A bet-hedging strategy for denitrifying bacteria curtails their release of N₂O

Pawel Lycus*, Manuel Jesús Soriano-Lagunab, Morten Kjoab, David John Richardsonb, Andrew James Gatesb, Daniel Aleksanteri Milliganb, Åsa Frostegårdb, Linda Bergaustb,1, and Lars Reier Bakkenb,1

*Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1430 As, Norway; and bSchool of Biological Sciences, University of East Anglia, NR4 7TJ, Norwich, United Kingdom

Edited by Donald E. Canfield, Institute of Biology and Nordic Center for Earth Evolution (NordCEE), University of Southern Denmark, Odense M., Denmark, and approved October 5, 2018 (received for review April 7, 2018)

When oxygen becomes limiting, denitrifying bacteria must prepare for anaerobic respiration by synthesizing the reductases NAR (NO₂⁻ → NO₂⁻⁻), NIR (NO₂⁻ → NO), NOR (2NO → N₂O), and NOS (N₂O → N₂), either en bloc or sequentially, to avoid entrapment in anoxia without energy. Minimizing the metabolic burden of this precaution is a plausibly fitness trait, and we show that the model denitrifier Paracoccus denitrificans achieves this by synthesizing NOS in all cells, while only a minority synthesizes NIR. Phenotypic diversification with regards to NIR is ascribed to stochastic initiation of gene transcription, which becomes autocatalytic via NO production. Observed gas kinetics suggest that such bet hedging is widespread among denitrifying bacteria. Moreover, in response to oxygenation, P. denitrificans preserves NIR in the poles of nongrowing persister cells, ready to switch to anaerobic respiration in response to sudden anoxia. Our findings add dimensions to the regulatory biology of denitrification and identify regulatory traits that decrease N₂O emissions.

Denitrifying organisms use nitrogen oxyanions and oxides as terminal electron acceptors to sustain respiration in the absence of oxygen. This plays a key role in the global nitrogen cycle, returning reactive nitrogen from the biosphere to the atmosphere (1). Although the final product of denitrification is harmless N₂, fractions are emitted to the atmosphere as the potent greenhouse gas N₂O. The increasing emission of N₂O over the past decades is primarily due to denitrification, ultimately driven by the anthropogenic escalation of the global nitrogen cycle (2). The concerns over climate forcing and destruction of stratospheric ozone by N₂O (3) have fueled increasing interest in the ecology and physiology of denitrifying organisms, with a strong emphasis on the phenomena that determine their N₂O production. Denitrifying organisms emit N₂O because it is a free intermediate in the reduction of nitrate to N₂, catalyzed by four enzymes encoded by nar, nir, nor, and nos gene clusters (Fig. 1). These are widespread among prokaryotes in soils, sediments, and biofilms (4), and analyses of bacterial genomes have revealed that ~30% of the genomes containing the nos genes lack genes encoding NIR (NirS or NirK, ref. 5). Such “truncated denitrifiers” have attracted attention because they are net sinks for N₂O, whereas organisms equipped with NIR, NOR, and NOS are both sinks and sources. This was taken to suggest that the abundance of the structural gene, nosZ, could predict the propensity of a denitrifying community to emit N₂O, but the search for evidence has not been successful (6). Genome analyses show that ~70% of all genomes with nosZ also carry the genes for NIR and NOR, thus regulation of denitrification in these organisms will play an important role in controlling N₂O emission.

Regulatory networks controlling the transcription of denitrification genes have been established for a number of organisms (7, 8). A common feature is the role of oxygen as a superordinate repressor. This is likely a strong fitness trait because oxygen respiration is energetically favorable over denitrification in terms of the generation of proton motive force per electron transferred (9). Organisms in soils, biofilms, and sediments are frequently challenged by fluctuating O₂ concentrations and anoxic spells of variable length (10). When confronted with oxygen depletion, they must synthesize a minimum complement of denitrification enzymes “in time,” i.e., before oxygen is completely depleted, to avoid entrapment in anoxia without sufficient energy to produce a viable denitrification respiratory chain (11, 12). Synthesis of the entire denitrification proteome would be a waste of energy if oxygen reappears within hours. Thus, they have a regulatory dilemma, which has its parallel in any organism that is forced by substrate deprivation to synthesize new enzymes. This was modeled by Chu (13), who concluded that leaky repression is an optimal adaptation. In the case of denitrification, this would mean a leaky oxygen repression of at least one denitrification gene.

