The ability to reduce atmospheric nitrogen (N$_2$) to ammonia, known as N$_2$ fixation, is a widely distributed trait among prokaryotes that accounts for an essential input of new N to a multitude of environments. Nitrogenase reductase gene (nifH) composition suggests that putative N$_2$-fixing heterotrophic organisms are widespread in marine bacterioplankton, but their autecology and ecological significance are unknown. Here, we report genomic and ecophysiology data in relation to N$_2$ fixation by three environmentally relevant heterotrophic bacteria isolated from Baltic Sea surface water: Pseudomonas stutzeri strain BAL361 and Raoultella ornithinolytica strain BAL286, which are gammaproteobacteria, and Rhodopseudomonas palustris strain BAL398, an alphaproteobacterium. Genome sequencing revealed that all were metabolically versatile and that the gene clusters encoding the N$_2$ fixation complex varied in length and complexity between isolates. All three isolates could sustain growth by N$_2$ fixation in the absence of reactive N, and this fixation was stimulated by low concentrations of oxygen in all three organisms (≈4 to 40 μmol O$_2$ liter$^{-1}$). P. stutzeri BAL361 did, however, fix N at up to 165 μmol O$_2$ liter$^{-1}$, presumably accommodated through aggregate formation. Glucose stimulated N$_2$ fixation in general, and reactive N repressed N$_2$ fixation, except that ammonium (NH$_4^+$) stimulated N$_2$ fixation in R. palustris BAL398, indicating the use of nitrogenase as an electron sink. The lack of correlations between nitrogenase reductase gene expression and ethylene (C$_2$H$_4$) production indicated tight posttranscriptional-level control. The N$_2$ fixation rates obtained suggested that, given the right conditions, these heterotrophic diazotrophs could contribute significantly to in situ rates.

The biological process of importing atmospheric N$_2$ is of paramount importance in terrestrial and aquatic ecosystems. In the oceans, a diverse array of prokaryotes seemingly carry the genetic capacity to perform this process, but lack of knowledge about their autecology and the factors that constrain their N$_2$ fixation hamper an understanding of their ecological importance in marine waters. The present study documents a high variability of genomic and ecophysiological properties related to N$_2$ fixation in three heterotrophic isolates obtained from estuarine surface waters and shows that these organisms fix N$_2$ under a surprisingly broad range of conditions and at significant rates. The observed intrinsic regulation of N$_2$ fixation for the isolates indicates that indigenous populations of heterotrophic diazotrophs have discrete strategies to cope with environmental controls of N$_2$ fixation. Hence, community-level generalizations about the regulation of N$_2$ fixation in marine heterotrophic bacterioplankton may be problematic.
trophic organisms to marine N₂ fixation, cell-specific rates of N₂ fixation should be connected to the identities of the responsible cells, either directly, e.g., by nanoscale secondary ion mass spectrometry in combination with fluorescence in situ hybridization (11), or alternatively, by indirect estimates based on cell-specific rates determined in vitro in combination with in situ abundance measures. For instance, using cell-specific rates from the soil-derived *Pseudomonas stutzeri* strain CMT.9.A, it was recently reported that the abundance of a predominant gammaproteobacterial phylotype in the eastern tropical south Pacific Ocean could not account for local N₂ fixation (12, 13). Such calculations are, however, potentially compromised by the lack of cell-specific N₂ fixation rates of marine heterotrophic diazotrophs, which in turn is caused by a scarcity of cultivated representative species. Attempts to cultivate heterotrophic diazotrophic bacteria from marine environments have generally been few and had varying degrees of success (14–17). Recently, alpha- and gammaproteobacterial representative diazotrophs have been cultivated from pelagic waters of the estuarine Baltic Sea (18–20), but further ecophysiological and genomic investigations elucidating the regulation of N₂ fixation in relation to environmentally relevant stimuli have not yet been undertaken.

