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N-fixation and related O_2 constraints on model marine diazotroph *Pseudomonas stutzeri* BAL361

Ryan W. Paerl*, Tobias N. G. Hansen, Nathalie N. S. E. Henriksen, Asmus K. Olesen, Lasse Riemann

Marine Biological Section, University of Copenhagen, 3000 Helsingør, Denmark

ABSTRACT: Marine non-cyanobacterial diazotrophs are widespread in the ocean and can be the dominant nitrogen (N) fixers in certain regions. Lagging behind distribution and diversity data for these diazotrophs is a fundamental understanding of their physiologies — particularly in regards to dealing with oxygen, a potential inhibitor of N-fixation present in most of the ocean. To address this constraint, we conducted multiple experiments with *Pseudomonas stutzeri* BAL361, a model marine planktonic non-cyanobacterial diazotroph previously isolated from the Baltic Sea. Here, we confirm that BAL361 uses nitrogenase to convert N_2 gas into biomass via N-fixation, reaching N-fixation rates upwards of 0.046 fmol N cell^{-1} h^{-1}. Planktonic BAL361 cells exhibited nitrogenase activity at ~54 µM O_2 or less — an O_2 threshold notably lower than that recently reported (~160 µM O_2) in experiments with BAL361 where large aggregates were observed. Provision of hydrophobic or hydrophilic particles or surfaces, used previously to stimulate N-fixation by aerobic natural communities, did not enhance N-fixation by aerobic BAL361 cultures. We empirically show that bulk N-fixation under aerobic conditions by BAL361 alone is possible by aggregation; however, it remains elusive how low numbers of solitary planktonic BAL361 cells in nature accomplish this same feat. Our findings draw new attention to the possibility that nutrient-rich conditions (including N-rich conditions) may be key to ultimately enable diazotrophs like BAL361 to overcome the 'O_2 problem' and perform N-fixation via microoxic zones within aerobic marine bulk waters.

KEY WORDS: Diazotrophs · N-fixation · *Pseudomonas* · Oxygen · Marine particles

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INTRODUCTION

Nitrogen (N)-fixation is regarded as an important input of N to the global ocean, as it enables sequestration of N, a key nutrient controlling planktonic primary production, from the atmosphere. Traditionally, N-fixing cyanobacteria (e.g. *Nodularia* spp., *Trichodesmium* spp. *Crocosphaera watsonii*, *Richelia* spp., and *Atelocyanobacterium thalassa*, UCYN-A) are viewed as the primary planktonic diazotrophs (N-fixers) contributing to N-fixation in marine and brackish waters (Stal et al. 2003, Zehr 2011, Farnelid et al. 2016). However, field-based surveys have reported detectable N-fixation in aphotic waters where cyanobacterial N-fixers are not present (Fernandez et al. 2011, Hamersley et al. 2011, Dekaezemacker et al. 2013, Rahav et al. 2013, Farnelid et al. 2013, Loescher et al. 2014, Benavides et al. 2016) and in photic layers where cyanobacterial sequences are not detectable (Sohm et al. 2011, Blais et al. 2012, Shiozaki et al. 2014). This finding, alongside the discovery that non-cyanobacterial diazotrophs are ubiquitous in marine waters (Zehr et al. 1998, Farnelid et al. 2011), has sparked interest in non-cyanobacterial N-fixation (reviewed in Bombar et al. 2016) — in particular, deciphering the genetic and physiological underpinnings that control their ability to perform N-fixation (Bentzon-Tilia et al. 2015a). Thus far, the majority of knowledge regarding physiological characteristics of non-cyanobacterial diazotrophs comes from experiments

*Corresponding author: rpaerl@ncsu.edu

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with soil isolates, (e.g. *Azotobacter* sp.; Poole & Hill 1997, Sabra et al. 2000).

A key environmental variable limiting N-fixation is oxygen, a potent inhibitor of the nitrogenase enzyme that drives N-fixation (Gallon 1981). In aerobic oceanic waters, oxygen is a major problem for non-cyanobacterial diazotrophs as they are thought to lack adaptations that diazotrophic cyanobacteria and select soil-associated non-cyanobacterial diazotrophs possess to avoid oxygen inhibition of nitrogenase, e.g. localizing N-fixation to oxygen-deplete cells (Haselkorn 2007), temporal segregation of photosynthesis and N-fixation (Berman-Frank et al. 2007), or encapsulation and high respiration rates (e.g. *Azotobacter vinelandii*; Poole & Hill 1997, Sabra et al. 2000, Inomura et al. 2017). Furthermore, cultivation of non-cyanobacterial marine diazotrophs appears most successful when utilizing highly reduced oxygen conditions (Guerinot & Colwell 1985, Boström et al. 2007, Farnelid et al. 2014). Reduced oxygen microzones occur in association with large (generally >1 mm) detrital or living (e.g. eukaryotic plankton) particles (Alldredge & Cohen 1987, Glud et al. 2015, Klawonn et al. 2015); hence, particle-associated microoxic zones may be habitats where non-cyanobacterial diazotrophs can carry out N-fixation in aerobic marine waters (Paerl 1985, Riemann et al. 2010, Bombar et al. 2016). Several experiments have directly demonstrated the efficacy of increased particle availability in facilitating N-fixation by natural populations — where additions of different particle types (e.g. glass fiber filter [GF/F], *Zostera* [eel grass], or transparent exopolymer particles [TEP] either stimulated nitrogenase activity or $^{15}N_2$ incorporation during short-term incubations; Paerl & Prufert 1987, Paerl & Carlton 1988, Rahav et al. 2013).

Recently, the Baltic Sea isolate *Pseudomonas stutzeri* BAL361 exhibited nitrogenase activity (acetylene reduction) under elevated extracellular oxygen concentrations (165 µM O_2) relative to other Baltic Sea isolates (~30 to 50 µM O_2) (Bentzon-Tilia et al. 2015a). Broadly, BAL361 is a representative of marine diazotrophic *Pseudomonas* found in diverse habitats (Farnelid et al. 2011, 2014, Turk-Kubo et al. 2014), and a representative of relatively abundant (at times reaching upwards of ~7×10^5 *nifH* gene copies l^{-1}) and transcriptionally active phylotypes common to diverse Baltic Sea waters (Farnelid et al. 2013, Bentzon-Tilia et al. 2014, 2015b). Putatively, BAL361 holds a competitive advantage over other non-cyanobacterial diazotrophs by performing N-fixation under intermediate oxygen concentrations in microenvironments, e.g. microzones on particles (Paerl 1985).

