension and which serves as a barrier for O₂ diffusion into the cells (Sabra et al., 2000).

In addition to the O₂ tension found in oceans, marine Cyanobacteria face another challenge when regarding nitrogen fixation, since many of them are photosynthetic and release O₂ as a by-product of oxygenic photosynthesis. Marine Cyanobacteria have therefore also developed various strategies to solve this metabolic constraint. Free-living filamentous Cyanobacteria like *Dolichospermum* sp. (previously named *Anabaena* sp.; Zhang et al., 2006) but also those living in symbiotic associations with photosynthetic diatoms like *R. intracellularis* and *C. rhizosoleniae* (Foster et al., 2011) phenotypically differentiate into distinct subpopulations: when DIN becomes limiting, some cells within the filament specialize to uniquely fix nitrogen. These cells, called heterocysts have a modified envelope containing additional layers (a glycolipid and a polysaccharide layer) which restricts O₂ diffusion in the cell cytoplasm (for a review, see Flores et al., 2019). Heterocysts commit to a dedicated developmental program (Flores et al., 2019), also marked by the loss of ability to perform oxygenic photosynthesis, which is restricted to the neighbouring vegetative cells (Zhang et al., 2006). Molecular exchanges of nitrogen and carbon compounds therefore occur between the vegetative cells and the heterocysts to sustain the growth of the filament (Nieves-Morion et al., 2021). As an alternative to this spatial regulation of diazotrophy, some unicellular Cyanobacteria such as *Crocospaera* perform oxygenic photosynthesis during the day and nitrogen fixation during the night (Tuit et al., 2004). Finally, non-heterocyst diazotrophs may protect their nitrogenase from O₂ by the production of hopanoid lipids (Cornejo-Castillo & Zehr, 2019).

In contrast, while strategies deployed by marine cyanobacterial diazotrophs to perform nitrogen fixation in oxic environment are documented, knowledge on those for marine NCD remain understudied (Bombar et al., 2016). This knowledge gap is due to the limited number of NCD isolated from marine environments (Farnelid et al., 2014; Martinez-Perez et al., 2018). Additionally, their physiology remains elusive because, to our knowledge, no genetic mutant of marine NCD has been obtained so far, hampering an in-depth physiological characterization of marine NCD.

Vibrio diazotrophicus is a marine bacterium, firstly isolated from gastrointestinal tract of sea urchins (Guerinot & Patriquin, 1981). It can be found in marine and estuarine environments (Guerinot et al., 1982) as well as in sediments (Castillo et al., 2018) and in enriched marine phytoplankton exometabolites (Fu et al., 2020). The type strain *V. diazotrophicus* NS1 (ATCC 33466) was shown by acetylene reduction assay (ARA) to produce nitrogenase and fix N_2 (Guerinot & Patriquin, 1981). The genome of *V. diazotrophicus* NS1 is available (accession number BBJY00000000.1, no associated publication or genomic characterization) and other isolates have been fully sequenced (Castillo et al., 2018) but those genomes have not been compared with the one of the type strain *V. diazotrophicus* NS1. Importantly, the presence of the *nif* cluster in these strains has never been investigated, nor it is known whether this *nif* cluster is conserved among members of this species or whether it varies according to their geographical isolation. Additionally, besides the description of its isolation in 1981 and its taxonomical characterization 1 year later (Guerinot et al., 1982), no further physiological or genetic characterization of any member of this species has been performed. Because many genetic and molecular tools are available for various species of this genus (Delavat et al., 2018; Morot et al., 2021; Tischler et al., 2018; Zhang et al., 2019; Zingl et al., 2020), we think that *V. diazotrophicus* NS1 can constitute an ideal model to assess the ecophysiology of marine NCD and their diazotrophic activity, provided that those tools are functional for this strain. Moreover, despite its isolation from various oceanic environments, it remains unknown whether *V. diazotrophicus* signature is found in existing marine metagenomic data, including the ones describing the presence of various marine NCD (Delmont et al., 2018, 2022).

In this manuscript, we report the genomic and physiological description of *V. diazotrophicus* NS1, together with the construction of the first genetic mutant of a marine NCD, impaired in nitrogen fixation. Importantly, we showed that under nitrogen-deficient conditions, *Vibrio diazotrophicus* produces more biofilm, suggesting the role of biofilm as a barrier of O_2 diffusion.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

The strains, plasmids and primers used in this study are shown in Tables S1–S3, respectively. Unless otherwise stated, *V. diazotrophicus* NBRC 103148 and *Escherichia coli* were grown with shaking in Luria Bertani (LB) at 30°C and 37°C, respectively. The nitrogen-deficient media used in this study is a modified diazotrophic medium for *Vibrio* (MDV), composed similarly to (Guerinot & Patriquin, 1981), with the following modifications: Solution 1 was prepared with NaCl 0.3 M, $MgSO_4 \cdot 7H_2O$ (0.05 M), $CaCl_2 \cdot 2H_2O$ (0.01 M), KCl (0.01 M), Tris (0.05 M) adjusted to pH 7.5, before the addition of 0.3 g/L yeast extract. Solution 2 was prepared in 500 ml H_2O with 0.005 g $Na_2MoO_4 \cdot 2H_2O$, 40 g glucose, 1 ml of a 3 g/L $FeNaEDTA \cdot 3H_2O$ stock solution. Solution 3 was prepared in 166 ml H_2O with 0.2 g KH_2PO_4 and 0.8 g K_2HPO_4 adjusted to pH 7.2. All three solutions were autoclaved, allowed to cool separately, and MDV was prepared by mixing 333 ml of Solution 1, 500 ml of Solution 2 and 166 ml of Solution 3. The 1 ml of a vitamin cocktail (stock solution containing 1 mg/L biotin, 1 mg/L vitamin B12 and 0.2 g/L thiamin-HCl) was subsequently added to 1 L MDV.

If necessary, trimethoprim (Trim, 10 µg/ml), kanamycin (Km, 50 µg/ml), diaminopimelic acid (DAP, 0.3 mM), glucose (0.3 g/L), L-arabinose (L-ara, 0.2%) or agar (1.5%) were added to the media.