A multitude of constraints presumably regulate N₂ fixation by heterotrophic bacteria in pelagic marine systems. The reduction of N₂ to NH₄⁺ is expensive in terms of reducing power and cellular energy, requiring at least 16 mol of ATP to reduce 1 mol of N₂ and, hence, demanding considerable amounts of organic carbon (C) compounds. In addition, the nitrogenase enzyme complex is usually highly sensitive towards O₂, and N₂ fixation is therefore supposedly tightly regulated by the presence of O₂, as well as reactive N (21). Despite these high energy requirements and stringent control mechanisms, *nifH* gene transcripts affiliated with heterotrophic diazotrophs have been detected in low-carbon, oxic, oligotrophic surface waters (7, 22), as well as in N-replete environments (8, 23), emphasizing that the extent and regulation of heterotrophic N₂ fixation are currently poorly understood.

In this study, we report the comparative investigation of the genomic traits relating to N₂ fixation in three heterotrophic bacterial isolates from Baltic Sea surface waters: *Pseudomonas stutzeri* strain BAL361, *Rhodopseudomonas palustris* strain BAL398, and *Raoultella ornithinolytica* strain BAL286. Two of the three isolates (*P. stutzeri* BAL361 and *R. palustris* BAL398) have been documented to occur at densities similar to those of other dominant diazotrophic groups (20), and close relatives of all three appear to be widely distributed in marine waters. *Pseudomonas*-like gammaproteobacterial *nifH* sequences are prevalent and often dominating in sequence data sets from a multitude of marine environments, including estuarine waters and the Pacific, Atlantic, and Indian Oceans (13, 24). Alphaproteobacterial diazotrophs of the order *Rhizobiales*, including the genus *Rhodopseudomonas*, are integral parts of the diazotrophic communities not only in the Baltic Sea (20, 24) but also in the Mediterranean Sea (25), South China Sea (26), and the Atlantic and Indian Oceans (13). The third isolate (*R. ornithinolytica* BAL286) represents the *Enterobacteriaceae*. This family has been found to dominate *nifH* gene sequence libraries from temperate estuarine surface waters in the summer season (24), and diazotrophic members of the genus *Klebsiella* have been recovered from estuarine eel grass root-associated communities (27). Thus, the three bacterial isolates collectively represent a subset of widely distributed and potentially important heterotrophic diazotrophs. Furthermore, we report data on nitrogenase activity in these bacteria as a function of different concentrations of O₂, dissolved inorganic N (NH₄⁺ and nitrate [NO₃⁻]), and dissolved organic C. Lastly, we evaluate the applicability of relative *nifH* transcript abundances as a proxy for N₂ fixation in the isolates examined.

**RESULTS AND DISCUSSION**

**Genomic traits.** The genomes of *P. stutzeri* BAL361, *R. palustris* BAL398, and *R. ornithinolytica* BAL286 were assembled into 398, 906, and 816 contigs, respectively. Based on these contigs, *P. stutzeri* BAL361 has a genome of 4.88 Mb comprising 4,514 coding sequences (CDSs), whereas *R. palustris* BAL398, and *R. ornithinolytica* BAL286 both have 6.13-Mb genomes comprising 5,860 and 5,720 CDSs, respectively (Table 1).

Consistent with the literature on the well-described *P. stutzeri* and *R. palustris* species (28–30), the genome sequences of *P. stutzeri* BAL361 and *R. palustris* BAL398 showed evidence of high metabolic versatility, including, for instance, metabolic subsystems related to diverse carbohydrate and fatty acid metabolisms, as well as RuBisCO-catalyzed C fixation and phototrophy in *R. palustris* BAL398. In contrast, *R. ornithinolytica* is not very well described, but it too seemed to be versatile, harboring genes relating to a multitude of catabolic subsystems, including monoo-, di-, oligo-, and polysaccharide utilization and fermentation. All three isolates seem to have allocated a substantial part of their genomes to aromatic hydrocarbon metabolism, with 49, 48, and 84 genomic features relating to this metabolic subsystem in *P. stutzeri* BAL361, *R. palustris* BAL398, and *R. ornithinolytica* BAL286, respectively (Table 1). Both *P. stutzeri* (see reference 28 and references therein) and *R. palustris* (31) can use aromatic compounds as sole C sources. Little is known about the aromatic hydrocarbon metabolism of *R. ornithinolytica*, but it has been isolated from oil-contaminated soil (32) and can grow with benzoic acid as the sole C source (33). We can only speculate on how this relates to the ecology of these organisms and whether it influences their role as diazotrophs, but we note that the genetic capacity to degrade aromatic compounds is widespread in Baltic Sea bacterioplankton (34) and that aromatic compounds may be particularly prevalent in waters influenced by river outflow (35), like the Baltic Sea.