Here, we build upon recent findings through several experiments testing new hypotheses pertaining to BAL361 physiology: (1) BAL361 nitrogenase activity under N-deplete conditions enables cell growth via N-fixation; (2) nitrogenase activity of planktonic BAL361 cultures is more sensitive to oxygen concentrations than aggregate-forming BAL361 culture (studied previously); and (3) increased availability of particles or surfaces enables planktonic BAL361 cells to carry out N-fixation in fully oxygenated, N-limited medium.

MATERIALS AND METHODS

Pseudomonas stutzeri BAL361 strain information

The non-pigmented gammaproteobacterium *Pseudomonas stutzeri* BAL361 was originally isolated from Landsort Deep (58° 36′ N, 18° 14′ E), 3 m depth, Baltic Sea water using diazotroph-selective medium and reduced oxygen conditions (Farnelid et al. 2014). Respective *nifH* gene, 16S rRNA gene, and high quality draft genome sequences are available via NCBI accession numbers: KC140355, KC140295, and NZ_JXXD00000000 (Bentzon-Tilia et al. 2015a).

BAL361 cultivation and pre-experimentation setup

All BAL361 cultures were handled in a laminar flow hood using aseptic technique and incubated at room temperature and under static (non-agitated) conditions unless noted otherwise. BAL361 cells were obtained from growth of cells from a single plate colony on marine broth 2216 (ZoBell 1941). Cells were centrifuged (3 min at 6000 × *g*) and washed with diazotrophic medium (described below), prior to use in experiments. The diazotrophic medium was made as previously (Farnelid et al. 2014) but with only 1.5 mM KH_2PO_4, no bicarbonate, and 0.5 or 1 mM glucose as the organic carbon source. Cells were added to medium at a dilution of 1:1000 or greater to initiate all experiments. Ammonium sulfate was added at the start of experiments in different concentrations (as marked in the text; maximally 200 µM) to increase BAL361 biomass for acetylene reduction assays, elemental analyses, and/or N-replete positive controls (non N-limited growth). All experiments included triplicate treatments and control cultures. Acid-washed and autoclaved glass 60 ml vials, sealed with rubber septa, were used to incubate BAL361 culture unless otherwise noted.

Experimental setups

N-fixation by BAL361 under reduced oxygen concentrations

N-fixation dependent growth of BAL361 was tested by adding cells to diazotrophic medium at ~30 to 35 µM O_2 (known to stimulate nitrogenase activity under N-deplete conditions (Bentzon-Tilia et al. 2015a), and comparing growth of the culture to fully aerobic (~200 µM O_2) cultures, with or without supplied ammonium (100 µM). Oxygen concentrations were determined using a Firesting O_2 oxygen meter and an optical microsensor (OXR50; Pyroscience), and adjusted via bubbling with high purity N_2 gas (Air Liquide). Subsamples for cell counts were fixed with 2% final concentration formaldehyde (Sigma Aldrich), then stored at −80°C. Bacterial cells were enumerated via SYBR Green I staining and flow cytometry (FCM)-based counting (Brussaard et al. 2010) using a FACS Canto II flow cytometer (Becton Dickinson).

Determining BAL361 N-fixation rate from growth and elemental analysis

Prior N-fixation rates for BAL361 were determined only by the acetylene reduction (AR) method (Bentzon-Tilia et al. 2015a); in contrast, the N-fixation rate of BAL361 was determined here based on growth rate under N-limitation and elemental analysis (particulate N measurements). Cells were added to 300 ml of diazotrophic medium (in sterile acid-washed Erlenmeyer flasks) containing 40 µM NH_4^+. Cell abundances in the experiment were monitored by flow cytometry. When the primary culture was N-limited (Day 4; determined by comparing the cell abundance of the primary N-limited culture to unamended and 200 µM NH_4^+ cultures), it was split into triplicate 90 and 10 ml cultures in sterile crimp-sealed vials. Oxygen concentrations were lowered to ~35 µM O_2 in the 90 ml cultures to facilitate N-fixation, while the 10 ml cultures were kept fully aerobic. Cells were collected (45 ml) from the ~35 µM O_2 cultures on Days 8 and 12 for elemental analysis via vacuum filtration (<127 mm Hg) of biomass onto pre-combusted 0.3 µm pore-size ADVANTEC glass fiber filters. Oxygen concentrations were maintained at ~35 µM O_2 post-Day 8 sampling. For contrast, cells were collected from N-replete 200 µM NH_4^+ aerobic cultures on Day 12 (stationary phase), as well as any particulates from the medium alone (medium blank). Triplicate filters (with biomass or filtered medium as blanks) were dried at 50°C, crimped in tin cups, and C and N content was measured on an elemental analyzer (CE 1110; Thermo Electron) using acetanilide (Merck) as a calibration source. N-fixation rate (mean and SD) was calculated for the experiment by dividing the increase in particulate N (minus the average of medium blank values) from Day 8 to 12 by the net increase in BAL361 cells for each replicate, and then dividing by 96 h (4 d). Maximum N-fixation rate for BAL361 was determined by multiplying the mean fmol N per N-fixing BAL361 cell (from elemental analysis samples and total filtered cells on Day 8, 12, n = 6) by the highest growth rate of N-fixing BAL361 culture, noted in the first experiment (0.65 d^{-1}).