Doubling times were determined by spectrophotometry using the TECAN Infinite M1000. Briefly, one colony of each studied strain was inoculated in LB + Trim and incubated overnight. The next morning, cells were washed three times to remove old LB from the overnight culture, OD_{600nm} was adjusted to 0.01 with fresh LB, and 200 µl was used to inoculate four wells from a polystyrene plate (Dutscher, ref 330035). Plate was left in the TECAN instrument set at 30°C with shaking, and OD_{600nm} was measured every 30 min for 48 h. Doubling times were calculated from the exponential growth phase of each culture.

Strain constructions and DNA techniques

Standard procedures were used for all molecular techniques, following the reagent suppliers recommendations. Deletion of *nifH* was performed by double homologous recombination between a suicide plasmid and the chromosome of *V. diazotrophicus*, following a previously established protocol (Morot et al., 2021), with some adaptations: *V. diazotrophicus* was grown overnight at 30°C (it does not grow at 37°C), cointegrates were incubated in LB + L-ara liquid before plating on LB + L-ara, and the temperature used to

incubate the filter-deposited LB + DAP plates was 30°C. The suicide plasmid pFD114 (Table S2) was constructed by amplifying around 750 bp of the upstream and downstream regions of the *nifH* gene of *V. diazotrophicus* (BBJY01_570151), using primers 210,210–210,213 (Table S3). Correct deletion of *nifH* was verified using primers 210,501 and 210,502.

Complementation of *nifH* was achieved by amplifying the *nifH* gene and its dedicated promoter, using primers 210,204 and 210,205 (Table S3). The P_{nifH} -*nifH* amplicon was cloned within the pGEM-T vector (Promega), verified by sequencing and cloned within pFD086 (Morot et al., 2021) to replace P_{lac} -*gfp*, using BamHI and XhoI. The resulting pFD120 plasmid was introduced by biparental mating as described above, using β 3914 containing pFD120 as a donor strain. All in silico plasmid maps are available upon request.

Electroporation assays were performed following an already published method (Delavat et al., 2018), except the growing temperature of *V. diazotrophicus*, set at 30°C.

Soft-gellan assay

A soft-gellan assay has been developed to test the capability of *V. diazotrophicus* NS1 or derivative mutants to grow under micro-oxic condition in the complete absence of nitrogen except N₂. A nitrogen-free MDV medium was prepared as follows (for one tube): 6.7 ml of Solution 1 (in which yeast extract was completely removed) plus 2 mg gellan gum were distributed in 18 cm glass tubes (Dutscher, ref 508232); 10 ml of Solution 2 containing 3 mg gellan gum in a separate bottle; 3.33 ml of Solution 3 containing 1 mg gellan gum in a separate bottle. All solutions were autoclaved separately, allowed to cool to 45°C, mixed in the glass tube, gently homogenized by inversion, before the addition of 20 μ l of vitamin and Trim. If necessary, 20 μ l of NO₃⁻ (stock solution NaNO₃ at 46.67 g/L) were added together with vitamins and Trim after autoclave. If necessary, 20 mg of NH₄Cl was added in the 3.33 ml of Solution 3 before autoclave.

Vibrio diazotrophicus containing the empty plasmid pFD085 and *V. diazotrophicus* Δ *nifH* containing either pFD085 or pFD120 were grown overnight in LB + Trim. The 1 ml of overnight cultures was subsequently washed with 1 ml nitrogen-free MDV (without yeast extract), and pellets were resuspended with 500 μ l nitrogen-free MDV. The 100 μ l of these washed suspensions were used to inoculate the soft-gellan tubes and tubes closed with a sterile blue rubber stopper, mixed by inversion and before being sealed with an aluminium capsule. Tubes were left for incubation at room temperature for 48–72 h, and growth was observed by the presence of a growing bacterial ring at a given depth within the tube. The experiment was performed in triplicates.

In order to quantify the O₂ concentration at the location of the growing ring, dissolved O₂ concentrations were measured with a retractable fibre oxygen microsensor (tip diameter 50–70 μ m; OXR50-OI, Pyro Science GmbH, Germany). The oxygen microsensor was connected to a FireSting-O₂ fibre-optic oxygen meter (FireSting-O₂, Pyro Science GmbH, Germany) and signals were recorded with a PC via the software Pyro Oxygen Logger (v3.319; Pyro Science GmbH). The sensor was linearly calibrated in distilled water by measuring in aerated water (100%) and in the same medium, in which 200 μ l of HCl-cysteine (5%) was added after media preparation to reduce any trace of O₂ (0%). The position of the sensor was controlled by a micromanipulator (MU1; Pyro Science, GmbH). Measurements were done at the medium–air interface and at the location of the bacterial ring. Additionally, the O₂ concentration profile was measured every 2 mm from the medium–air interface until 4 mm below the bacterial ring and resazurin (1 μ g/ml final concentration, added in Solution 2 from a 1 mg/ml stock solution) was used as an indicator for the presence of O₂ (Figure S1).

Biofilm production in microplates

Biofilm production was quantified from *V. diazotrophicus* NS1 and derivative mutants, under various experimental conditions. To do so, strains carrying either pFD085 or pFD120 were inoculated from a single colony in 5 ml LB + Trim and incubated overnight at 30°C with shaking. Part of the cultures were washed three times with LB, diluted to an initial OD_{600nm} of 0.01 with LB + Trim, and 200 μ l of this OD-adjusted suspension was inoculated in quadruplicate in a 96-polystyrene-well microplate (Dutscher, ref 330035). The remaining overnight cultures were washed three times with MDV, adjusted to an initial OD_{600nm} of 0.01 with MDV + Trim, with MDV + Trim containing 46.67 mg/L NO₃⁻ or with MDV + Trim containing 20 mg/LNH₄Cl, and 200 μ l of these OD-adjusted suspensions were inoculated in quadruplicate in the same microplate. Plates were placed in a microplate reader (TECAN Infinite M1000) for 48 h at 30°C without shaking, and OD_{600nm} was measured every 30 min. After 48 h, plates were treated following an already established protocol (Morot et al., 2021). Briefly, cell suspensions were removed, 220 μ l of crystal violet (0.1%) was added into the wells, incubated for 10 min before wells being washed twice with distilled water. Microplates were dried for 24–48 h at room temperature, and crystal violet was dissolved with 30% acetic acid for 10 min before OD_{550nm} measurement. Biofilm production was corrected by dividing the OD_{550nm} measured after crystal violet staining by the maximal OD_{600nm} measured during the growing time of the corresponding well.

