

N₂ fixation in marine heterotrophic bacteria: Dynamics of environmental and molecular regulation

dinitrogen reductase/nifHDK operon/nitrogenase/in situ hybridization/Vibrio natriegens

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ABSTRACT Molecular and immunological techniques were used to examine N₂ fixation in a ubiquitous heterotrophic marine bacterium, the facultative anaerobic Vibrio natriegens. When batch cultures were shifted from aerobic N-replete to anaerobic N-deplete conditions, transcriptional and post-translational regulation of N₂ fixation was observed. Levels of nifHDK mRNA encoding the nitrogenase enzyme were highest at 140 min postshift and undetectable between 6 and 9 h later. Immunologically determined levels of nitrogenase enzyme (Fe protein) were highest between 6 and 15 h postshift, and nitrogenase activity peaked between 6 and 9 h postshift, declining by a factor of 2 after 12-15 h. Unlike their regulation in cyanobacteria, Fe protein and nitrogenase activity were present when nifHDK mRNA was absent in V. natriegens, indicating that nitrogenase is stored and stable under anaerobic conditions. Both nifHDK mRNA and Fe protein disappeared within 40 min after cultures were shifted from N₂-fixing conditions (anaerobic, N-deplete) to non-N₂-fixing conditions (aerobic, N-enriched) but reappeared when shifted to conditions favoring N₂ fixation. Thus, unlike other N₂-fixing heterotrophic bacteria, nitrogenase must be resynthesized after aerobic exposure in V. natriegens. Immunological detection based on immunoblot (Western) analysis and immunogold labeling correlated positively with nitrogenase activity; no localisation of nitrogenase was observed. Because V. natriegens continues to fix N₂ for many hours after anaerobic induction, this species may play an important role in providing "new" nitrogen in marine ecosystems.

Biological N₂ fixation is the reduction of atmospheric N₂ to ammonium. The reaction is catalyzed by the enzyme nitrogenase and requires an input of energy via photosynthesis or the metabolism of organic carbon compounds. Nitrogenase and the ability to fix N₂ are present in a wide variety of eubacteria and some methanogenic archaeabacteria (1, 2). Several genera of N₂-fixing heterotrophic eubacteria have been isolated from marine sediments and have been suggested to be important sources of "new" nitrogen in some systems (3). Planktonic N₂-fixing heterotrophic species also have been described, but their importance as a source of "new" nitrogen is unclear because of difficulties in enumeration and/or quantifying nitrogenase activity (4–6). Thus, a reliable estimate of the abundance of heterotrophic N₂-fixing bacterioplankton in the oceans, their potential for N₂ fixation, and the environmental factors that regulate this potential are either unavailable or obscure.

Nitrogenase is rapidly and irreversibly inactivated by O₂, which particularly targets the Fe protein (dinitrogen reductase) (7). Consequently, even though a diverse assemblage of heterotrophic marine bacteria with the potential to fix N₂ may be present, the potential can be realized only when a suitable carbon source is available and when localized O₂-depletion zones are present (4). The capacity for N₂ fixation in natural populations of marine eubacteria is beginning to be evaluated using molecular and immunological techniques (5, 6, 8).

The eubacterial heterotrophic genus Vibrio is ubiquitous in marine and estuarine environments. Most species are free-living with some forming a major portion of the surface plankton; others are symbiotic with teleost fishes and squid or are pathogenic in fish and shellfish (9–13). Some free-living and symbiotic species are bioluminescent; others are capable of N₂ fixation (9, 14). Little is known, however, about N₂ fixation or nitrogenase activity in any of the four species of N₂-fixing Vibrio, and virtually nothing is known about their abundance (5, 14).