Experiments with Paracoccus denitrificans have provided some kinetic evidence for leaky repression of NAR and NOS, but not of NIR and NOR synthesis (14). Moreover, P. denitrificans displays a depression of respiratory electron flow in response to oxygen depletion, and this diauxie suggested that only a fraction of the cells can synthesize active NIR in time. Modeling provides support to the hypothesis that the phenomenon could be ascribed to a low probability for the initiation of nirS transcription, but with a positive feedback via NO and the NO sensor NnrR (12). Inspired by the fact that a similar diauxie in the transition from aerobic to anaerobic respiration is observed in other denitrifying organisms (12, 15), we have investigated the mechanisms in more detail in

Significance

Denitrifying microorganisms reduce nitrate to N₂ via nitrite, NO, and N₂O under hypoxic/anoxic conditions. Since these organisms are the main sources and sinks for N₂O in the environment, their regulatory biology controls the emission of this potent greenhouse gas. We demonstrate bet hedging in Paracoccus denitrificans when facing hypoxia: a minority of the cells synthesize NIR + NOR, the N₂O source, while all synthesize NOS, the N₂O sink, hence the population becomes a strong net sink for N₂O. Bet hedging is prominent below 20 °C and data indicate that it is widespread in soil organisms. This suggests a prominent role of bet hedging in controlling N₂O emissions that can now be tested for in other strains and in natural environments.

Author contributions: P.L., M.J.S.-L., D.J.R., A.J.G., Å.F., L.B., and L.R.B. designed research; P.L., M.J.S.-L., A.J.G., L.B., and L.R.B. performed research; M.K. contributed new reagents/analytic tools; P.L., M.K., D.A.M., L.B., and L.R.B. analyzed data; and L.B. and L.R.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

1To whom correspondence may be addressed. Email: linda.bergaust@nmbu.no or lars.bakken@nmbu.no.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1805000115/-/DCSupplemental.
Denitrifying organisms sustain anaerobic respiration by the stepwise reduction of NO$_3^-$ to N$_2$, catalyzed by four metallo-enzymes:

![Diagram of denitrification process]

Hypothesized cell diversification in response to oxygen depletion:

1. Early expression of NOS in all cells
2. Early expression of NIR in most cells
3. Stochastic expression of NIR + NOR, with low probability $r$, h$^{-1}$

Strong net sink for N$_2$O

$\text{O}_{\text{Oxic}} \xrightarrow{\text{Hypoxic}} \text{Anoxic}$

**Fig. 1.** Hypothesized cell diversification of *P. denitrificans* in response to oxygen depletion, corroborated by modeling the diauxic electron flow kinetics (Fig. 2). The model assumptions are that all cells synthesize NOS, while NAR and NIR + NOR synthesis is stochastic, occurring with a specific probability which is moderate for NAR (0.03-0.04 h$^{-1}$), very low for NIR + NOR (~0.004 h$^{-1}$), but with a positive feedback via NO (12). The different probabilities for a cell to synthesize NAR and NIR + NOR implies that after 30 h with critically low oxygen concentrations, 70-77% of the cells have synthesized NAR, while only 11% of the cells have synthesized NIR + NOR. Cells without NOS (but with NOS) can rarely by reduce the N$_2$O produced by the cells with NOS. Thus, the entire population effectively avoids entrapment in anoxia.

*P. denitrificans*, using a chromosomal mCherry–NirS fusion to track NirS, and immunocytostaining to track NOS synthesis and localization in bacterial cell populations. We decided to focus on these two enzymes because: (i) as homodimeric soluble periplasmic proteins, they are experimentally more tractable than the heterooligomeric integral membrane complexes NAR and NOR; (ii) NIR defines the denitrification process as it performs the conversion of an aqueous N-oxygen, nitrite, to a gaseous N-oxide, nitric oxide; and (iii) NOS defines the destruction of a potent greenhouse gas which is an obligate intermediate in the full denitrification process.