Biofilm production on flowcells

The thickness and biovolume of the biofilm produced by *V. diazotrophicus* NS1 were quantified using confocal laser scanning microscope after incubation of cells in flowcells. *Vibrio diazotrophicus* biofilms were grown at 30°C under hydrodynamic conditions in a three-channel flow cell (1 × 4 × 44 mm; Biocentrum, DTU, Denmark; Pamp et al., 2009). The flow system was assembled, prepared and sterilized as described previously (Tolker-Nielsen & Sternberg, 2011). The substratum consisted of a microscope glass coverslip (24 × 50 mm; KnittelGlaser, Braunschweig, Germany). Each channel was inoculated with 250 µl of an overnight LB + Trim culture of *V. diazotrophicus* pFD086 diluted (after three washing steps in MDV) to an OD_{600nm} of 0.1 in MDV. A 2 h-attachment step was performed without any flow of medium. Then a 5 ml/h flow of MDV was applied for 24 h using a Watson Marlow 205 U peristaltic pump (Watson Marlow, Falmouth, UK). Biofilms were observed by monitoring the green fluorescence protein (GFP) fluorescence with a LSM 710 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) using a 40× oil immersion objective. GFP was excited at 488 nm and fluorescence emission was detected between 500 and 550 nm. Images were acquired at intervals of 1 µm throughout the whole depth of the biofilm. ZEN 2.1 software (Zeiss, Oberkochen, Germany) was used for visualization and image processing. Quantitative analyses of image stacks were performed using COMSTAT software (<http://www.imageanalysis.dk/>) (Heydorn et al., 2000). Analysis was performed from three biological replicates (independent experiments) and eight technical replicates, every replicate comprising three to five stacked images.

Statistical analyses

All statistical analyses were performed using GraphPad Prism; *p* values < 0.05 were considered statistically significant.

Phylogenomics, pangenomics and metagenomics detection of *V. diazotrophicus* NS1

In order to determine the genomic relatedness of *V. diazotrophicus* NS1 with the MAGs of heterotrophic diazotrophs unveiled in *Tara* metagenomes (Delmont et al., 2022), we used anvi'o 7.1 (Eren et al., 2021) and the contigs snakemake (Koster & Rahmann, 2018) workflow. Briefly, the workflow created a contigs database with 'anvi-gen-contigs-database', which used Prodigal version 2.6.3 (Hyatt et al., 2010) to identify open reading frames. It used 'anvi-run-hmm' to detect the single-copy core genes from bacteria (*n* = 71,

modified from Lee, 2019), ribosomal RNAs (rRNAs) (*n* = 12, modified from <https://github.com/tseemann/barmap>). For the phylogenomic tree of *V. diazotrophicus* NS1 and the MAGs from (Delmont et al., 2022), we selected 16 bacterial SCGs present in at least 40 out of the 41 genomes, which we concatenated and aligned using Muscle v3.8.1551 (Edgar, 2004) and used FastTree 2.1.11 (Price et al., 2010) to generate a tree. We used 'anvi-interactive' to visualize the phylogenomic tree.

To compare *V. diazotrophicus* NS1 with publicly available genomes of the same species, we used 'ncbi-genome-download' (<https://github.com/kblin/ncbi-genome-download>) to get all *V. diazotrophicus* genomes from GenBank. We used 'anvi-compute-genome-similarity' to compute the average nucleotide identity (ANI) using pyANI v0.2.11 and discarded two genomes for their very small size (the others having an estimated completion value of 100%), which would otherwise impact computed alignment values. We used anvi'o Pangenomics snakemake workflow to compare the gene content between the genomes. The workflow used Prodigal to identify open reading frames, DIAMOND v2.0.14 (Buchfink et al., 2015) to quantify the similarity between each pair of genes, the Markov clustering algorithm (MCL) to identify gene clusters, and Muscle v3.8.1551 to align amino acid sequences. We used 'anvi-compute-genome-similarity' to compute the Average ANI using pyANI v0.2.11 and visualized the pangenome using 'anvi-display-pan'. We used the 'functional homogeneity' index to filter for more divergent gene clusters (max functional homogeneity of 0.95), which we used to generate a *V. diazotrophicus* specific phylogenomic tree (*n* = 99 genes) using the same approach as described above (Muscle v3.8.1551 and FastTree 2.1.11).

In order to search, extract and compare the *nif* cluster of *V. diazotrophicus* genomes, we used COG20 (Galperin et al., 2021), KOfam (Aramaki et al., 2020) and the InterPro website (Jones et al., 2014) to identify the nitrogen fixation associated genes. We extracted three loci from each genome using 'anvi-export-loci' and the anchor genes *nifH* (K02588), *nifU* (K02594) and *nifQ* (K15790) and used the R package 'gggenes' (<https://wilcox.org/gggenes/>) to visualize the gene synteny.

We have also used metagenomes from the *Tara ocean* project to recruit reads onto *V. diazotrophicus* NS1, in order to assess its occurrence from *Tara* metagenomes. Briefly, we use the 'metagenomics' snakemake workflow in anvi'o and mapped metagenomics short-reads using Bowtie2 (Langmead & Salzberg, 2012) and summarized the results with 'anvi-summarize'. We used the 'detection' value to estimate the presence/absence of *V. diazotrophicus* NS1 in both the prokaryotic and large size fraction metagenomes.

RESULTS

Optimization of genetic tools for *V. diazotrophicus* NS1

Vibrio diazotrophicus NS1 is a marine strain, for which no genetic mutant has been described. Because this strain might serve as an ideal model for genetic and ecophysiological characterization of marine NCD, we sought to develop and optimize a genetic protocol for *V. diazotrophicus* NS1. To achieve this aim, we first applied and modified an electroporation protocol, originally developed for *Vibrio harveyi* and *Pseudoalteromonas* sp. (Delavat et al., 2018). We tested different variants of the protocol, by using either 1- or 2 mm electroporation cuvettes, modifying the voltage from 1500 to 2500 V in the presence or absence of 15% glycerol in the sucrose buffer (Delavat et al., 2018), from stationary-phase cultures grown in LB or LBS (LB containing 20 g/L NaCl final concentration) at 30°C. The best electroporation protocol yielded 81 CFU/μg DNA, and was obtained when the following protocol was applied: glycerol-containing buffer was used to wash LB-grown cultures, and the electroporation voltage was set at 2000 V in a 2 mm electroporation cuvette (data not shown). This demonstrates that, although being at low efficiency, electroporation can be applied for this strain.