The facultatively anaerobic and N₂-fixing Vibrio natriegens is found in marine and estuarine environments throughout the world and has one of the shortest doubling times (9.8 min at 37°C) of any bacterium (12, 14–17). As a first attempt to characterize and understand the regulation of N₂ fixation in this genus, we manipulated cultures of V. natriegens to evaluate the effects of O₂ and combined nitrogen availability on (i) the induction time and stability of nifHDK mRNA and Fe protein abundance, (ii) nitrogenase activity, and (iii) cellular abundance and distribution of dinitrogenase. We anticipated that understanding temporal and environmental regulation of nitrogenase gene expression and enzyme activity would expand our knowledge of the role of this genus in the ocean nitrogen cycle.

MATERIALS AND METHODS

Culture of Bacteria. Batch cultures of V. natriegens (ATCC 14048) were grown at 27°C in (i) FL medium, 1 g each of peptone and yeast extract per liter (S) (N-replete); (ii) UR-FE medium without peptone and yeast extract (5, 14) (N-deplete); (iii) UR-FE medium, 100 mM NH₄Cl, 0.05 g of yeast extract per liter (N-enriched); or (iv) UR-FE medium, 0.01 or 0.05 g of yeast extract per liter (N-deficient).

Cells were grown in aerobic N-replete or N-enriched media, then shifted to an anaerobic environment (N₂/CO₂, 99:1) with N-deficient or N-deplete media. Initial cultures (100 ml) were grown overnight in aerobic media, diluted into 1 liter of fresh medium, and grown aerobically to OD₅₅₀ = 0.4–0.5. The 0 time point sample was collected, and the remaining cells were washed twice in either a N-deficient or N-deplete medium, added anaerobically to a N-deficient or N-deplete medium, and grown anaerobically. Samples were collected anaerobically to determine cell density (OD₅₅₀), steady-state levels of nifHDK mRNA and Fe protein, nitrogenase activity, and immunological reactivity.

RNA Transcript Abundance. Sodium azide (final conc., 0.02 M) was added to cell samples, and RNA was extracted (18, 19).

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Equal amounts of total RNA (20 μg) were resuspended, electrophoresed, and hybridized at 42°C; filters were washed twice in 5 × standard saline phosphate/EDTA (SSPE; 1 × = 180 mM NaCl/10 mM Na2HPO4, pH 7.4/1 mM EDTA)/0.2% SDS for 15 min at 50°C, 1 × SSPE/0.75% SDS for 30 min at 42°C, and 0.1 × SSPE/1% SDS for 10 min at 42°C (19).

**Probe Preparation.** The probe for nifHDK mRNA was a 1.9-kb restriction fragment containing nifH and a 5′ portion of nifD from Klebsiella pneumoniae double-digested from pSA30 (23) with EcoRI and BamHI at 37°C for 3 h (New England Biolabs). After electrophoresis on a 0.6% agarose gel, the 1.9-kb restriction fragment was excised by centrifugation (24), and random-primed labeled (Prime-a-Gene, Promega) with [α-32P]dCTP (Amersham).

**Western Blot Analysis.** Lysates were sonicated, cleared by centrifugation, electrophoresed in SDS/15% polyacrylamide gels, and transferred to nitrocellulose membranes (19). Polyclonal Fe protein antiserum (raised against the protein from Azotobacter) and goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase were the primary and secondary antibodies, respectively. Colorimetric detection was by a standard alkaline phosphatase reaction (Promega).

**Nitrogenase Activity.** Nitrogenase activity was assayed by the acetylene reduction method (25, 26). A sample (5 ml) was drawn anaerobically after the shift from aerobic to anaerobic conditions and injected into crimp-sealed 20-ml serum bottles previously flushed with N2. Acetylene was generated from calcium carbide (27) and injected (1 ml) into the sample bottles. Duplicate experiments and anaerobic negative controls (no acetylene added) were conducted. Gas phase samples (50 μl) were withdrawn with a gas-tight syringe (Hamilton) within 1 h of incubation. Ethylene content was determined using a Shimadzu flame ionization gas chromatograph (model GC-14A) equipped with a GS-O capillary column (J & W Scientific, Folsom, CA; 30 m × 0.546 mm); ethylene production rates were determined by use of a linear regression.