The number of N metabolism-related features also suggests a high degree of versatility in this metabolic subsystem in all three isolates compared to the nondiazotrophic reference strains *Can- didatus Pelagibacter* sp. strain IMC9063 and *Flavicola taffensis* DSM16823 (Table 1). In addition to N₂ fixation and ammonium assimilation, all three isolates seemed to be able to respire NO₃⁻ through ammonification, as well as denitrification. If these genetic subsystems are used in situ, these organisms can conceivably switch between being a source and a sink of N depending on the growth conditions. Taken together, the diverse potential metabolic strategies observed among the isolates indicate that high metabolic flexibility is a key trait in heterotrophic, N₂-fixing bacteria inhabiting Baltic Sea surface waters.

Nitrogenase reductase gene (*nifH*) sequences were obtained from *P. stutzeri* BAL361 and *R. palustris* BAL398 in connection with their isolation (20). Upon the isolation of *R. ornithinolytica* BAL286, an iron-only (FeFe) nitrogenase-associated reductase gene (*anfH*) sequence, similar to those known from *Azotobacter vinelandii* and *Azomonas macrozygotes* (18), was recovered, but not a conventional molybdenum-iron (FeMo) nitrogenase-
associated reductase gene (nifH) sequence. In congruence with the observation that alternative nitrogenases seem to complement the FeMo nitrogenase when Mo is depleted (36), rather than being autonomously regulated, a FeMo nitrogenase-associated reductase-encoding gene was recovered from the genome sequence of *R. ornithinolytica* BAL286. It was related to the nifH gene from *Klebsiella pneumoniae* (92% nucleotide sequence similarity; GenBank accession number V00631).

*R. palustris* has previously been reported to harbor alternative nitrogenases (29), but this was not the case for *R. palustris* BAL398, confirming a previous PCR-based analysis of this strain (20).

The nifH and anfH gene sequences suggested that the isolates represented the alphaproteobacterial and gammaproteobacterial parts of the canonical nifH cluster I (37), as well as the archaean and FeFe nitrogenase-containing cluster II (Fig. 1).

The genetic information related to N₂ fixation was clustered in distinct regions in all three genomes. These regions contained genes encoding the nitrogenase complexes, as well as genes involved in FeMo cofactor synthesis and transcriptional regulation. In *P. stutzeri* BAL361, this region consisted of 49.3 kb encoding 61 genes (Fig. 2A). This region had a GC content considerably higher than that of the rest of the genome (66.2% versus 62.6%), supporting the notion that the N₂ fixation region in *P. stutzeri* is part of a genomic island acquired by horizontal gene transfer (HGT) (38). Several CDSs were interspersed between the genes identified as being part of the N₂ fixation subsystem, contributing to the rather large size of the N₂ fixation region in this genome. Despite their unknown function, these CDSs seem to be conserved among organisms fixing N₂ under microaerobic and aerobic conditions, and their transcription is increased under conditions favoring N₂ fixation, suggesting that they do play a role in aerobic N₂ fixation (38).