Regulation of BAL361 nitrogenase activity by oxygen

Oxygen-limitation of nitrogenase activity was evaluated in cultures of N-limited BAL361 cells. A 1 l aerobic culture of N-limited BAL361 (started with 40 µM NH_4^+ as described above) was dispensed into 24 sterile glass vials, sealed with septa, and their oxygen concentrations were altered (~11 to 200 µM O_2) as described above. AR was measured (Stewart et al. 1967) and cells were enumerated at the end of the AR assays. Triplicate 20 ml BAL361 cultures (or deionized water for blanks; 40 ml of headspace) were used for all AR assays. Ten percent of headspace in sealed vials was replaced with acetylene (Air Liquide). Headspace samples were collected after ~20 h (unless noted otherwise) and injected into Exetainer (Labco) vials (for later analysis) or directly into either a SRI 310C or Shimadzu GC2010 gas chromatograph equipped with a Poropak T column. Ethylene quantities were determined from an ethylene standard curve (0.5% ethylene in N_2; Mikrolab). The average ethylene detected in blanks was subtracted from incubations with BAL361 cells.

Particle addition experiments

The potential for (hydrophobic and hydrophilic) particle addition to stimulate N-fixation of aerobic BAL361 cultures was tested. N-limited BAL361 cultures (obtained as above) were initially amended with different particles (in some cases whole filters). AR assays were run (as described above, unless noted otherwise) after 1 to 2 d when cultures were N-limited based on cell growth — identified by con-

trasting growth of no NH_4^+ addition and 200 µM NH_4^+addition cultures. Reduced oxygen (achieved as described above in oxygen manipulation experiments) and full oxygen (~200 µM unless noted otherwise) controls were run alongside particle treatment cultures and assessed for nitrogenase activity using AR. Pre-combusted GF/F filters (GE Life Sciences) were added (as the 'particle') to N-limited BAL361 in an initial particle experiment.

More diverse particles were used in a second experiment. GF/F particles (from pre-combusted filters) generated by sonication were collected on 47 mm, 10 µm pore-sized polycarbonate (PC) filters (Millipore) and sterilized (on the filter) by autoclaving. Autoclaved *Zostera marina* (common eel grass) blades (from Øresund waters off Helsingør, Denmark) were used to generate particles <1 mm in width via cutting with surgical scissors. These were incubated in diazotrophic medium for ≥1 d (as leaching of organics occurred in preliminary experiments), then collected on 47 mm, 10 µm pore-size PC filters, and autoclaved prior to use in experiments. Transparent exopolymer particles (TEP) were added in experiments in the form of autoclaved Xanthan gum (G1253; Sigma Aldrich). GF/F and *Zostera* particles were added at a final concentration of 0.1 g l^{-1} to specific BAL361 cultures (in sealable glass vials), matching quantities used in prior experiments with natural communities (Paerl & Carlton 1988). TEP was added at 0.01 g l^{-1}, matching maximal concentrations used in bioassay experiments with natural communities (Rahav et al. 2013). Vials were incubated on a slow-rotating plankton wheel. AR assays were run (started Day 1) as above but also incubated and sampled for a longer period of time (2 to 5 d) to allow for maximum interaction between cells and particles. Cells were enumerated on Days 0, 1, and 5; the Day 5 abundances were used to calculate per cell ethylene production of cultures on Days 2 to 5. Cultures amended with 200 µM NH_4^+ (positive controls for growth) were enumerated on Days 3 and 4 as well, and were not used in AR assays.

A third experiment tested if addition of a natural microbial community would facilitate nitrogenase activity in BAL361 plus particles and light shaking (~100 rpm) to facilitate encounters of cells and/or particles. The natural community was obtained by serial filtration of (300 ml) Øresund surface water on a 45 mm, 0.8 µm pore-sized PC prefilter (Millipore) and then on a 45 mm, 0.2 µm pore-sized PC filter. Cells were washed off the 0.2 µm filter with 3 ml diazotrophic medium by pipetting and added at a volumetric ratio of 1:100 to a BAL361 culture, with or without (GF/F or TEP) particles. Qualitative visualization of particles and BAL361 cells in particle experiments was done at 100 to 200× magnification using dark-field microscopy (Olympus BX61 microscope).

Induced associations of BAL361 with filters

Forced aggregation was tested as a means to stimulate BAL361 nitrogenase activity under fully aerobic conditions, in contrast with provision of particles. In an initial experiment, aerobic N-limited BAL361 culture (diazotrophic medium plus 40 µM NH_4^+ as done before) was sampled in early exponential phase and the cells were concentrated onto pre-combusted 25 mm GF/F filters using autoclaved Swinnex (Millipore) cartridges and dropwise syringe-driven filtration. Select filters were coated with 1% low melting point agarose (A9414; Sigma Aldrich). Filtrate generated in the cell-concentration process was captured and added to sterile scintillation vials plus filters with concentrated cells. Vials containing bulk culture alone were also set up in parallel. Oxygen concentrations on the surface of filters and/or bulk culture were measured in all vials after 24 h of incubation. In a follow-up experiment, 1 l of aerobic N-limited BAL361 culture (diazotrophic medium plus 40 µM NH_4^+ as done before) was sub-sampled in early and late stationary phase for cells and concentrated using the same procedure as that used before, but instead cells or cells on filters were added to glass vials (20 ml final volume) for running AR assays. Flasks with only the 20 ml of the N-limited primary culture were also set up in parallel. AR assays were run as before (oxygen being reduced in positive control flasks) but headspace was sampled after 20 and 40 h of incubation. Cell abundances were enumerated by flow cytometry as before.

In a final experiment, small numbers of BAL361 cells were 'seeded' upon GF/F (hydrophilic) or polyethersulfone filters (hydrophobic) (0.2 µm pore-size; Pall) to test if they could ultimately enable detectable nitrogenase activity by BAL361 under fully aerobic conditions. Late-exponential phase BAL361 cells from a marine broth 2216 culture were centrifuged, washed (as above), and diluted using diazotrophic medium. Approximately 2000 cells were gently vacuum filtered (<127 mm Hg) upon each filter type (in triplicate). Filters were added to 20 ml of diazotrophic medium plus 40 µM NH_4^+ in flasks (not sealed, to keep conditions aerobic) for running the AR assay downstream once cultures entered stationary phase (due to N-limitation). Two sets of triplicate flasks con-

taining diazotrophic medium, 40 µM NH_4^+, and ~2000 cells (directly added, not on filters) were also set up at the start of the experiment for later use in AR assays (one set being incubated under reduced oxygen conditions, 35 µM O_2). AR assays began on Day 5, and the injected vials were incubated for 5 d; headspace samples were collected on Days 6, 8, and 10 of the experiment.