**In Situ Detection of Fe Protein.** Cells of V. natriegens were collected in aerobic and anaerobic conditions; negative control cells included Escherichia coli (JM109) and baker's yeast. All cells were fixed overnight in 4% formalin/phosphate-buffered saline-M (PBS-M; 10 mM Na2HPO4/10 mM NaH2PO4/200 mM NaCl, pH 7.2); subsequent steps are modified from Orellana and Perry (28). Cells were permeabilized in 100 μl of lysozyme at 1 mg/ml-1 in 10 mM Tris-HCl/1 mM EDTA (pH 8.0) for 2 min on ice, washed, and blocked for 15 min at room temperature with 1% blotto [50 mM Tris-HCl, pH 7.4/100 mM NaCl/5% (wt/wt) Carnation nonfat dry milk] in PBS-M. Polyclonal Fe protein (1:100) and goat anti-rabbit conjugated to CY-2 were the primary and secondary antibodies, respectively. After a washing in dim light, cells were smeared onto coated slides (29), air dried, and viewed with epifluorescent microscopy (Olympus, (New Hyde Park, NY) model BH2; fluorescein isothiocyanate filter set).

**Immunogold Localization of Fe Protein.** Cells were fixed in 4% glutaraldehyde/sorbitol buffer (0.6 M sorbitol/5 mM potassium phosphate, pH 7.8/30 mM Hepes-KOH, pH 7.8) for 2 h at 4°C and resuspended in sorbitol buffer. Loosely packed cell pellets were dehydrated in ethanol and embedded in Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, Germany). Silver sections were mounted in presoaked (0.5% bovine serum albumin/0.1 M Tris-HCl, pH 7.4) carbon-coated...
nickel grids and floated on 20-μl drops of polyclonal Fe
protein antisera (1:10) for 1 h. Goat anti-rabbit IgG tagged
with 20-nm colloidal gold was the secondary antibody. Sections
were washed and stained for 15 min in 1% uranyl acetate.

By use of enlarged micrographs and a computer-based
imaging system (JAVA, Jandel, San Rafael, CA), cell area in the
electron micrograph of 10 cells for each treatment was deter-
mined, and the number of particles within each cell was
counted by eye. Elongated cells were selected to ensure
consistent cell orientation.

**RESULTS**

The plasmid pSA30 contains the nifHDK operon from *K.
pneumoniae* (23) that hybridize to nifHDK sequences from
everal all *N*-fixing bacteria examined to date (30, 31). Southern blot analysis confirmed that a 1.9-kb restriction
fragment containing nifH and partial nifD sequences from
pSA30 hybridized to a 6.3-kb EcoRI insert from pSA30 that
carries the nifHDK operon and the EcoRI insert from pRmR2,
which contains the nifHD genes from *Rhizobium meliloti* (21)
(data not shown). Northern blot analysis demonstrated that the
1.9-kb nifHD insert from pSA30 bound to RNA ~5 kb in
size from *V. natriegens* actively fixing nitrogen, but not to a
similarly sized RNA from the non-*N*-fixer *Vibrio harveyi* (data
not shown). As the nifHDK operon in *K. pneumoniae* is 4 kb (7),
the 1.9-kb nifHD probe undoubtedly hybridized to a nifHDK
mRNA in *V. natriegens*. The gene nifH expresses the Fe protein
subunit of nitrogenase (detected by Western blot analysis in the
present study), whereas nifD expresses the α subunit of the
nitrogenase Mo–Fe protein (7).

The doubling time of *V. natriegens* grown in N-replete FL
medium was ~1 h at 27°C, decreasing to 4–5 h when shifted
to an N-deficient UR-Fe medium (Fig. 1A). Both growth rates
were essentially identical to a previous study of *N* fixation in
*V. natriegens* that used the same media and temperatures (14).

The nifHDK mRNA appeared between 120 and 140 min
after the shift from aerobic N-replete FL medium to anaerobic
N-deficient UR-Fe medium, as indicated by binding of the
nifHD probe (Fig. 1B). After the cultures were shifted back to
an aerobic N-enriched medium, the nifHDK mRNA disappeared
within 40 min, but reappeared between 120 and 140
min after the culture was reshifted to an anaerobic N-deficient
condition (Fig. 1B).