**Results**

Many studies investigating the regulation of denitrification have focused on gene transcription, despite active enzymes being products of gene transcription, translation, and posttranslational modification. To enable tracking of NIR, we constructed a strain where nirS was replaced by a chimeric mCherry–nirS fusion gene under the control of the native promoter (*SI Appendix, section 1.2*), allowing visualization of NirS positive cells by red fluorescence. We established that mCherry–NirS fusion proteins were correctly localized in the periplasm (*SI Appendix, Fig. S15*) and active, confirming correct posttranslational localization and cofactor insertion. The phenotype of the mCherry–NirS strain with respect to specific rate of oxygen consumption, aerobic growth rate, oxygen concentration at which denitrification is initiated, and anaerobic growth rate were practically identical to the parent strain (*SI Appendix, Table S2*). Estimating the apparent probability for cells to synthesize NirS (12) showed that (Fig. 2): (i) the denitrification phenotype of the mCherry–NirS construct is very similar to the wild type at all temperatures tested; (ii) the apparent probability for NirS synthesis increases with temperature for both strains, as predicted by the Arrhenius equation ($V = Ae^{-Ea/kT}$) with high apparent activation energy; and (iii) the NO concentration maximum increases with temperature for both strains.

To detect NOS in single cells, we developed an immunofluorescence staining method (*SI Appendix, section 1.3 and Fig. S2*). To differentiate between growing and nongrowing cells, we tried a number of published methods, either by positive staining of growing cells or by detecting growth as a dilution of stain, but they were unsuccessful for *P. denitrificans* under our experimental conditions, and we decided to design our own method (*SI Appendix, section 1.4*). Fluorescein isothiocyanate cell tracking (FITCCT). In short, cells are exposed for 10 min to fluorescein isothiocyanate and, after removal of excess stain, inoculated into fresh medium and their growth is monitored (*SI Appendix, section 1.4*). The staining was found to have negligible effects on the phenotype with respect to aerobic and anaerobic respiration and growth (*SI Appendix, Table S2*) and the fluorescence of cells was reduced by 50% for each cell division, while nongrowing cells retained the fluorescence (*SI Appendix, Figs. S9 and S10*). These tools allowed us to stringently test the hypothesized cell diversification summarized in Fig. 1. Stochastic initiation of NirS transcription, leading to two subpopulations, was verified by the observed fraction of red fluorescent, i.e., mCherry–NirS positive, cells throughout batch cultivation at 17 °C (Fig. 3). The fraction of red fluorescent cells increased as predicted by the model, which assumes a low probability for the initiation of nirS transcription once the repression by oxygen is relieved, and that the NirS positive cells grow exponentially throughout the anoxic phase. Moreover, the immunocytostaining of NOS demonstrated that all cells synthesized N$_2$O reductase (Fig. 3).

We also used FITCCT to investigate the anaerobic growth of cells with and without NirS. These experiments were conducted with 10% acetylene to inhibit NOS (for details, see *SI Appendix, section 2.2*). The results demonstrated that the FITC fluorescence was reduced gradually in the cells with NirS (mCherry

**Fig. 2.** Denitrification phenotype of the mCherry–NirS strain and the wild type as a function of temperature. (A) Gas and electron flow kinetics, measured and modeled (model: Hassan et al. (12)) for single vials (wild type) at the two extreme temperatures tested; 30 °C and 17 °C. The characteristic depression in electron flow rate after oxygen depletion at 17 °C is hardly detectable at 30 °C. The experiment was performed with three to six replicate vials at different temperatures, and the probability for initiation of nirS transcription ($r$, h$^{-1}$) was estimated for each individual vial, and ln(r) plotted against the inverse temperature (K$^{-1}$, B, Bottom), showing reasonably linear decline (wild type: $y = 64.38-20113x$; $R^2 = 0.9549$ and mCherry–NirS strain: $y = 41.95-13396x$; $R^2 = 0.8904$). The estimated apparent activation energy is 111 (standard error 26) and 166 (standard error 35) kJ mol$^{-1}$ for the mCherry–NirS strain and the wild type, respectively ($P < 0.01$ for the difference between strains). The maximum NO concentrations at the different temperatures (nanomoles of NO) are shown in the B, Top. The entire dataset and the model fits are shown in *SI Appendix, Figs. S4 and S5*. 