The N₂ fixation region of *R. palustris* BAL398 was in general similar to the composition of the FeMo N₂ fixation cluster of *R. palustris* strain CGA009 (29), comprising 27 CDSs within a single 22.0-kb region and lacking the negative transcriptional regulator-encoding gene, nifL (Fig. 2B). And yet, no genes related to alternative nitrogenase complexes were observed, supporting the idea that these may have been acquired recently by HGT in *R. palustris* CGA009 (39) or lost secondarily in *R. palustris* BAL398.

Despite containing the genetic information of both the FeMo and the FeFe nitrogenase complexes, the N₂ fixation region of *R. ornithinolytica* BAL286 constituted only 28.8 kb and comprised

| Genomic feature | P. stutzeri BAL361 | R. palustris BAL398 | R. ornithinolytica BAL286 | “Candidatus Pelagibacter“ sp. IMC9063 | F. taffensis DSM16823 |
|-----------------|-------------------|-------------------|-------------------|---------------------------------|-------------------|
| Genome size (Mb) | 4.88              | 6.13              | 6.13              | 1.28                            | 4.63              |
| No. of coding sequences | 4514             | 5860              | 5720              | 1439                            | 4132              |
| % GC content     | 63                | 65                | 56                | 30                              | 37                |
| No. of RNA genes | 57                | 49                | 85                | 35                              | 44                |
| No. of mobile genetic elements | 4               | 9                 | 16                | 6                               |                   |
| Nitrogen metabolism subsystem (no. of defined features) | | | | | |
| Nitrogen fixation | 22                | 22                | 22                | 22                              | 22                |
| Denitrification | 22                | 12                | 9                 | 9                               | 9                 |
| Nitrate and nitrite ammonification | 26               | 9                 | 20                | 20                              | 20                |
| Ammonia assimilation | 13               | 19                | 12                | 12                              | 6                 |
| Dissimilatory nitrite reduction | 13               | 2                 | 6                 | 2                               | 2                 |
| Nitroreductive stress | 5                | 2                 | 6                 | 2                               | 2                 |
| Cyanate hydrolysis | 4                 | 8                 |                   |                   |                   |
| Total | 105               | 72                | 69                | 6                               | 2                 |
29 CDSs. It was hence the most condensed N₂ fixation region of the three (Fig. 2C). The part of the region comprising the FeMo nitrogenase complex was similar to that of *Klebsiella pneumoniae* strain M5a1 (40), but following the *nifQ* gene, the FeFe complex was encoded in the opposite transcription orientation. Bacteriophage-associated sequences were found just upstream from the FeFe nitrogenase gene cluster, and a distinct change in GC content occurred at the intersection between the FeMo and FeFe clusters (Fig. 2C). It seems, therefore, likely that the alternative nitrogenase complex of *R. ornithinolytica* BAL286 has been acquired horizontally, as in *P. stutzeri* BAL361, in this case by bacteriophage-mediated transfer.

It is generally believed that the congruence between *nifH* gene phylogeny and 16S rRNA gene phylogeny is an indication that HGT of entire nitrogenase gene clusters is a rare event (3). Transfer events within phylogenetic groupings would, however, not affect phylogenetic congruence and it seems to have happened in *Pseudomonas stutzeri*, as well as in other diazotrophs (39, 41, 42). *R. palustris* CGA009 harbors alternative nitrogenase gene clusters, which seem to have been acquired through HGT (39). This indication of an HGT event is consistent with the fact that our *R. palustris* BAL398 strain does not harbor any alternative nitrogenase gene clusters. In combination with our deduction that *R. ornithinolytica* BAL286 has acquired an FeFe nitrogenase system horizontally, we speculate that alternative, accessory nitrogenase systems may be particularly prone to HGT.