Cultures of *V. natriegens* grown in aerobic N-enriched
UR-Fe medium displayed a doubling time of ~1 h, similar to
growth in aerobic FL medium (Figs. 2A, 3A, and 4A). When
cultures were shifted from N-enriched UR-Fe medium to
either anaerobic N-deficient or anaerobic N-deplete UR-Fe
medium, nifHDK mRNA appeared between 60 and 80 min
(Fig. 2B) and between 100 and 120 min (Fig. 3B) after the shift.

**FIG. 3.** Transcription and translation of the nif operon in *V.
natriegens*. (A) An inoculum from the aerobic N-enriched culture
(solid symbols) was transferred to the anaerobic N-deplete culture
(open symbols). Circles (solid or open) correspond to sampling times
in *B* and *C*. (B) Northern blot analysis and detection of polypeptides cross-
reacting with Fe protein antiserum. In *B* and *C*, the time course was set to 0 upon
shift to the anaerobic N-deplete condition.

![Translation of the nif operon and corresponding nitrogenase activity in V. natriegens.](image)
The steady-state level of the message declined between 6 and 9 h after the shift (Fig. 3B). Additionally, nifHDK mRNA declined to undetectable levels within 10 min after cultures growing anaerobically in a N-deficient medium were exposed to air (Fig. 2B).

Nitrogenase Fe protein antiserum resolved three polypeptides; all three were in the range of molecular masses (35–40 kDa) reported by others using nitrogenase antisera (5, 26), and all varied in time of appearance during the experiments. One polypeptide of ~35 kDa was absent until between 100 and 120 min after shifting to anaerobic conditions (Figs. 1C, 3C, and 4B). Levels of the 35-kDa polypeptide increased from 120 to 160 min and remained high for the period 6–15 h. The polypeptide was undetectable within 40 min of shifting back to an aerobic N-enriched medium, then reappeared 120 min after reshifting to an anaerobic N-deficient medium (Fig. 1C). Thus, the 35-kDa polypeptide was present only after induction of nifHDK mRNA, as would be expected because nifH encodes the Fe protein subunit of nitrogenase (35 kDa; see ref. 5).

Two additional polypeptides of ~37 and ~38 kDa cross-reacted with Fe protein antiserum between 100 min and 15 h after shifting from aerobic N-enriched to anaerobic N-deplete media (Figs. 1C and 3C; merged to form one band in Fig. 4B). During early postshift periods (0–100 min), however, the 37-kDa polypeptide was not detected, and the 38-kDa polypeptide either was not detected or was present at low levels. Both, however, were present at lower levels after N2-fixing cells were shifted from anaerobic to aerobic N-enriched conditions (Fig. 1C).

Nitrogenase activity appeared 100 min after the aerobic/anaerobic shift, corresponding to the first whole-cell immunological detection of Fe protein, as well as to detection of the protein (35-kDa polypeptide; Fig. 4B, see also Figs. 1C and 3C). Although equivalent and high levels of Fe protein were detected from 180 min to 15 h postshift, nitrogenase activity increased linearly to peak levels at 6–9 h, then declined twofold from 12 to 15 h (Fig. 4B). No nitrogenase activity was detected in any of the anaerobic controls (data not shown).

Immunofluorescence demonstrated presence of the Fe protein antiserum in 4 and 8 h V. natriegens cells, but not in 0 h V. natriegens, E. coli, or yeast cells (Fig. 5). Immunogold detection revealed significantly more gold particles within the 4-h [mean/μm2 (SEM) = 5.90 (0.58)] and 8-h [6.50 (0.51)] cells than within the 0-h cells [0.38 (0.06)] (one-way ANOVA, n = 10 for each cell type, df = 2, F = 56.411, P < 0.001; Tukey test, 0 h ≠ 4 h = 8 h; P < 0.001) (Fig. 6). Localization of gold particles within specific areas of the cytoplasm was not appar-