Lycus et al. 20113
fluorescence), while the cells that lacked NirS (Fig. 4) retained a strong FITC fluorescence. In theory, if not inhibited by acetylene, the cells without NirS (but with NOS) would be able to grow slowly by reducing N₂O provided by the cells with NirS. We have no FITC-based evidence for this, but the N₂O kinetics provide compelling evidence (12), and an experiment with N₂O in anoxic vials with nitrate/nitrite-free medium demonstrated the potential for growth by N₂O as the sole electron acceptor (SI Appendix, Figs. S8–S10).

Our model (12) assumed stochastic initiation of nirS transcription, with a very low probability, which then turns autocatalytic by NO via the NO sensor NnrR. In theory, this could imply that NO produced by the first few cells carrying active NirS would induce nirS transcription in the rest of the population, but this is evidently not the case. A tentative explanation is that the bulk concentrations of NO are too low (10–30 nM in the liquid), due to the high-affinity NO reductase present within actively denitrifying cells (16). A crude test of this was conducted by injecting NO to the culture at the time of oxygen depletion. The result was that nearly 100% of cells synthesized NirS and nitrite reduction to N₂O was much faster than in the control vials without NO exposure (SI Appendix, Fig. S17). Another prediction of the model is that in the absence of any usable electron acceptor, no cells would be able to synthesize Nir due to lack of metabolic energy; hence NIR-free cells would be entrapped in anoxia even in the presence of nitrite. However, if provided with N₂O, they would have the energy to synthesis Nir, even in the absence of nitrogen oxanions, albeit to a very low level due to lack of the positive regulatory feedback loop via NO and NnrR to promote synthesis of the full denitrification enzyme pathway. To explore this we used aerobically raised FITC-stained mCherry–nirS cells to inoculate anoxic vials with growth medium that was effectively stripped for nitrogen oxanions (see ref. 17), and cultures were provided with N₂O as the only electron acceptor. Controls without N₂O were also included. The results demonstrated that practically all cells were able to grow by reducing N₂O, as evidenced by dilution of the FITC fluorescence, while cells not provided with N₂O did not (SI Appendix, Figs. S8–S10). The cells provided solely with N₂O synthesized NirS, but only to a level of one to two orders of magnitude lower than in fully active denitrifying cells. However, when nitrite was injected after growth on N₂O for 45 h, NirS was synthesized to high levels in all cells. This contrasts the results for the transition from oxic to anoxic conditions, where only a low fraction of cells synthesized NirS. It could be taken to suggest a regulatory effect of prolonged exposure to anoxia and N₂O as the sole electron acceptor, enabling all of the cells to synthesize NirS when provided with nitrite. To our knowledge, no regulatory effect of N₂O on denitrification has been proven (18).

Little is known about the fate of the denitrification enzymes once oxygen returns. They could either be diluted by aerobic growth, degraded, or localized in aging cells by asymmetric distribution among daughter cells, as has been demonstrated both with cytotoxoplastic (19, 20) and periplasmic proteins (21). As a first approach to investigating the fate of the denitrification proteome, we designed an “entrapment assay” in which cells without intact Nir would be unable to grow: the cell suspensions to be tested were transferred to anoxic media without nitrogen oxanions, to which nitrite was added after depletion of the last traces of oxygen. Cells without NIR (raised through >10 generations of aerobic growth) were unable to initiate anaerobic respiration, while cells with NIR (anaerobically raised cells) were active immediately. We used this assay to assess the fate of a denitrification proteome during aerobic growth. P. denitrificans was raised by >12 generations of anaerobic growth on nitrite, ensuring that all cells carried a full set of denitrification enzymes. These denitrifying cultures were then exposed to fully oxic conditions in medium without nitrogen oxanions and allowed to grow by aerobic respiration up to ~12 generations. At intervals, cells were tested with the entrapment assay (SI Appendix, Fig. S3) using the kinetics of nitrite reduction to N₂O to assess the fraction of entrapment assay competent cells (EACs), i.e., cells that were able to initiate anaerobic respiration and growth in this assay.