In order to improve the efficiency of exogenous DNA transfer to *V. diazotrophicus* NS1, we also performed bi- and triparental mating as recently developed (Morot et al., 2021). Both conjugations were successful for transferring the replicative plasmid pFD086 to *V. diazotrophicus* NS1, giving rise to bright GFP-fluorescent cells (Figure 1). Finally, applying this protocol, we successfully inserted a mini-Tn7 in *V. diazotrophicus* NS1 containing the unstable pFD052 plasmid expressing the Tn7-transposase (Delavat et al., 2018), the latter being subsequently cured (data not shown).

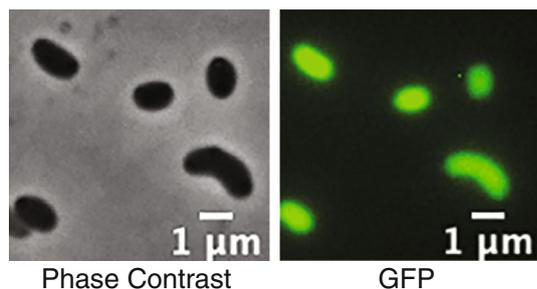


FIGURE 1 Microscopy picture showing the obtention of *V. diazotrophicus* carrying the GFP-expressing plasmid pFD086. Images were acquired from LB-grown overnight cultures.

Construction of a *nifH* deletion mutant and physiological characterization

Unveiling the genetics and ecophysiology underlying diazotrophy in *V. diazotrophicus* requires the deletion of genes and the subsequent physiological characterization of the obtained mutants. In this study, we focused on the deletion of the *nifH* gene (BBJY01_570151), applying an adapted protocol successfully used for *V. harveyi* ORM4 (Morot et al., 2021). Using this protocol, a clean *nifH* deletion mutant was obtained (Figure 2A). This mutant grows as well as the wild type when growing in LB-rich medium (Figure 2B).

In order to characterize the diazotrophic activity of the *nifH* mutant, we developed a soft-gellan assay, characterized by the use of a nitrogen-free medium containing 0.3% gellan gum. Inoculating tubes sealed with a blue rubber stopper and an aluminium capsule (Figure 3) with *V. diazotrophicus* NS1 containing the empty plasmid pFD085 led to a growth of this strain

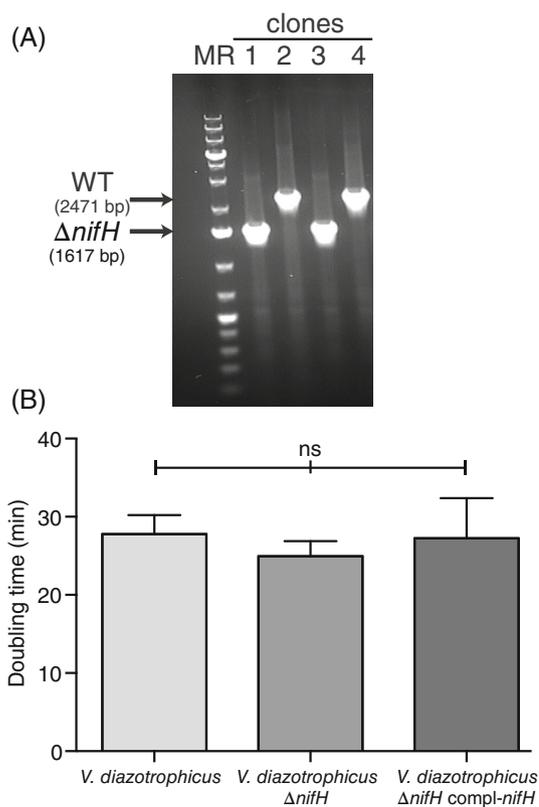


FIGURE 2 Genetic and physiological characterization of the *V. diazotrophicus* NS1 and the $\Delta nifH$ mutant. (A) Gel electrophoresis showing the deletion of *nifH* in *V. diazotrophicus* NS1 (Lanes 2 and 4). (B) Generation time of *V. diazotrophicus* NS1 and derivative mutants. *Vibrio diazotrophicus* NS1 and *V. diazotrophicus* NS1 $\Delta nifH$ contained the pFD085 plasmid and *V. diazotrophicus* NS1 compl-*nifH* corresponds to *V. diazotrophicus* NS1 $\Delta nifH$ containing pFD120. A one-way ANOVA with Tukey test was done to compare the different mutants. Depicted here are the mean + SEM of four replicates.

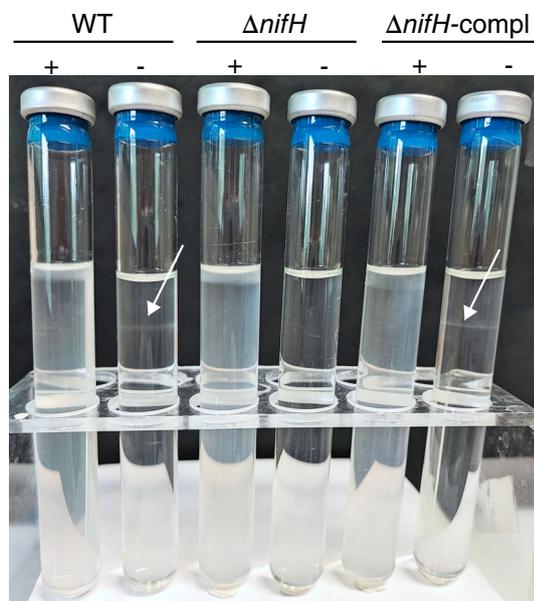


FIGURE 3 Soft-gellan assay demonstrating bacterial growth under appropriate microoxic conditions. The '+' and '-' indicate the presence or absence of NH_4^+ , respectively. White arrows indicate the location of the growing ring. Note the absence of ring in the $\Delta nifH$ mutant and in the presence of NH_4^+ . Similar results were obtained using NO_3^- as a bioavailable inorganic nitrogen source (see Figure S2).

throughout the entire tube height (with a higher biomass closer to the surface) when NH_4^+ is added. Importantly, in the absence of NH_4^+ , a single ring is visible after 48 h, located around 15 mm below the surface of the medium. Applying this assay for the *nifH* mutant containing pFD085 gave a similar pattern as the one of the wild type when grown with NH_4^+ . However, in the complete absence of nitrogen, no band nor growth was detectable in the *nifH* mutant. Additionally, no band was visible when tubes were left for 2 more weeks, indicating that the observed phenotype does not correspond to a longer lag phase in this medium. *In trans* complementation of the *nifH* deletion mutant by the introduction by conjugation of a replicative plasmid containing the entire *nifH* gene and its P_{nifH} promoter restored the wild type phenotype, with the formation of a clear band located roughly 15 mm below the surface of the medium (Figure 3). We subsequently repeated the experiment, using NO_3^- as an alternative bioavailable nitrogen source, and the results were identical (see Figure S2). Thus, we demonstrated that growth of *V. diazotrophicus* NS1 in nitrogen-free medium is dependent on an intact nitrogenase.