Data analyses

Significant differences between treatments and controls were determined using *t*-tests in Prism (GraphPad). Plots (scatter, bar) as well as linear and non-linear fits of experimental data were also generated using Prism. Calculations of growth rate, elemental composition per cell, and N-fixation rate were performed using Excel (Microsoft).

RESULTS

N-fixation by BAL361 under reduced oxygen concentrations

Under reduced oxygen (35 µM) conditions, BAL361 utilized N-fixation to grow on (N-limited) diazotrophic medium (Fig. 1). Under these conditions, maximum BAL361 yields were just under half of that seen in cultures supplied with 100 µM NH_4^+. The N-fixing culture also reached an estimated maximum growth rate of ~0.65 d^{-1} (r^2 = 0.94, n = 9, sigmoidal fit) under N-limited, reduced oxygen conditions (Fig. 1).

An AR-independent estimate of N-fixation by BAL361 was determined by coupling growth measurements with elemental analysis. A BAL361 culture grown on diazotrophic medium amended with 40 µM NH_4^+ became N-limited (relative to a 200 µM NH_4^+ amended culture) by Day 4 (Fig. 2), and lowering of oxygen concentrations in the culture to ~35 µM (Day 4) promoted net cell growth by Day 12, while no net growth was observed in the N-limited culture left at atmospheric oxygen concentrations (Fig. 2B). Mean N per BAL361 cell for the N-fixing culture on Days 8 and 12 was 23.6 ± 3.34 fg N $cell^{-1}$ (Table 1). In contrast, BAL361 cells collected (Day 12; stationary phase) from the 200 µM NH_4^+ (full oxygen) culture contained 11.1 ± 0.27 fg N $cell^{-1}$ and showed a lower C:N ratio (~5.7 vs. 10.4; Table 1).

Dividing the net increases in particulate N and total cells (cell concentration × 45 ml; Fig. 2) for replicate N-limited, low oxygen (~35 µM) BAL361 cultures over 4 d (Day 8 to 12) gives an N-fixation rate of 0.016 ± 0.012 fmol N $cell^{-1}$ h^{-1}. Using the maximum growth rate for N-fixing BAL361 of 0.65 d^{-1} from the first experiment (Fig. 1) and 23.6 fg N $cell^{-1}$ for N-fixing cells (mentioned above; Table 1), a maximum N-fixation rate for BAL361 was estimated to be 0.046 fmol N $cell^{-1}$ h^{-1} (converting fg to fmol and days to hours).

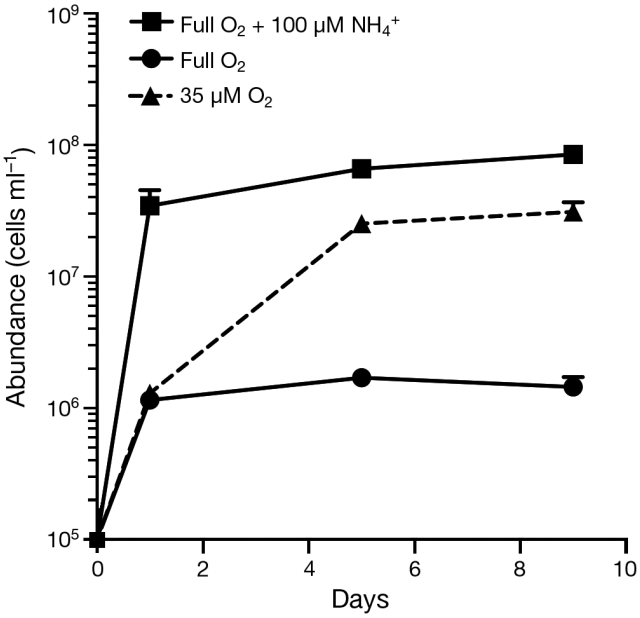

Fig. 1. *Pseudomonas stutzeri* BAL361 grows to higher yields under reduced bulk oxygen conditions (35 µM O_2; dashed line) in N-limited diazotrophic medium versus full O_2 (~200 µM O_2; solid line with circle data points). Increased yields in full O_2 + 100 µM NH_4^+ cultures (solid line with square data points) confirms N as the primary limiting nutrient. Data points and error bars are mean ± SD of measurements from triplicate cultures

Oxygen threshold for nitrogenase activity of planktonic N-limited BAL361 cultures

BAL361 cultures did not form visible aggregates in initial experiments, including those amended with 200 µM NH_4^+ (Figs. 1 & 2). In contrast, BAL361 formed extensive biofilms when grown on nutrient-rich marine broth 2216 under static conditions (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a081p125_supp.pdf). In previous experiments, aerobic BAL361 culture formed 1 to 4 mm cell aggregates, potentially due to cultivation and experimental conditions (Bentzon-Tilia et al. 2015a). Considering the lack of aggregates in our experiments, O_2 limita-