The experiment had three alternative outcomes: (i) If cells actively degrade NIR, we would observe a rapid decline in EACs throughout the first generations of aerobic growth. (ii) If NIR is not actively degraded, but evenly distributed among daughter cells, the fraction of EACs would remain constant throughout the first generations of aerobic growth until the NIR content reached a critically low concentration (diluted by growth). (iii) If NIR is not actively degraded, but localized to the old poles of the cells during aerobic growth, the fraction of EACs would decline by 50% for each generation of aerobic growth. The result (Fig. 5) suggested the latter. Here, the number of EACs per milliliter remained practically constant throughout 12 generations of aerobic growth (Fig. 5, Inset 1), resulting in a gradual decline in percent of EACs as predicted (Fig. 5, Inset 2).

Differential localization of certain virulence proteins at the old cell poles has been established previously in Shigella flexneri (21). To examine the nature of NirS localization and distribution in P. denitrificans, time lapse microscopy of anaerobically raised cells was performed during aerobic growth on agar pads. Here, the following patterns were observed (Fig. 6): In almost all cells, mCherry–NirS migrated to the cell poles within minutes after exposure to oxygen (Movie S1). Some cells did not grow at all, and for this cell population NirS remained at the poles (Movie S2). The cells that did grow, first redistributed their NirS to the entire periplasm and started to grow, diluting their NirS by even distribution among daughter cells (Fig. 6, SI Appendix, Fig. S16, and Movie S3). However, some cells within microcolonies of growing cells stopped growing after one to three generations, and in these cells, NirS migrated back to the poles (Fig. 6 and Movie S4).
This pattern provides a plausible explanation of the observed result of the entrapment assay: the arrested growth by some of the cells qualifies for the term persister cells, because they are cells that retain their NirS in a potentially active form, hence enabling them to tackle sudden anoxia. Conversely, the cells that became engaged in aerobic respiration lost the ability to switch to anaerobic respiration in the entrapment assay.

Control experiments were conducted to exclude artifacts regarding the migration of NirS to the cell pole. A strain with inducible expression of mCherry that was transported to the periplasm was constructed (SI Appendix, section 1.2.2), and growth experiments demonstrated even distribution of mCherry throughout the periplasm and no migration to the cell pole in response to oxygen was observed (SI Appendix, Fig. S11). Thus, the migration of mCherry–NirS to the cell pole in response to oxygen is clearly due to a property of NirS, not mCherry. In addition, cells inactivated by NaN3 showed no migration to the pole (SI Appendix, Fig. S12); hence the migration depends on some metabolic integrity. To exclude artifacts created by the agar pad conditions, we corroborated the polar localization by transferring anaerobically grown cells to aerobic vials which were sampled for microscopy (fixed by formalin immediately after sampling). Samples taken throughout the first 30 min confirmed rapid migration to the poles, and samples taken after two to five generations of aerobic growth demonstrated that the persister cells had NirS localized at the cell poles. We also observed migration to the cell poles under anoxic conditions, but only in response to depletion of electron acceptors (SI Appendix, Fig. S13).

Discussion

Cell diversification in isogenic cultures has been described in a wide variety of prokaryotes, and the phenomenon is ascribed to noise and bistability of the regulatory networks (22, 23). Well-documented cases are endospore formation, chemotaxis, expression of genes for substrate utilization (lac operon in Escherichia coli), and the formation of persister cells (24). Some such phenomena are termed bet hedging because the population spreads the risks when responding to fluctuating conditions, in effect accepting a penalty for a fraction of the population, in exchange for a long-term fitness advantage for the entire population (25).

This present study reveals that P. denitrificans cells display a bet-hedging strategy with respect to the synthesis of NIR when challenged with imminent anoxia. Most likely, the synthesis of NOR is coordinated with NIR, as indicated by the NO kinetics during denitrification (16). The hypothesized mechanism was a constant, low probability of initial nirS transcription, but with a positive feedback loop via NO and NnrR (12, 26), and this is supported by our experimental results. Moreover, we observed a strong effect of temperature on the probability for NirS synthesis (Fig. 2). The probability of expressing the entire denitrification proteome increased sharply with temperature, resulting in pronounced bet hedging at low temperatures, while a high fraction of the cells express the entire denitrification proteome at high temperature. This would make sense if the probability of severe/long-lasting anoxic spells increase with increasing soil temperature (since this would penalize the cells without NIR + NOR). This is indeed plausible if we think of anoxic spells induced by transient waterlogging of soil (after heavy rain): The oxygen consumption rate will increase with temperature, while the rate of drainage (thus restoring oxic conditions) will not. Hence, the probability of severe/long anoxic spells would increase with increasing temperature. The temperature effect explains why the bet-hedging phenomenon has gone undiscovered until now because, in general, P. denitrificans has been studied at temperatures ≥30 °C wherein the phenomenon is almost undetectable (Fig. 2A). This underscores the importance of conducting physiological experiments under environmentally relevant conditions.