The O_2 concentration in the soft-gellan tube was subsequently measured in the headspace and at the location (or corresponding depth) of the growing ring. Uninoculated soft-gellan tubes showed a high O_2 concentration at both locations, with values ranging from 173.6 μM (sd 20.1) at a depth corresponding to the

growing ring to 245.5 μM (sd 1.9) in the head space. In contrast, after 72 h growth of *V. diazotrophicus* in nitrogen-free MDV, the O_2 decreased to a concentration of 1.6 μM (sd 1.1) at the location of the ring, while this concentration was 238.8 μM (sd 1.4) in the headspace. To better approximate the O_2 concentration at a depth where N_2 fixation can occur, we quantified this concentration in 12 h-inoculated tubes, where the growing ring is not yet formed. Under this condition, a O_2 concentration of 14.8 μM (sd 7.7) is quantified at a similar depth as the one where the ring will form 2 days later. Thus, we demonstrated here that diazotrophy occurs under microaerobic conditions. Moreover, a subsequent soft-gellan experiment using *V. diazotrophicus* NS1 constitutively expressing GFP (thanks to the pFD086 plasmid it contains) showed that cells growing under microaerobic conditions within the ring are free-living and isolated and do not form aggregates (Figure S3).

***Vibrio diazotrophicus* produces more biofilm in MDV**

In order to determine the possible regulatory mechanism deployed by *V. diazotrophicus* NS1 to avoid the nitrogenase inhibition by O_2 , we sought to quantify the ability of this strain to produce biofilm. Microplates containing either LB, MDV or MDV supplemented with either NO_3^- or NH_4^+ , plus trimethoprim were inoculated with either *V. diazotrophicus* NS1 (pFD085), *V. diazotrophicus* NS1 $\Delta nifH$ (pFD085) or *V. diazotrophicus* NS1 $\Delta nifH$ (pFD120) and left without shaking at 30°C. Neither *V. diazotrophicus* NS1 nor their derivatives do produce any biofilm when grown in LB. However, when grown in MDV, biofilm production significantly increased in the three tested strains (Figure 4A), and this biofilm was located at the bottom of the wells. Of note, MDV medium contains 100 mg/L yeast extract, which provides low-but-necessary bioavailable nitrogen for the *nifH* mutant to grow, allowing direct comparison of the three tested strains. Importantly, when MDV was supplemented with NO_3^- or NH_4^+ , the biofilm production significantly decreased as compared with the one obtained in MDV medium. Additionally, we observed only subtle differences between the different tested strains, all three of them producing more biofilm in MDV as compared to LB and to MDV supplemented with either NO_3^- or NH_4^+ . This increase in biofilm formation in MDV suggests that in a nitrogen-limited medium, the production of biofilm by *V. diazotrophicus* NS1 might protect its nitrogenase from an excess in O_2 .

Furthermore, the thickness and biovolume of the biofilm produced in MDV by *V. diazotrophicus* were quantified by confocal microscopy, using flow cells. Under this condition, *V. diazotrophicus* produced a