Collectively, our results show that the genetic regions comprising the *nifH* gene phylogeny and 16S rRNA gene phylogeny is an indication that HGT of entire nitrogenase gene clusters is a rare event (3). Transfer events within phylogenetic groupings would, however, not affect phylogenetic congruence and it seems to have happened in *Pseudomonas stutzeri*, as well as in other diazotrophs (39, 41, 42). *R. palustris* CGA009 harbors alternative nitrogenase gene clusters, which seem to have been acquired through HGT (39). This indication of an HGT event is consistent with the fact that our *R. palustris* BAL398 strain does not harbor any alternative nitrogenase gene clusters. In combination with our deduction that *R. ornithinolytica* BAL286 has acquired an FeFe nitrogenase system horizontally, we speculate that alternative, accessory nitrogenase systems may be particularly prone to HGT.

**Growth kinetics, N₂ fixation potential, and regulation.** To ensure that C₂H₄ production in the acetylene reduction assays (ARAs) was measured on actively growing cultures, the growth of the three isolates was monitored over a 20-day period. In ARAs, the acetylene was added after 14 days, when all three isolates were still actively growing (Fig. 3). Growth under standard conditions,
i.e., in carbonate-buffered microoxic diazotroph medium devoid of reactive N and containing $37 \pm 4 \mu$mol O$_2$ liter$^{-1}$ and 20 mmol glucose liter$^{-1}$, yielded rather slow growth with generation times of 15, 4, and 30 days for $P$. stutzeri BAL361, $R$. palustris BAL398, and $R$. ornithinolytica BAL286, respectively. These rates are much lower than growth by marine bacterioplankton, which generally exhibit generation times of 9 to 18 h (43).

Growth in parallel cultures supplemented with dissolved inorganic N (60 $\mu$mol NO$_3^-$ or NH$_4^+$ liter$^{-1}$) was also monitored. Cell counts showed that the addition of NO$_3^-$ and NH$_4^+$ decreased the generation times to 3 and 19 h for $P$. stutzeri BAL361, 9 and 22 h for $R$. palustris BAL398, and 22 and 19 h for $R$. ornithinolytica BAL286, respectively. This suggested that the slow growth in the cultures devoid of dissolved inorganic N was due to N limitation.

When fixing N$_2$ under oxic conditions, aerobic and facultative aerobic microorganisms are faced with the problem of synthesizing ATP from oxidative phosphorylation while at the same time protecting the nitrogenase complex from O$_2$ inhibition. Azotobacter vinelandii can fix N$_2$ under aerobic conditions, and soil-derived $P$. stutzeri strains are known to fix N$_2$ microaerobically (44). The genomic features of the nitrogenase gene cluster of $P$. stutzeri BAL361 seem to support the idea that this organism is capable of microaerophilic or aerobic N$_2$ fixation as well (38). Indeed, the growth of this isolate was stimulated by an increased O$_2$ concentration at the time of inoculation, with a generation
time of 3.6 days compared to 15 days under low initial O₂ concentrations (Fig. 3A versus 4A). ARA showed that ethylene (C₂H₄) was produced at 4 to 165 μmol O₂ liter⁻¹, with the highest cell-specific C₂H₄ production rates, 0.0034 fmol C₂H₄ cell⁻¹ h⁻¹, observed at 165 μmol O₂ liter⁻¹ (Fig. 4D). Interestingly, *P. stutzeri* BAL361 formed aggregates 1 to 4 mm in diameter when grown under oxic conditions, as indicated by the resazurin dye signal (see Fig. S1 in the supplemental material), suggesting that these bacteria excrete extracellular polymeric substances in order to control O₂ diffusion and facilitate N₂ fixation in an oxic environment. While this is consistent with a previous observation (19), the frequency of this trait in indigenous bacterioplankton is unknown.