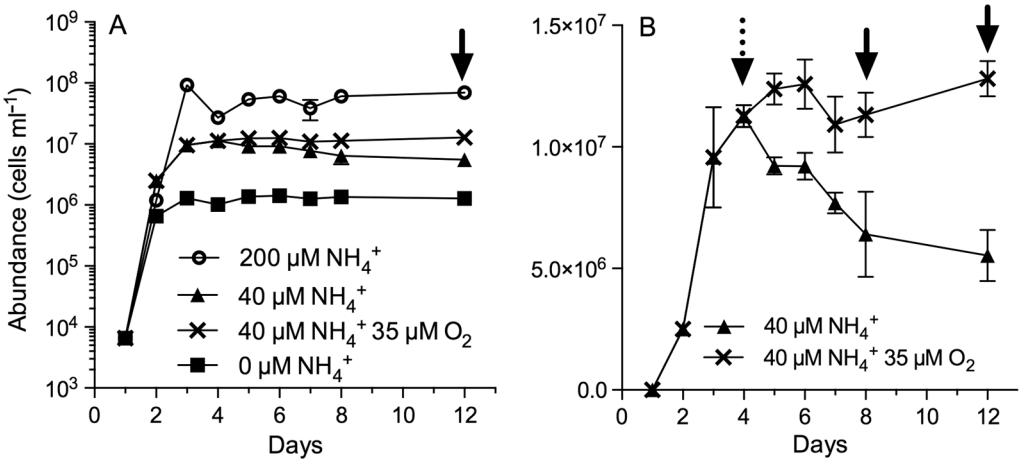

Fig. 2. Growth curves of *Pseudomonas stutzeri* BAL361 cultures sampled for elemental analyses (see 'Materials and methods'). Solid arrows indicate where cells were harvested on glass filters. Cell abundances are shown for (A) all BAL361 cultures and (B) only data for 40 µM NH_4^+ cultures. Dashed arrow indicates where a primary 40 µM NH_4^+ culture was divided into triplicate aerobic and low oxygen (35 µM O_2) cultures — the latter exhibiting net growth from Day 8 to 12. Note the linear scale on the *y*-axis in (B). Data points (±SD) represent data from triplicate subsamples, except for 40 µM NH_4^+ data after Day 4, which are from biological triplicates

tion of BAL361 nitrogenase activity was re-evaluated in order to resolve O_2-physiology of planktonic BAL361 cells. The exposure of planktonic BAL361 to varying O_2 concentrations in our experiments revealed that nitrogenase activity was restricted to ~54 µM O_2 (27% atmosphere) or lower (Fig. 3).

Surface or particle additions to aerobic cultures of N-limited BAL361

The addition of whole GF/F filters (large 'particles') (Fig. 4A) or hydrophilic or hydrophobic particles (e.g. GF/F particles, TEP) (Fig. S2 in the Supplement) did not significantly stimulate nitrogenase activity of aerobic N-limited BAL361 cultures. Supplementing N-limited BAL361 cultures including particles with a natural microbial community from Baltic Sea water (see 'Materials and methods') also did not stimulate notable nitrogenase activity in aerobic BAL361 cultures (Fig. S3). BAL361 cells were attached to added

Table 1. Nitrogen (N) per cell and C:N ratios for *Pseudomonas stutzeri* BAL361 cells collected from stationary phase (Day 12, 200 µM NH_4^+; Fig. 2A) or fixing N (Days 8 and 12, lowered oxygen 40 µM NH_4^+) (Fig. 2B), as well as mean values ±SD for multiple marine bacterial isolates collected from exponential phase or stationary (stat.) phase limited due to N, C, or P availability (Vrede et al. 2002)

Strain(s)	fgN cell^{-1}	C:N ratio	n
BAL361 stat. 200 µM NH_4^+	11.1 ± 0.27	5.7 ± 0.04	3
BAL361 N-fixing	23.6 ± 3.34	10.4 ± 0.34	6
Marine bacteria (stat. N-limited)	18 ± 1	7.5 ± 1.2	34
Marine bacteria (stat. C-limited)	12 ± 1	3.8 ± 0.1	37
Marine bacteria (stat. P-limited)	12 ± 2	9.5 ± 1.0	19
Marine bacteria (exponential)	35 ± 2	5.2 ± 0.2	49

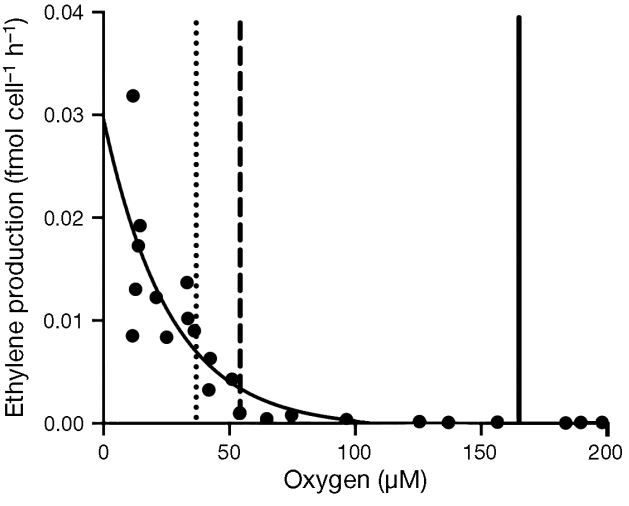

Fig. 3. Nitrogenase activity (determined via acetylene reduction assay) of planktonic *Pseudomonas stutzeri* BAL361 cells requires O_2 concentrations below ~54 µM. Plotted points represent data for single incubations (n = 24) and their respective starting oxygen concentrations. Solid line: non-linear regression for the entire dataset (r^2 = 0.75). Bentzon-Tilia et al. (2015a) reported an upper O_2 concentration limit of nitrogenase activity in BAL361 of 165 µM (solid vertical line); upper O_2 limits for other diazotrophic strains *Rhodopseudomonas palustris* BAL398 (dashed line) and *R. ornithinolytica* BAL286 (dotted line) (from Bentzon-Tilia et al. 2015a) are also included

particles (Fig. S4), but this did not lead to detectable reduced oxygen concentrations (TEP particles could not be easily probed by an O_2 sensor) near particle surfaces (data not shown), and no significant increase in nitrogenase activity was detected (Figs. 4, S2 & S3).