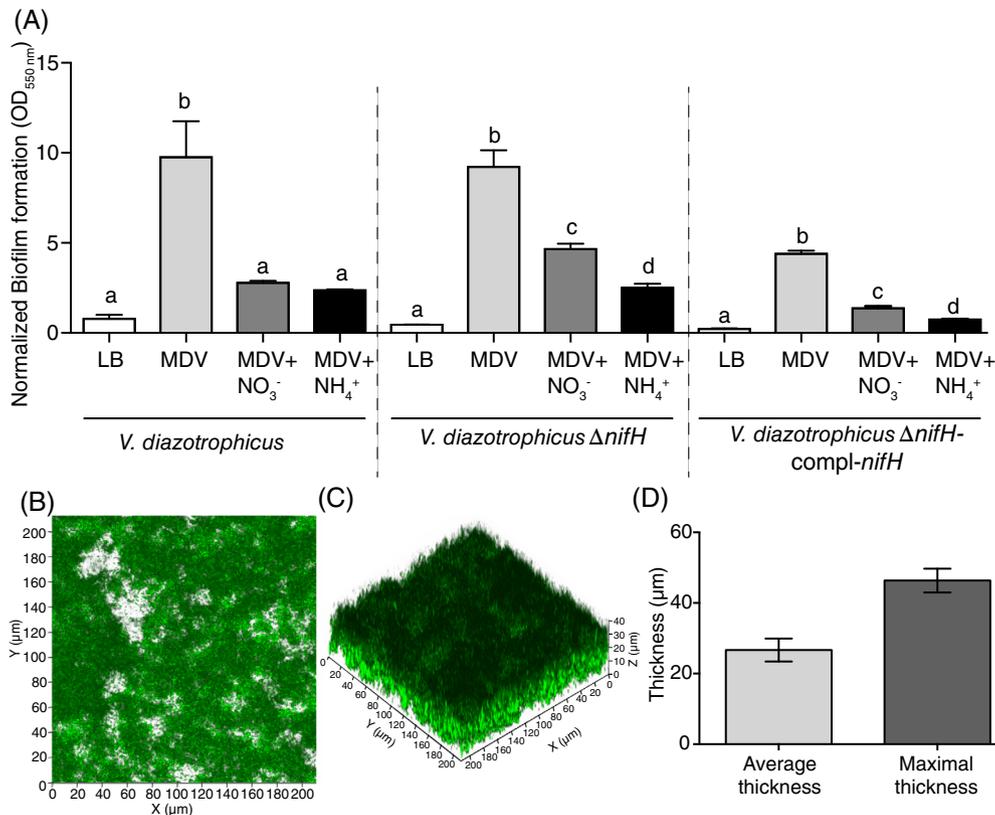


FIGURE 4 Biofilm formation characteristics of *V. diazotrophicus* NS1. (A) Corrected biofilm formation in *V. diazotrophicus* NS1 and derivative mutants in different media. *Vibrio diazotrophicus* NS1 and *V. diazotrophicus* NS1 $\Delta nifH$ contained the pFD085 plasmid and *V. diazotrophicus* NS1 compl-*nifH* corresponds to *V. diazotrophicus* NS1 $\Delta nifH$ containing pFD120. Presented here are the mean + SEM of at least three replicates. Significance was assessed by one-way ANOVA with Tukey test for each mutant in the different media. (B) View of the top of the biofilm produced by *V. diazotrophicus* NS1 pFD086 after 24 h of incubation in a flow cell. (C) Stacked microscopy image showing the thickness of the biofilm produced after 24 h. (D) Average and maximal thickness of the biofilm produced by *V. diazotrophicus* NS1 pFD086 after 24 h. See Figure S4 for images corresponding to timepoint 48 h.

large amount of biofilm (average biovolume of $11.70 \mu\text{m}^3 \cdot \mu\text{m}^{-2}$, standard error of the mean [SEM] $1.64 \mu\text{m}$). The thickness of the biofilm was also measured, reaching on average $26.65 \mu\text{m}$ (SEM $3.25 \mu\text{m}$) and a maximal thickness of $46.35 \mu\text{m}$ (SEM $3.34 \mu\text{m}$) after 24 h (Figure 4B–D). After 48 h, these values increased even more, but the biofilm matrix was too dense and too heterogeneous, probably because of the maturation of the biofilm, causing early dispersal (Figure S4).

***Vibrio diazotrophicus* is different from recently discovered heterotrophic bacterial diazotrophs**

The genome of *V. diazotrophicus* NS1 was compared with the 40 MAGs of HBDs recently retrieved from *Tara Ocean* metagenomes (Delmont et al., 2022). Expectedly, *V. diazotrophicus* NS1 falls in a group containing all MAGs affiliated to *Gammaproteobacteria* (Figure 5A). However, the closest MAG (called HBD_Gamma_02

here and in the study by Delmont et al., 2022) seems to be affiliated to the order *Pseudomonales* and not to *Vibrionales*, and ANI comparison (value of 0.68, see Table S4) confirmed their phylogenomic distance. In addition, the genome of *V. diazotrophicus* NS1 was not detected in the *Tara Ocean* metagenomes (max detection was 8% of the genome, Table S5). We subsequently compared the genomic relatedness of *V. diazotrophicus* NS1 to the genomes of six strains isolated from deep subsurface sediments of the Baltic Sea (Castillo et al., 2018), to the one isolated from phytoplankton exometabolite-enrichments (Fu et al., 2020) and to strain *V. diazotrophicus* 99A isolated in Pennsylvania (unpublished data). Pangenomic and phylogenomic analyses showed that *V. diazotrophicus* NS1 is closely related to six of these eight strains (Figure 5B), with *V. diazotrophicus* 60.6B and HF9B standing a bit apart, possibly belonging to new species (ANIb comparison below 0.95, see Figure 5B and Table S4). Of note, all of these nine *V. diazotrophicus* isolates carry a single copy of the *nifH* gene (Figure 5B,C) and a similar *nif* cluster (Figure 5C).