*R. palustris* BAL398 and *R. ornithinolytica* BAL286 showed limited or no growth at approximately 200 μmol O₂ liter⁻¹ (Fig. 4B and C); however, low-O₂ conditions stimulated N₂ fixation in these isolates (Fig. 4D). C₂H₄ production was low (~0.001 fmol C₂H₄ cell⁻¹ h⁻¹) in the 0 to 5 μmol O₂ liter⁻¹ range but increased at 38 μmol O₂ liter⁻¹ to 0.0067 fmol C₂H₄ cell⁻¹ h⁻¹ in *R. palustris* BAL398. The facultative anaerobic *R. ornithinolytica* BAL286 displayed the same overall pattern, reaching a peak C₂H₄ production...
rate of 0.0058 fmol C₂H₄ cell⁻¹ h⁻¹ in the presence of 14 μmol O₂ liter⁻¹, supporting observations that N₂ fixation in enterobacterial diazotrophs is stimulated by the presence of low concentrations of O₂ (45, 46). Hence, these diazotrophs are likely faced with the challenge of balancing the detrimental effects of O₂ on the nitrogenase complex with the need to produce sufficient energy to fuel it.

Increasing C concentrations were found to stimulate N₂ fixation in P. stutzeri BAL361. C₂H₄ production increased steadily from <0.001 fmol C₂H₄ cell⁻¹ h⁻¹ in cultures supplemented with 0.020 μmol glucose liter⁻¹ to 0.089 fmol C₂H₄ cell⁻¹ h⁻¹ in cultures with 2,000 μmol glucose liter⁻¹ (Fig. 5). A decrease in C₂H₄ production at 20,000 μmol glucose liter⁻¹ coincided with a change in the resazurin dye signal, indicating that the increased respiration had reduced the O₂ concentration to a level impairing N₂ fixation. Hence, interactions between factors controlling N₂ fixation may complicate the interpretations made from culture-based estimates of N₂ fixation. C₂H₄ production was only observed at the highest C concentration (20,000 μmol glucose liter⁻¹) for R. palustris BAL398. Here, cell-specific C₂H₄ production rates reached 0.0044 fmol C₂H₄ cell⁻¹ h⁻¹, suggesting that this organism required more energy to sustain N₂ fixation. R. palustris is known to produce substantial amounts of H₂ when fixing N₂ (29, 47), implying a significant waste of ATP (45). This could potentially be overcome by this photoheterotroph by acquiring energy from anoxygenic photosynthesis, as suggested for Rhodopseudomonas capsulata (48), but we chose not to include light as a factor in these assays to reduce complexity. R. ornithinolytica BAL286 did increase C₂H₄ production in response to increasing glucose concentrations, and yet the cell-specific rates dropped, suggesting that other factors controlling N₂ fixation interfered in these treatments.

Due to the high energy consumption by the N₂ fixation reaction, it is generally believed that N₂ fixation shuts down in response to available reactive N. All three isolates decreased N₂ fixation in response to increasing NO₃⁻ concentrations, with the C₂H₄ production rates dropping 1 to 2 orders of magnitude (Fig. 6A). N₂ fixation decreased significantly in response to increasing NH₄⁺ concentrations in P. stutzeri BAL361 and R. ornithinolytica BAL286 cultures as well, but in R. palustris BAL398 cultures, N₂ fixation increased dramatically upon the addition of 5 μmol NH₄⁺ liter⁻¹ or more (Fig. 6B), reaching the highest cell-specific C₂H₄ production rates measured in any of the ARAs (0.515 fmol C₂H₄ cell⁻¹ h⁻¹). The cell-specific C₂H₄ production rates were not significantly different between P. stutzeri BAL361 and R. ornithinolytica BAL286 in the presence of NH₄⁺ (P = 0.90), whereas the C₂H₄ production rates were significantly higher in R. palustris BAL398 cultures (P = 0.01). We therefore speculate that the nitrogenase complex has a function in addition to N acquisition in R. palustris BAL398. In fact, it was recently proposed...
that *R. palustris* may use CO₂ and N₂ fixation to maintain a balanced redox state when growing on lactate, which has the same oxidation/reduction value as glucose, which was used in this study (49). Hence, *R. palustris* BAL398 likely uses N₂ fixation as an electron sink when grown at suboxic conditions on glucose and NH₄⁺. This falls in line with the many spurious findings of marine N₂ fixation at NH₄⁺ concentrations of up to 200 μmol liter⁻¹ (8, 50) and highlights that regulation of N₂ fixation by reactive N is presently far from understood.