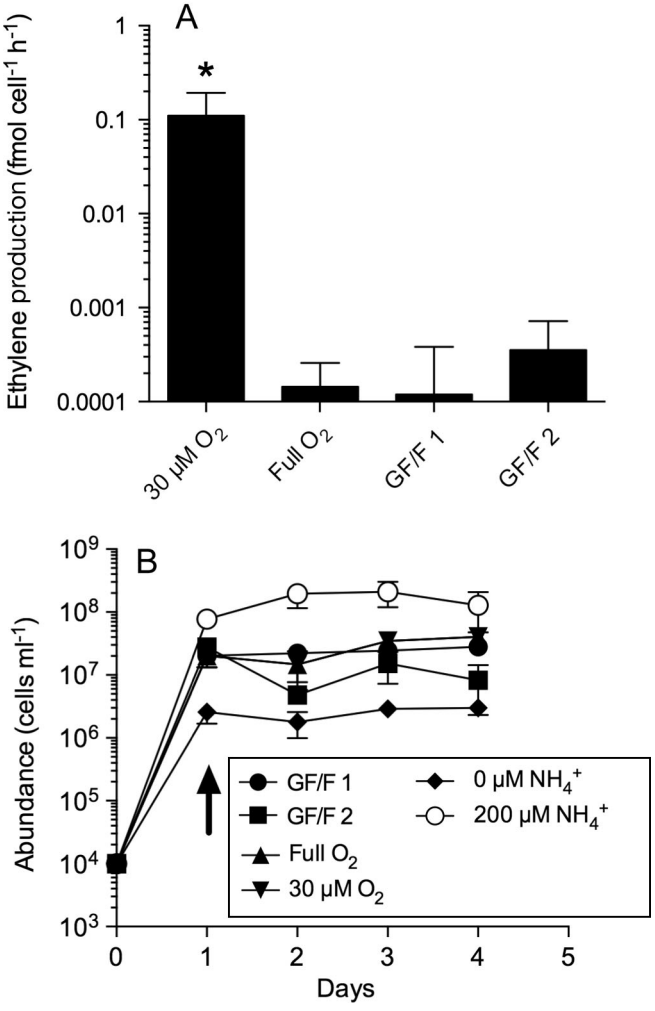

Fig. 4. The addition of 1 (GF/F 1) or 2 (GF/F 2) glass fiber filters did not stimulate nitrogenase activity of aerobic N-limited *Pseudomonas stutzeri* BAL361 cultures. (A) No aerobic cultures incubated with GF/F filters exhibited significantly higher nitrogenase activity (ethylene production cell^{-1}) versus aerobic cultures without added particles (full O_2). Nitrogenase activity was significantly higher in 30 µM O_2 cultures (*; *t*-test, df = 5, $p < 0.05$). (B) Abundances of planktonic BAL361 cells in particle-free aerobic (full O_2), as well as treatment cultures during the GF/F filter addition experiment. AR assays were initiated on Day 1 (marked by an arrow). Higher cell yields in the 200 µM NH_4^+ cultures confirm that the 40 µM NH_4^+ amended cultures were N-limited at the time of sampling. Data points and error bars: mean ± SD of measurements from triplicate cultures

Artificial associations of BAL361 to surfaces as a means to enable nitrogenase activity under aerobic conditions

To test if active BAL361 cells can at all engage nitrogenase under bulk aerobic conditions via aggregation, we conducted artificial aggregations of the diazotroph on filters. Initial tests revealed that concentrated BAL361 cells upon pre-combusted GF/F filters, with or without a covering of 1% agarose, were able to generate microoxic zones on filters (25 µM O_2 near filters versus ~145 to 155 µM O_2 in surrounding waters; see 'Materials and methods' and Fig. S5). In the follow-up experiment testing nitrogenase activity of concentrated cells, the concentration of BAL361 cells onto GF/F filters, with or without an agarose layer, significantly increased bulk nitrogenase activity under aerobic conditions (Fig. 5). After 40 h of incubation, elevated activity could no longer be detected in the cultures with concentrated cells without agarose covering (Fig. 5B). Concentrated late stationary phase BAL361 cells exhibited no significant nitrogenase activity in cultures with concentrated cells or lowered oxygen (Fig. 5C,D).

Lastly, we tested if BAL361 could form filter-associated aggregations (like those forced in the prior experiment) by 'seeding' BAL361 cells (~2000) via filtration onto (hydrophilic) GF/F filters, or (hydrophobic) PC filters, and ultimately exhibit significant nitrogenase activity under aerobic conditions. No significant nitrogenase activity was detected in these 'filter-seeding' experiments after 6, 8, and 10 d of incubation. Surface-localized oxygen concentrations only slightly decreased on filters over 10 d of incubation (Fig. S7B). Planktonic cell abundances in cultures with seeded filters increased after 2 to 3 d (Fig. S7C), suggesting cell detachment from filters during the beginning of the incubation.

DISCUSSION

N-fixation by BAL361 under low oxygen, N-deplete conditions

Here, we show that BAL361 employs N-fixation for growth under low oxygen and N-deplete conditions (Fig. 1), substantiating that it can utilize nitrogenase for N acquisition. Our estimate of maximum N-fixation derived from growth and elemental analysis (0.046 fmol N cell^{-1} h^{-1}) is only slightly above the highest AR-derived estimates for BAL361 (0.037 fmol N cell^{-1} h^{-1}, 30 µM O_2; Fig. 4A), assuming a tradi-