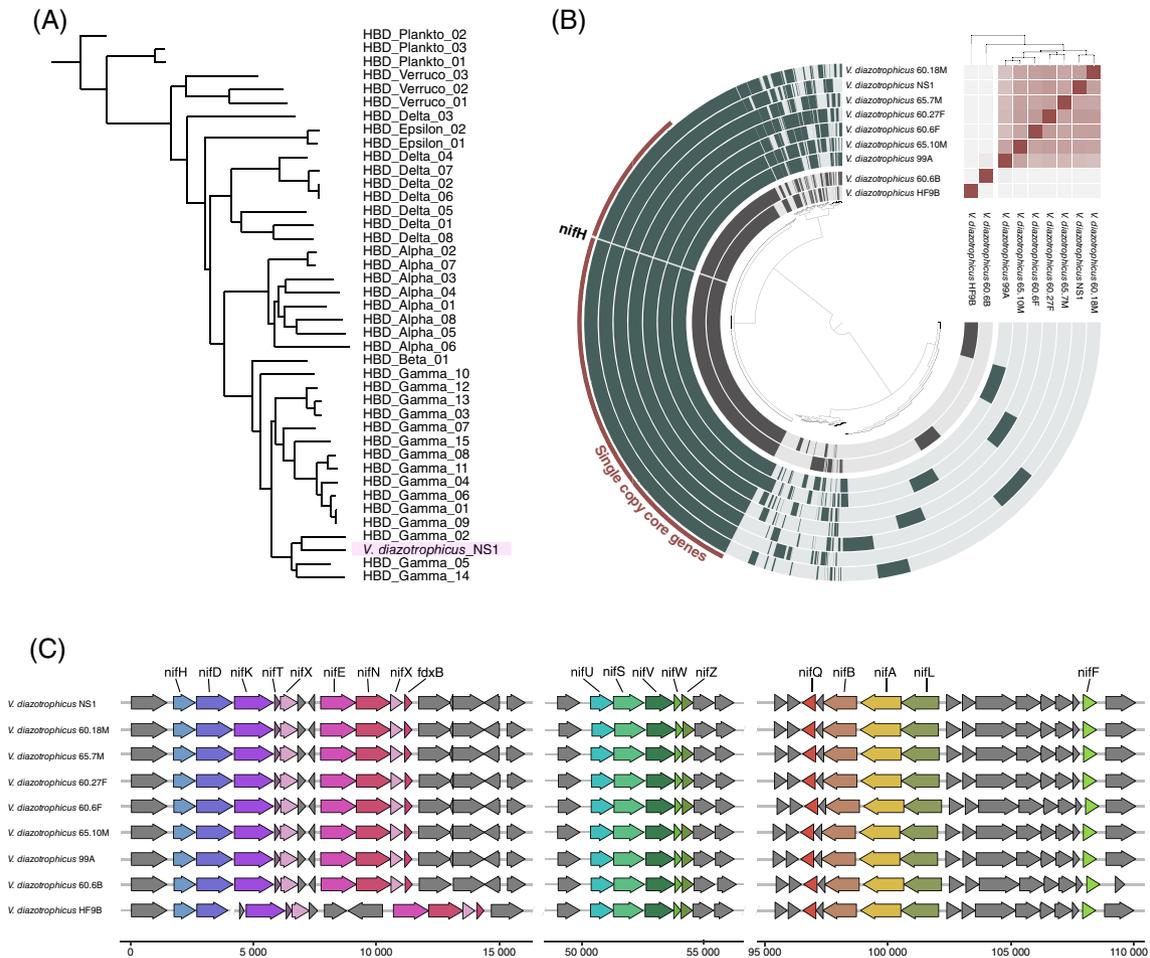


FIGURE 5 Comparative genomics of *V. diazotrophicus* NS1. (A) Phylogenomic tree of *V. diazotrophicus* NS1 and the 40 diazotroph MAGs from (Delmont et al., 2022) using 16 single-copy core genes occurring in at least 40 of the 41 genomes. We used FastTree to compute a phylogenomic tree and anvio (Eren et al., 2021) for the visualization. (B) Pangenomics analysis of publicly available *V. diazotrophicus*. A total of 6928 gene clusters are represented in the figure with each darker colour in the genome's layer indicating the presence of gene cluster in the corresponding genome. The red selection highlights the 2884 single-copy core genes. The heatmap corresponds to the ANI computed between all genomes and it is scaled from below 0.95 ANI (white) to 1 ANI (dark red). We generated a phylogenomics tree (shown above the ANI heatmap) using a subset of set of 99 single copy core gene with a maximum functional homogeneity of 0.95 using anvio. (C) Conserved synteny of the *nif* gene locus across multiple *V. diazotrophicus* genomes. The scale is based on the total size of the locus in *V. diazotrophicus* NS1. The double bars indicate that the locus continues on a different contig. Acc. Num: 65.7 M (POSL00000000), 65.10 M (POSM00000000), 60.27F (POSK00000000), 60.18 M (POSJ00000000), 60.6F (POSI00000000), 60.6B (POSH00000000), HF9B (JAATOR00000000) and 99A (PRJNA456207)

DISCUSSION

Nitrogen fixation is a major metabolism in many ecosystems, supplying living micro- and macro-organisms with otherwise scarce bioavailable nitrogen. In oceans, diazotrophy can account for half of the annual new production in euphotic zones in oligotrophic oceans (Karl et al., 1997). The diversity of marine diazotrophs has long been thought to be restricted to members of the phylum Cyanobacteria. However, culture-independent approaches, like those targeting the *nifH* gene or metagenomic and metatranscriptomic analyses from various oceanic samples demonstrated the presence in abundance of non-cyanobacterial diazotrophs (NCD; Delmont et al., 2018, 2022; Farnelid et al., 2011, 2013,

2019; Geisler et al., 2019; Kapili et al., 2020; Rahav et al., 2013; Zehr et al., 1998). Despite these observations, few marine NCD have been brought to culture (Farnelid et al., 2014; Martinez-Perez et al., 2018), hampering in depth understanding of their ecophysiology and contribution to global N budget in oceans. The goal of this study was to unveil the physiology of *V. diazotrophicus* NS1, a marine NCD isolated already 40 years ago (Guerinot & Patriquin, 1981) but surprisingly overlooked for in-depth analysis of its physiology.

The genome of *V. diazotrophicus* NS1 is available, and its analysis shows that it contains one *nif* cluster, separated in three chromosomal locations and spanning some 110 Kbp (Figure 5C). This cluster is found in all *V. diazotrophicus* strains whose genomes have

been sequenced, with only minor differences. The presence of only one *nif* cluster and one *nifH* gene (Figure 5C) is unlike other soil NCD like those belonging to the *Bradyrhizobium* genus, which can be endowed with two *nif* clusters (de Matos et al., 2021).

Because *V. diazotrophicus* NS1 belongs to one of the few marine NCD isolated, we sought to optimize for this strain some genetic tools, which are already existing for other *Vibrio* species (Delavat et al., 2018; Morot et al., 2021; Tischler et al., 2018; Zhang et al., 2019; Zingl et al., 2020). Results show that *V. diazotrophicus* is genetically tractable, both by electroporation and conjugation. Electroporation efficiency was albeit low, as compared with the one reported for other *Vibrio* or *Pseudoalteromonas* species (Delavat et al., 2018). Improving the electroporation efficiency will require trials and errors, but having an already functional method reinforce its high potential for rapid introduction of exogenous DNA, strengthened by its functionality in other marine bacteria like *Algoriphagus machipongonensis* (Xu et al., 2022) or human pathogens like *Vibrio parahaemolyticus* (Soree et al., 2022). Conjugation was more efficient, both for the transfer of replicative plasmids and for the insertion of mini-transposons. Mating is however more time-consuming and requires efficient counterselection of the donor. This counterselection is achieved here thanks to the use of strain β 3914 (Le Roux et al., 2007), a RP4⁺ and DAP⁻auxotroph strain and thanks to a protocol recently optimized for other *Vibrio* species (Morot et al., 2021).

In order to study the ecophysiology of marine NCD, we next focused on the targeted deletion of genes. The *nifH* was the ideal candidate for gene deletion since it is found in only one copy in *V. diazotrophicus* NS1, it is conserved in diazotrophs and considered as a biomarker for diazotrophy and it codes the nitrogenase-reductase, and essential component of the nitrogenase multiprotein complex. Adapting an already existing protocol (Morot et al., 2021), we were able to perform a scarless deletion of the *nifH* gene in this strain. To our knowledge, this mutant represents the first marine NCD mutant described in the literature, opening an avenue for a better characterization of their ecophysiology and role in the N and C biogeochemical cycles. Its nitrogen-fixation capability was subsequently tested thanks to a strain- and medium-adapted soft-gellan bioassay (Hara et al., 2009; Hashidoko et al., 2002). This soft-gellan assay allows the inoculated bacteria to 'find' their best appropriate O₂-tension for optimized growth, characterized by a visible ring formed within the semi-solid medium (Hashidoko et al., 2002). When a nitrogen-free medium is used, the location of this growing ring corresponds to the delicate balance between O₂ tension-necessary for aerobic respiration- and diazotrophy inhibition by O₂. Additionally, gellan gum allows a complete transparency of the medium and better visualization of bacterial growth (Hashidoko et al., 2002),

frequently allows faster growth and a higher bacterial diversity recovered (Delavat et al., 2012, 2013; Tamaki et al., 2009) and, importantly, bacteria grown in soft-gellan show a much higher diazotrophic activity compared to the same bacteria grown in soft-agar (Hara et al., 2009).