The regulation of NIR synthesis in P. denitrificans can be seen as a beneficial energy-conserving strategy, i.e., minimizing the cost of protein synthesis in all cases when oxygen quickly reappears to adapt to changing environmental conditions. It could prove fatal, however, if it results in complete entrapment of the majority of cells in long-term anoxia. This penalty is evidently avoided by a leaky repression of nosZ (and possibly nar). By synthesizing NOS in 100% of the population in response to...
The entrapment assay demonstrates the ability of cells to switch to anaerobic respiration in the entrapment assay (sudden anoxia, ensuring that cells without NIR are effectively entrapped in anoxia). The main panel shows the electron flow to N-oxides ($\text{Ve}_\text{red}^\text{ox}$) during the entrapment assay for cells sampled after 0, 1, 2, 4, and 12 generations of aerobic growth (initial cell number was $10^{10}$ in each assay). The cumulative $\text{N}_2$ production is shown in Inset 3 (logarithmic time scale). Continuous lines (both in the main panel and Inset) show the model (12) fitted to the data (least square) by adjusting the initial number of cells that switched to anaerobic respiration and growth (EACs). Inset 1 is a reconstruction of aerobic growth through 12 generations, plotting population size relative to initial number ($N/N_0$), while EACs remained essentially constant, the total number of cells increased by a factor of 4,000. Inset 2 shows the %EAC in each sample, together with prediction (solid line), assuming asymmetric distribution of NirS during aerobic growth, i.e., that all NirS migrates to one daughter cell. These results were taken to suggest asymmetric distribution, but the time lapse imaging of aerobically growing cells provided a more plausible explanation (Fig. 6).

An important question is “How does bet hedging contribute to the survival of the population?” The entrapment assay demonstrates that organisms can become entrapped in anoxia if exposed to sudden disappearance of oxygen. This is a rare phenomenon in natural environments, however, where most anoxic spells are plausibly initiated by a more gradual depletion of oxygen. Thus, the advantage of bet hedging would primarily be to save energy by synthesizing only NAR and NOS in the majority of cells. NOS could possibly provide better protection to save energy by synthesizing only NAR and NOS in the majority of cells growing (indicated by arrowheads), retaining mCherry–NirS, growing fast from the very beginning. However, a single cell among the third generation cells stopped growing (indicated by arrowheads), retaining mCherry–NirS, while the rest of the population continued to divide NirS by growth. This is further illustrated by the cell lineage and mCherry intensity of individual cells (see also Movie S4) in the two Bottom panels. (Scale bars: 2 μm.) Full-frame pictures are shown in SI Appendix, Fig. S16, and a corresponding time lapse video (Movie S2) is available. See also time lapse movies of the growing cells in A and B (Movies S3 and S4). The total fluorescence signal for microcolonies and for nongrowing cells remained practically constant throughout the experiment (SI Appendix, Fig. S18).
have been described (30). However, only a few examples of spatially organized periplasmic proteins have been reported (21). Spatiotemporal organization of cytoplasmic proteins is often intimately linked to cell cycle and proton motive force (31), and it is reasonable to assume that periplasmic enzymes associating with the membrane or membrane-bound factors, may be governed by similar rules. The activity of NirS is linked to a range of factors and it makes little sense for the enzyme to exist as a detached entity floating freely in the periplasm. On the contrary, it is likely to interact intimately with the other denitrification enzymes, such as the membrane-embedded NorBC during active denitrification (32). Membrane-associated factors may in turn interact with cytoplasmic proteins in a manner dependent on the electrostatic state of the membrane. In this scenario, NOR and NirS may engage in a “capture and release” cycle driven by the proton motive force. Once detached from NOR, NirS may diffuse passively to the poles and/or interact with a secondary partner with polar localization. Alternatively, and perhaps more likely, NirS may migrate in complex with its membrane-embedded partners in a manner dependent on their interaction with the cytoplasm. A link between the proton motive force and NirS localization is supported by the observation of migration of NirS to the cell poles under anaerobic conditions in response to exhaustion of NO (SI Appendix, Fig. S13). In contrast, there was no evidence of migration of NirS to the cell pole under any of the conditions tested (Fig. 3 and SI Appendix, Fig. S14). NosZ may form a complex with the integral membrane protein NosR rather than NorBC (32), which could explain the divergent localization response of NosZ and NirS.