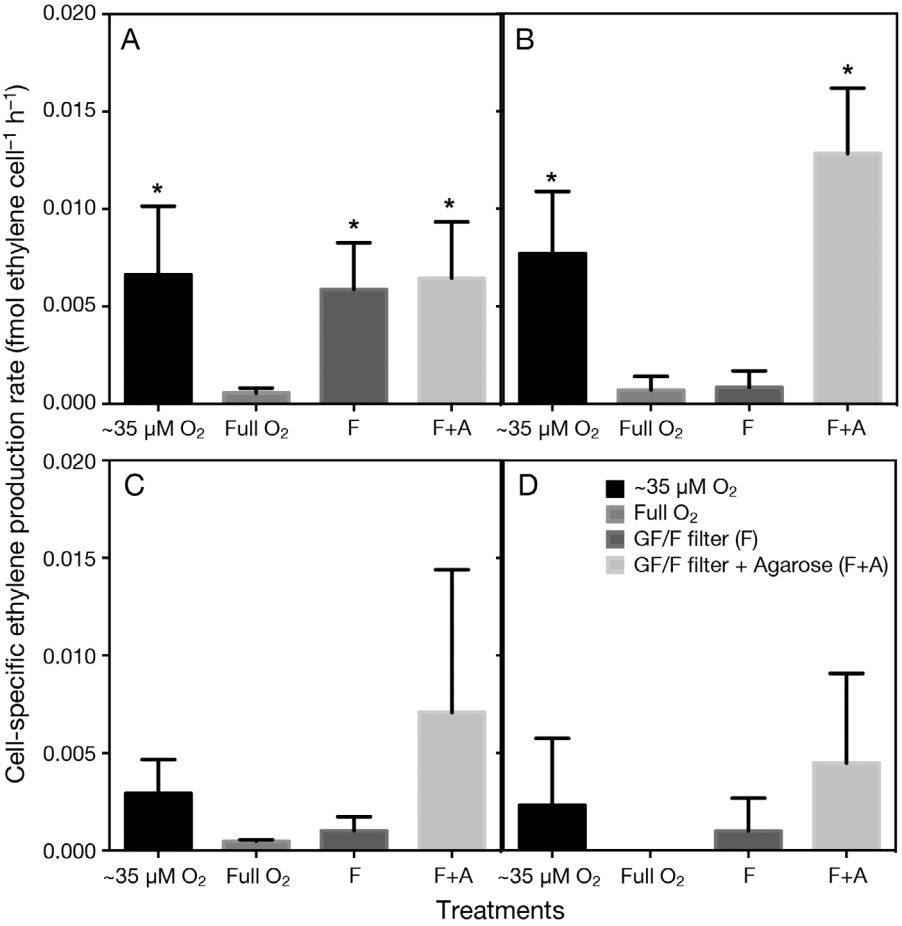

Fig. 5. Nitrogenase activity is detectable in aerobic cultures when *Pseudomonas stutzeri* BAL361 cells are concentrated on GF/F filters (F) or concentrated and covered with agarose (F+A), under N-limited conditions. Cultures with non-concentrated cells incubated under ~35 µM O_2 (~35 µM O_2) or non-concentrated cells and fully aerobic (full O_2) were also assayed for nitrogenase activity in parallel. Nitrogenase activity of cultures after (A) 20 h and (B) 40 h is presented for concentration experiments using N-limited BAL361 cells from early stationary phase. Data from experiments using N-limited cells from late stationary phase are shown after (C) 20 h and (D) 40 h. The growth curve of the primary BAL361 culture sampled for experiments is plotted in Fig. S6 in the Supplement. Means (columns) and SD (error bars) of assayed triplicate cultures are plotted. (*) indicates means were significantly higher than full O_2 cultures (*t*-test; $p < 0.05$)

tional ethylene to fixed-N conversion factor of 3:1 (Capone 1993).

The maximum N-fixation rate of 0.046 fmol N $cell^{-1}$ h^{-1} for BAL361 determined here is ~4× higher than that previously reported for the strain (Bentzon-Tilia et al. 2015a) and higher than reported rates for several soil- or sediment-associated non-cyanobacterial diazotrophs, aside from *Azotobacter vinelandii* (Table 2). Estimating the contribution of BAL361-like cells to bulk N-fixation rates in nature from our laboratory-determined rate values is challenging; however, we estimate an upper limit contribution to be 0.77 nmol N l^{-1} d^{-1} based on the maximum cell-specific N-fixation rate (above) and the highest reported abundance of

Table 2. Maximum N-fixation rates for *Pseudomonas stutzeri* BAL361 and rates reported for other non-cyanobacterial diazotrophs. Values are in fmol N $cell^{-1}$ h^{-1}, using a protein to cell conversion factor of 5×10^{-12} mg protein $cell^{-1}$ (Simon & Azam 1989), and an ethylene to N conversion ratio of 3:1 (Capone 1993)

Strain	Max. N-fixation rate	Reference
Geobacter metallireducens ATCC 53774	1.2×10^{-3}	Bazylinski et al. (2000)
Magnetospirillum magnetotacticum ATCC 31632	2.2×10^{-3}	Bazylinski et al. (2000)
Rhodopseudomonas sulfidophila W4	5.4×10^{-4}	Kelley et al. (1979)
Rhodopseudomonas capsulata B10	1.3×10^{-2}	Colbeau et al. (1980)
Beggiatoa spp. 81-1c	1.2×10^{-3}	Nelson et al. (1982)
'Estuarine' *Azotobacter*	1.4×10^{-2}	Dicker & Smith (1981)
Azotobacter chroococcum	4.0×10^{-3}	Drozd & Postgate (1970)
A. vinelandii sp. OP[a]	3.4×10^{0}	Bellenger et al. (2011)
P. stutzeri BAL361	4.6×10^{-2}	This study

[a]Rates determined via $^{15}N_2$ incorporation

BAL361-related *nifH* phylotypes from local Baltic Sea water (7 × 10^5 cells l^{-1}, assuming 1 *nifH* gene copy equates to 1 cell) (Farnelid et al. 2013). This rate lies on the low end of reported field estimates from the Baltic Sea (Farnelid et al. 2013, Bentzon-Tilia et al. 2015b); hence, the largest contribution of BAL361 (and close genetic relatives) to bulk N-fixation is expected during periods of low level bulk N-fixation.

The maximum N-fixation dependent growth rate of BAL361 (0.65 d^{-1}) reported here is much lower than that of soil diazotroph *A. vinelandii*, but higher than several 'model' cyanobacterial diazotrophs, suggesting BAL361 more efficiently uses N-fixation for growth (under optimum conditions) (Table 3). Notably though, the maximum growth rate of BAL361 on NH_4^+ is much faster than the N-fixation dependent growth rate (Fig. 1). Larger (e.g. >0.5 mm) particles rapidly sink out of the water column (e.g. 10 m d^{-1} and up to >100 m d^{-1}) (Alldredge & Gotschalk 1988, Ploug & Bergkvist 2015). Hence, if colonization is to enable notable amounts of aerobic N-fixation by BAL361, then high growth rates on fixed N, e.g. ~5.8 d^{-1} full O_2 + NH_4^+ (Fig. 1), are initially needed to rapidly increase biomass prior to the onset of N-limited conditions. Select large particles are viewed as habitats rich in fixed N (Ploug & Bergkvist 2015); however, the proportion of larger particles that ultimately facilitate N-fixation (for example, through rapid remineralization, cell growth, and nitrogenase activity by diazotrophic inhabitants) is unclear.