When grown in a nitrogen-free soft-gellan medium, we clearly showed that, unlike *V. diazotrophicus* NS1 wild type, the Δ *nifH* mutant is unable to grow (Figure 3 and Figure S2). Moreover, *in trans* complementation of the *nifH* mutant restores its capacity to grow in a nitrogen-free medium, demonstrating that growth in this medium is dependent on an intact nitrogenase. We show here that the developed soft-gellan bioassay is powerful to screen for diazotrophic activity and to phenotypically differentiate *V. diazotrophicus* mutants, for example, those related to N₂ fixation (catalytic or regulatory genes) or related to protection from O₂. This experiment and the fact that no growth was obtained in the Δ *nifH* mutant even after prolonged incubation in these soft-gellan tubes also confirm the presence of only one copy of the *nifH* gene in the genome *V. diazotrophicus* NS1 and no paralog being able to partially complement this mutation (Figure 5B).

Because of the inhibition of diazotrophy by O₂, nitrogen fixation occurs at an intermediate O₂ tension, where bacterial respiration can occur without inhibiting diazotrophy. We demonstrated here with a nitrogen-free soft-gellan medium that *V. diazotrophicus* NS1 grows under microoxic conditions, ranging from 1 to 15 μ M O₂ to achieve a sufficient N₂ fixation activity to sustain its growth (Figure 3). Decreasing the local O₂ tension is therefore important and can be achieved by different means (for a review, see; Gallon, 1992). As the ecophysiology of marine NCD is basically unknown, we tested whether *V. diazotrophicus* NS1 is able to produce a biofilm, which would restrict the diffusion of O₂ in the cells. Indeed, this strain and the Δ *nifH* mutant produce more biofilm when grown in a nitrogen-limited medium, compared with the biofilm formed in LB-rich medium, or when NO₃⁻ or NH₄⁺ is present (Figure 4A). This demonstrates that the biofilm production is a physiological response of *V. diazotrophicus* when N is limiting, that the biofilm production is not dependent on the presence of an intact nitrogenase and that both processes are genetically decoupled, at least at this genetic hierarchy. Biofilm production is a common trait in *Vibrio* species and is implicated in many bacterial processes such as virulence (Gallego-Hernandez et al., 2020; Rodrigues et al., 2018) or symbiosis (Visick, 2009). It is typically regulated by quorum sensing, which controls individual and collective behaviours depending on cell density. Whether the quorum sensing system branches at a given genetic level to control both biofilm production and N₂-fixation remains to be discovered.

The thickness of the biofilm was quantified using flow cells, showing that *V. diazotrophicus* NS1 produces a

substantial biofilm after 24 h of growth (Figure 4B–D). The thickness of the biofilm measured in this experiment (on average of 26.65 μm) probably serves as a barrier for O_2 -diffusion. Indeed, this value is coherent with previous data, showing the rapid decrease in O_2 in thick biofilms, which ultimately turn anoxic (Stewart et al., 2016). Thus, *V. diazotrophicus* NS1 produces biofilm when grown under nitrogen-limited condition, which might restrict O_2 diffusion, thus help solving the dilemma of nitrogenase inhibition by O_2 . This behaviour is analogous to the one of the terrestrial strain *Pseudomonas stutzeri* A1501, which produces biofilm under nitrogen-starved conditions (Wang et al., 2017). Marine non-cyanobacterial diazotrophs are in contrast less studied. Cell aggregates of 1–4 mm in diameter were observed for the marine diazotroph *P. stutzeri* BAL361 when grown in nitrogen-free media under oxic condition, which was hypothesized to be an adaptive mechanism for *P. stutzeri* BAL361 to fix N_2 despite high surrounding O_2 -tension (Bentzon-Tilia et al., 2015). Cell aggregation and exopolysaccharide production is also suspected to play a role in the diazotrophic activity of the marine NCD *Sagittula castanea*, even though cell aggregation was also observed under anoxic conditions in this strain (Martinez-Perez et al., 2018). In this study, we showed that *V. diazotrophicus* cells grown under microaerobic conditions in nitrogen-free media do not form aggregates (Figure S3) and demonstrated an increase in biofilm density and biofilm thickness of a marine NCD when grown under nitrogen-limited conditions as compared to LB-grown culture, constituting an additional level of evidence that biofilm formation is important for these bacteria when grown under oxic condition. This hypothesis is reinforced by the recent demonstration of the large prevalence of marine NCD in high size fractions of marine metagenomic samples (Delmont et al., 2022), suggesting NCD aggregation on particles and planktonic aggregates (Farnelid et al., 2019; Geisler et al., 2019).

Vibrio diazotrophicus is a bacterial species, which has been isolated from different marine environments, including sea water (Guerinot et al., 1982), gastrointestinal tractus of sea urchin (Guerinot & Patriquin, 1981) or deep-sea sediments (Castillo et al., 2018), and from enriched-phytoplankton exometabolites (Fu et al., 2020). However, this species is barely detected from metagenomes coming from *Tara Ocean* expeditions (Table S5) and is only distantly related to newly discovered MAGs (Delmont et al., 2022). This almost absent distribution from those metagenomic samples despite their effective presence in various marine environments suggests that additional NCD live in alternative ecological niches that have been overlooked by most metagenomic studies, like those from macroorganisms (e.g. see urchins) or those from deep-sea sediment (Castillo et al., 2018).

In conclusion, this study allowed unveiling the eco-physiology of a marine NCD belonging to the species

V. diazotrophicus, which might serve as an ideal model to decipher their ecological importance related to the N and C biogeochemical cycles and their genetic and physiological adaptations to their environments, including the O_2 -tension and the presence of dissolved inorganic nitrogen.

AUTHOR CONTRIBUTIONS

FD designed the work. AJD, KT, PC, AM, SR, BJ, FT and FD performed the experiment. FD wrote the manuscript, which was revised and edited by every co-author.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding this work.

DATA AVAILABILITY STATEMENT

The genome sequence of NS1 is available on NCBI. Any additional information will be made available upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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