Much like rapid transitions from aerobic to anaerobic growth, the abrupt return of oxygen in the absence of N-oxides can be viewed as a crisis with profound regulatory challenges. To grow, cells must reassemble their aerobic respiromye, and this may require de novo protein synthesis dependent on existing energy reserves. Thus, the conservation of NIR in nongrowing persister cells, may be a result of energy depletion, i.e., “entrainment in oxia” in a select fraction of cells fully invested in an anaerobic lifestyle. We can only speculate as to the mechanisms involved in the formation of persister cells at this point, and more work is needed to verify their actual role in persistence of NIR during oxia spells.

Conclusion

Bet hedging with respect to NIR, coupled with early and complete onset of NOS, bears environmental implications because organisms with this regulatory setup become strong net sinks of N2O. Moreover, at the risk of unduly anthropomorphizing nonsentient organisms, such phenotypic heterogeneity can be seen as an ingenious strategy for safeguarding one’s interests without exhaustive investments. Placing wagers on multiple near–future outcomes nullifies the risk on population level, at lower costs than full synthesis of all enzymes.

Materials and Methods

Batch cultivation, monitoring of gas kinetics, and modeling of recruitment to anaerobic respiration during oxygen depletion are described in SI Appendix, section 1.1. The construction of the mCherry–NirS strain, and the control strain with naked periplasmic mCherry are described in SI Appendix, section 1.2. The complementation of immunity plasmid-containing NOS is described in SI Appendix, section 1.3. Development of the FITC method and the testing of phenotypic effects of the staining are described in SI Appendix, sections 1.4 and 2.3. Fluorescence microscopy and time lapse imaging of cells on agar slabs are described in SI Appendix, section 1.5. The entrapment assay, designed to assess the number of cells that are able to switch to anaerobic respiration in response to sudden anoxia, is described in SI Appendix, section 1.6.

Acknowledgments

P.L. and M.I.S.-L. were financed by the Marie Skłodowska Curie Initial Training Network, Nitrous Oxide Research Alliance (NORA), Grant 316472, under EU’s 7th framework programme. A.I.G. acknowledges support from the Biotechnological and Biological Sciences Research Council, United Kingdom (Grant BB/M00256X/1). L.B. was financed by the Research Council of Norway (Grants 231282/F20 and 275389/F20). M.K. acknowledges support from Research Council of Norway (Grant 250976).