BAL361 does not easily overcome full aerobic conditions and perform N-fixation

Our results show that planktonic BAL361 cells are challenged to readily perform aerobic marine N-fixation. This is because of the following:

Firstly, the oxygen threshold for nitrogenase activity in planktonic BAL361 cells is ~54 µM O_2; 27% atmosphere), a value closer to that attributed to other non-cyanobacterial diazotrophs (Fig. 3). Hence, we suspect aggregation in prior experiments (Bentzon-Tilia et al. 2015a) enabled increased nitrogenase activity under elevated bulk oxygen concentrations, akin to artificial aggregation in our experiments (Fig. 5). This physiological response is, however, only likely under nutrient-replete conditions due to the substantial energetic costs of cellular oxygen protection mechanisms (Inomura et al. 2017).

Secondly, planktonic BAL361 cells did not sufficiently colonize, proliferate, and perform appreciable amounts of N-fixation on hydrophobic or hydrophilic particles (Fig. 4; Figs. S2, S3 & S7 in the Supplement), covering contrasting surface properties considered to impact bacterial cell adhesion (Cunliffe et al. 1999). In contrast, natural plankton communities are able to use these same particles, leading to increased bulk N-fixation (Paerl & Carlton 1988, Rahav et al. 2013); perhaps specific community interactions are needed to facilitate particle-stimulated N-fixation in the water column. Simplistically, we can show that forced aggregation of BAL361 cells alone stimulates N-fixation under bulk aerobic conditions (Fig. 5); however, it is unclear how planktonic BAL361 cells can accomplish this using artificial or natural particles.

We offer some hypotheses to explain how BAL361 might perform N-fixation under aerobic conditions.

Firstly, BAL361 may need to initially grow under nutrient-rich conditions to form robust aggregates (with or without particle association) to ultimately generate microoxic conditions, and then be exposed to N-limited conditions. This is partly counter-intuitive to traditional thinking that N-deplete conditions (especially relative to P) are prerequisite for N-fixation by marine plankton (Carpenter & Capone 2008, Zehr & Paerl 2008). A shift from nutrient-rich to N-deplete conditions is feasible in surface benthic sediment, where resuspension could shuttle aggregates with attached and active diazotrophs from the sediment environment into an N-limited overlying water column, or in organic planktonic aggregates as their C/N ratios decrease over time or with depth (Smith et al. 1992).

Secondly, BAL361 may require interactions with other organisms, e.g. particle-associated microbes and/or larger plankton, to encounter microoxic, N-deplete conditions suitable for N-fixation (Paerl & Kuparinen 2003). Bacterial colonization of particles is thought to be rapid (Kiørboe et al. 2003); hence, it is expected that colonized particles are prevalent in nature. Compounds re-

Table 3. Maximal N-fixation dependent growth rates for *Pseudomonas stutzeri* BAL361 and other cultivated diazotrophs

Strain	Growth rate (d^{-1})	Reference
Trichodesmium sp. IMS101	0.14	LaRoche & Breitbarth (2005)
Anabaena flos aqua UTEX 2557	0.26	Berman-Frank et al. (2007)
Cyanothece sp. WH8904	0.28	Berman-Frank et al. (2007)
Azotobacter vinelandii sp. OP	7.2	Bellenger et al. (2011)
P. stutzeri BAL361	0.65	This study

leased by high enzymatic activity uncoupled from uptake by particle-associated bacteria (e.g. Smith et al. 1992) may attract motile BAL361 cells to low-oxygen microenvironments where fixed N is unavailable to BAL361, but available to other bacteria that respire and maintain microoxic conditions. Alternatively, associations with larger live plankton (living 'particles') may be needed. Indeed, non-cyanobacterial diazotrophs do associate with diverse larger plankton (e.g. copepods, dinoflagellates) (Proctor 1997, Braun et al. 1999, Farnelid et al. 2010, Scavotto et al. 2015), and microoxic regions are known to occur in/on larger plankton (Tang et al. 2011); however, N-deplete microzones in/on larger plankton are not yet well resolved. Regardless, colonization of such microoxic zones likely requires collaborative or competitive interaction with existing microbial consortia.

Overall N-fixation in aerobic marine waters devoid of cyanobacterial diazotrophs typically exhibits low (<1 nmol l^{-1} d^{-1}) and/or patchy rates of N-fixation (Sohm et al. 2011, Blais et al. 2012, Rahav et al. 2013, Shiozaki et al. 2014, Benavides et al. 2016) — likely due to the ephemeral nature of non-cyanobacterial N-fixation in the water column. Oxygen is a key regulator of non-cyanobacterial N-fixation in aerobic waters, in addition to energy demands and fixed N availability in microhabitats (e.g. large particles) (Ploug et al. 1997, Ploug & Bergkvist 2015). Nonetheless, non-cyanobacterial N-fixation fascinatingly occurs in aerobic waters, but replicating the *in situ* environmental conditions that enable this phenomenon in the laboratory remains a significant challenge.

CONCLUSIONS

The BAL361-specific results presented here emphasize the significant 'oxygen problem' facing planktonic non-cyanobacterial N-fixers in aerobic marine waters — arguably more than conveyed by recent research findings. Future investigations should examine the physiological characteristics of other non-cyanobacterial diazotrophs, as it may be that populations other than BAL361 employ unique strategies to cope with fully aerobic conditions and perform N-fixation. In particular, particle-associated microoxic zone formation and the initiation and progression of non-cyanobacterial diazotrophs aggregations are research topics that deserve future attention and could help better explain how non-cyanobacterial N-fixation occurs in aerobic marine waters. Lastly, our findings draw attention to microbial consortia, as opposed to single diazotrophic strains, as the putative functional unit of non-cyanobacterial N-fixation in planktonic marine environments.

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