![Image](image_url)
ent. Particles also were associated with the cell membrane of both \( \text{N}_2 \)-fixing and non-\( \text{N}_2 \)-fixing cells, perhaps because of nonspecific binding of the polyclonal Fe protein antiserum to membrane-associated proteins and/or a contaminating antegen. \( \text{N}_2 \)-fixing cells accumulated intracellular refractive granules (low electron density areas 0.6–0.7 \( \mu \text{m} \) in diameter) and were smaller than non-\( \text{N}_2 \)-fixing cells [mean length and diameter and volume (\( \mu \text{m}^3 \), SEM): 0 h = 3.8 \( \times \) 1.6 \( \mu \text{m} \), (37.9, 0.10), 4 h = 2.8 \( \times \) 1.2 \( \mu \text{M} \) (16.9, 0.9), 8 h = 2.6 \( \times \) 1.2 \( \mu \text{M} \) (15.2, 1.0); \( n = 10 \) for each cell type; one-way ANOVA for volumes, df = 2, \( F = 110.74, P < 0.001; \) Tukey test, 0 h \( \neq \) 4 h = 8 h; \( P < 0.001 \)].

**DISCUSSION**

The use of both molecular probes to detect the relative levels of \( \text{nif} \) transcripts (mRNA) and antibodies to detect the presence of nitrogenase can provide a more detailed understanding of how \( \text{N}_2 \)-fixing bacteria respond to environmental changes and can resolve spatial and temporal dynamics of \( \text{N}_2 \) fixation in the sea. Such studies can also provide insights into the coupling and interactions of \( \text{N}_2 \) fixation with the biogeochemical cycles in the ocean.

\( \text{N}_2 \) fixation in heterotrophic bacteria can be regulated at several molecular levels. For example, transcriptional regulation may be modulated by specific environmental factors such as \( \text{O}_2 \) combined nitrogen, and/or the availability of a carbon source to support energetic requirements (7, 32–34). Posttranslational regulation may involve the inactivation of proteins by degradation or by modifications that influence the abundance and/or activity of nitrogenase (for review, see refs. 7 and 35). We demonstrate that both types of regulation occur in the heterotrophic, marine \( \text{N}_2 \)-fixing bacterium \( V. \ natriegens \). Whether this is a general feature of marine heterotrophic bacteria remains to be determined.

Transcriptional control of \( \text{N}_2 \)-fixation potential was suggested by the failure to detect \( \text{nifHDK} \) mRNA after 10 min of shifting cells from anaerobic to aerobic conditions (see Fig. 2B). However, as the relative abundance of the message also decreased between 6 and 9 h later under anaerobic conditions during mid-growth phase, factors other than anoxia may influence transcription.

Posttranslational control by degradation also was suggested, as a 35-kDa polypeptide cross-reacting with Fe protein antiserum was abundant in anaerobic conditions but undetectable within 40 min of shifting cells to aerobic conditions. The disappearance of Fe protein was independent of \( \text{nifHDK} \) mRNA levels (and transcriptional control) because prior to the shift, Fe protein increased when \( \text{nifHDK} \) mRNA levels decreased. Degradation or inactivation undoubtedly was due to the presence of \( \text{O}_2 \), which inactivates nitrogenase (7). Additionally, the response of the 35-kDa polypeptide to the anaerobic–aerobic–anaerobic shift indicates that it may be crucial to nitrogenase activity and serve as a more important marker for \( \text{N}_2 \)-fixation capacity than the 37- and 38-kDa polypeptides, both of which vary in a less predictable fashion. The 37- and 38-kDa polypeptides may correspond to some of the multiple bands reported by others for the Fe protein (5, 36).