1. Galloway JN, et al. (2004) Nitrogen cycles: Past, present and future. Biogeochemistry 70:153–226.
2. Schlesinger WH (2009) On the fate of anthropogenic nitrogen. Proc Natl Acad Sci USA 106:203–208.
3. Ravishankara AR, Daniel JS, Portmann RW (2009) Nitrous oxide (N2O): The dominant ozone-depleting substance emitted in the 21st century. Science 326:123–125.
4. Shapleigh JP (2013) Denitrifying prokaryotes. The Prokaryotes, eds Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (Springer, Berlin).
5. Graf DRE, Jones CM, Halin s (p 2014) Interpreter heat shocking highlight modulation of the denitrification pathway and underpin the importance of community structure for N2O emissions. Proc Natl Acad Sci USA 110:9626–9631.
6. Bakken LR, Frostegård Å (2017) Sources and sinks for N2O, can microbiologist help to mitigate N2O emissions? Environ Microbiol 19:4801–4805.
7. Van Spanning RIM, Richardson DJ, Fergusson S (2007) Introduction to the biochemistry and molecular biology of denitrification. The Biology of the Nitrogen Cycle, eds Bothe H, Fergusson S, Newton WE (Elsevier, Amsterdam), pp. 3–21.
8. Zumft WG, Krones PM (2007) Respiratory transformation of nitrous oxides (N2O) to dinitrogen by Bacteria and Archaea. Adv Microb Physiol 52:107–227.
9. Chen J, Strous M (2013) Denitrification and aerobic respiration, hybrid electron transport chains and co-evolution. Biochim Biophys Acta 1827:136–144.
10. Marchant HK, et al. (2017) Denitrifying community in coastal sediments performs aerobic and anaerobic respiration simultaneously. ISME J11:1799–1812.
11. Haiberg O, Binnewijden SJ, Sørensen J (1997) Growth of silicon immobilized bacteria on polycarbonate membrane filters, a technique to study microcolony formation under anaerobic conditions. Appl Environ Microbiol 63:2920–2924.
12. Hassan J, Ou Z, Bergaust L, Bakken LR (2016) Transient accumulation of N2O; and N2O during denitrification explained by assuming cell diversification by stochastic transcription of denitrification genes. PLoS Comput Biol 12:e1004621.
13. Chu D (2017) Limited by sensing-A minimal stochastic model of the lag-phase during diazotrophic growth. J Theor Biol 414:137–146.
14. Ou Z, Bakken LR, Molstad L, Frostegård Å, Bergaust LL (2016) Transcriptional and metabolic regulation of denitrification in Paracoccus denitrificans allows low but significant activity of nitrous oxide reductase underoxic conditions. Environ Microbiol 18:2951–2963.
15. Lycus P, et al. (2017) Phenotypic and genotypic richness of denitrifiers revealed by a novel isolation strategy. ISME J 11:2219–2232.
16. Hassan J, Bergaust L, Molstad L, de Vries S, Bakken LR (2016) Homeostatic control of nitric oxide (NO) in nanomolar concentrations in denitrifying bacteria-Modelling and experimental determination of NO reductase kinetics in vivo in Paracoccus denitrificans. Environ Microbiol 18:2964–2978.
17. Bergaust L, van Spanning RIM, Frostegård A, Bakken LR (2012) Expression of nitrous oxide reductase in Paracoccus denitrificans is regulated by oxygen and nitric oxide through Fnr and NNR. Microbiology 158:826–834.
18. Sullivan MJ, Gates AJ, Appia-Ayme C, Rowley G, Richardson DJ (2013) Copper control of bacterial nitrous oxide emission and its impact on vitamin B12-dependent metabolism. Proc Natl Acad Sci USA 110:19926–19931.
19. Lindner AB, Madden R, Demarez A, Stewart EW, Taddei F (2008) Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. Proc Natl Acad Sci USA 105:3076–3081.
20. Macara IG, Milii S (2008) Polarity and differential inheritance-Universal attributes of life? Cell 135:801–812.
21. Scribano D, et al. (2014) Polari localization of PhoN2, a periplasmic virulence-associated protein of Shigella flexneri, is required for proper IcsA expression at the old bacterial pole. PLoS One 9:e90030.
22. Ackermann M (2013) Microbial individuality in the natural environment. ISME J 7:465–476.
23. Veening JW, Smits WK, Kuipers OP (2008) Bistability, epigenetics, and bet-hedging in bacteria. Annu Rev Microbiol 62:193–210.
24. Levin K (2007) Persister cells, dormancy and infectious disease. Nat Rev Microbiol 5:48–56.
25. de Jong IG, Haccou P, Kuipers OP (2011) Bet hedging or not? A guide to proper classification of microbial survival strategies. BioEssays 33:215–223.
26. Hassan J, Bergaust LL, Wheat ID, Bakken LR (2014) Low probability of initiating nir transcription explains observed gas kinetics and growth of bacteria switching from aerobic respiration to denitrification. PLoS Comput Biol 10:e1003933.
27. Flechard CR, Neftel A, Jocher M, Neftel A, Fuhrer J (2005) Bi-directional soil/aerobic respiration to denitrification. Biogeochemistry 74:299–319.
28. Davis BM, Waldor MK (2013) Establishing polar identity in gram-negative rods. Nat Rev Microbiol 11:179–182.
29. Flechard CR, de Jong IG, Haccou P, Kuipers OP (2011) Bet hedging or not? A guide to proper classification of microbial survival strategies. BioEssays 33:215–223.
30. Hassan J, Bergaust LL, Wheat ID, Bakken LR (2014) Low probability of initiating nir transcription explains observed gas kinetics and growth of bacteria switching from aerobic respiration to denitrification. PLoS Comput Biol 10:e1003933.