The disappearance of the 35-kDa polypeptide within 40 min of exposure to aerobic conditions and its simultaneous reappearance with \( \text{nifHDK} \) mRNA between 120 and 140 min after shifting to anaerobic conditions suggests an inability to protect nitrogenase from \( \text{O}_2 \) inactivation and subsequent degradation. Consequently, new nitrogenase must be synthesized after each \( \text{O}_2 \) exposure. In contrast, other \( \text{N}_2 \)-fixing heterotrophic bacteria recover nitrogenase activity slowly (\( \text{Desulfovibrio} \) \( \text{gigas} \)) or rapidly (\( \text{Azotobacter} \)) after \( \text{O}_2 \) exposure and without protein synthesis (37, 38). Whether an inability to protect nitrogenase from \( \text{O}_2 \) degradation implies a primitive or recent development is not clear (39).

Cyclic synthesis of nitrogenase in a freshwater cyanobacterium (\( \text{Synechococcus} \)) required the continued presence of nitrogenase mRNA; when the mRNA was absent or transcription was inhibited, no nitrogenase activity was detected (40). In the present study, however, three polypeptides cross-reacting with Fe protein antiserum continued to be detected several hours after \( \text{nifHDK} \) mRNA was absent or below detectable levels. Furthermore, maximum nitrogenase activity was measured during the period when \( \text{nifHDK} \) mRNA declined to undetectable levels and significant nitrogenase activity (albeit twofold lower) remained for several hours after \( \text{nifHDK} \) mRNA was undetectable. Thus, the strong correlation between levels of nitrogenase mRNA and nitrogenase activity reported for cyanobacteria (40), was not observed in \( V. \ natriegens \).

The inability to detect \( \text{nif} \) mRNA after 9 h does not necessarily mean that transcription of \( \text{nif} \) genes ceased. The data do suggest, however, that \( V. \ natriegens \) produced a large amount of nitrogenase shortly after exposure to an anaerobic N-deplete environment, possibly by overexpressing \( \text{nifHDK} \) mRNA (which became diluted on a per cell basis as the cells achieved balanced growth); synthesizing many polypeptides per message, resulting in detectable amounts of protein being present before detectable amounts of message are detected.

**FIG. 6.** Immunogold localization of Fe protein in \( V. \ natriegens \). Cells from an aerobic N-replete culture (0 h) \( (A) \) and 4 h \( (B) \) and 8 h \( (C) \) after shifting to anaerobic N-deplete conditions. R, refractive granules. (\( \times \)10,400.)
(Fe protein was detected slightly before nilHDK mRNA); and/or producing nitrogenase that was very stable under anaerobic conditions.

The presence of Fe protein and nitrogenase activity when nilHDK mRNA was undetectable suggests that nitrogenase is a relatively stable protein and can be "stored" under anaerobic conditions. Consequently, *V. natriegens* may retain N\(_2\)-fixing capacity over a broad temporal scale, and this retention may convey an ecological advantage when levels of combined nitrogen oscillate under anaerobic conditions. The presence of a relatively stable nitrogenase throughout anaerobic periods when levels of combined nitrogen are high may provide cells with the capacity to rapidly fix N\(_2\) if combined nitrogen levels suddenly decline. Indeed, nitrogenase is not inhibited by ammonium in *vitro*, but ammonium can result in a rapid, but reversible, inhibition of nitrogenase activity in some diazotrophs (7, 41).

Immunogold detection revealed nitrogenase throughout the cytoplasm of *V. natriegens* cells actively fixing N\(_2\), rather than sequestered in specific locations. N\(_2\)-fixing cells also contained intracellular refractive granules. Similar granules have been noted in cells from aerobic cultures of *V. natriegens* that were carbon-replete, but nitrogen and/or phosphorous-starved, and are believed to contain the storage product poly-~H~2~O~ hydroxy~butyrate (12, 42, 43). Carbon reserves may be formed under high C/P ratios (43); similarly, high C/N ratios may trigger formation of carbon reserves in N\(_2\)-fixing cells.

The present study has demonstrated the utility of molecular probes to detect the presence and temporal dynamics of N\(_2\)-fixation capacity in heterotrophic marine bacteria. Refinement and adaptation of these approaches and techniques to natural bacterial assemblages should advance our understanding of the ecological role of heterotrophic N\(_2\) fixation in marine ecosystems.

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