To investigate whether nitrogenase activity was regulated on the transcriptional level, cell-specific *nifH* and *anfH* mRNA transcript abundances were determined by extracting RNA from the same cultures as those used in the ARAs at the point of C₂H₄ quantification and subsequently running reverse transcriptase quantitative PCRs (RT-qPCRs). The transcript abundances were then normalized to the flow cytometry-determined cell numbers. It was, however, not possible to identify any clear patterns in transcript abundances, and the correlations between cell-specific nitrogenase reductase gene transcript abundances and cell-specific C₂H₄ production rates were never significant (Spearman’s rank-order correlation test, α = 0.05) (see Table S1 in the supplemental material). Hence, posttranscriptional regulation is likely important here. It has previously been reported that the nitrogenase enzyme complex of *Rhodospirillum rubrum*, among others, is subject to posttranscriptional modifications that stringently regulate enzyme activity by covalent attachment of ADP-ribose moieties to the Fe protein (51–53). This reversible ADP-ribosylation inhibits the association of the Fe protein with the MoFe protein and acts as a second layer of control on the energy-consuming reduction process. Hence, *nifH* and/or *anfH* transcript numbers are likely not good proxies for N₂ fixation under the circumstances tested here.

Furthermore, normalization to the transcripts of housekeeping genes may give a better indication of the relative levels of transcription of *nifH* and *anfH* genes, as flow cytometry data also include inactive cells and cells containing multiple chromosomes.

With some exceptions (*R. palustris* BAL398 in the presence of NH₄⁺, N₂ fixation was highest in cultures where O₂ concentrations were reduced (5 to 165 μmol liter⁻¹), C concentrations were high (2,000 to 20,000 μmol liter⁻¹), and reactive N was absent. Converting C₂H₄ production to N₂ fixation in these cultures using the theoretical conversion factor of 3 (54), the heterotrophic isolates fixed N₂ at rates of 0.0007 to 0.03 fmol N cell⁻¹ h⁻¹. These numbers are similar to but generally higher than the rates used in previous estimates of the contribution by heterotrophic diazotrophs to N₂ fixation in *in situ* (13). *R. palustris* BAL398 and a *Pseudomonas*-like isolate similar to *P. stutzeri* BAL361 have previously been quantified at abundances of up to 4.7 × 10⁴ and 7.9 × 10⁴ cells liter⁻¹, respectively (20). Applying the measured rates, cells at these densities could contribute with N₂ fixation rates of 0.790 to 56.9 pmol N liter⁻¹ day⁻¹.

Converting the C₂H₄ production rates obtained from *R. palustris* BAL398 cultures subjected to NH₄⁺ additions to N₂ fixation rates as described above, significantly higher N₂ fixation rates are obtained (0.17 fmol N cell⁻¹ h⁻¹). Hence, under conditions low in O₂ and replete in C and NH₄⁺, *P. stutzeri* BAL361 and *P. stutzeri* KC140355) and *R. palustris* BAL398 (56°37′N, 18°14′E) in March 2009 (20). *R. norisihlolytica* BAL286 (56°37′N, 18°14′E) in April 2005 using semisoloid diazotroph medium (18).

**Genome sequencing and comparisons.** The isolates were grown in ZoBell broth (60) to an optical density of 0.5 to 1.0 and harvested, and genomic DNA was extracted (EZNA tissue DNA kit; Omega Bio-Tek, Norcross, GA, USA). DNA from *P. stutzeri* BAL361 and *R. palustris*
BAL398 was sheared using a Bioruptor (Diagenode, Liege, Belgium), and a paired-end (PE) sequencing library (~450-bp inserts) for Illumina was constructed as previously described (61) using short indexing primers (62). To generate the 100-nucleotide (nt) PE data, approximately 1/10 of one lane on an Illumina HiSeq 2000 was run. Sequences were assembled using Velvet de novo assembler version 1.2.08 with scaffolding switched off and a k-mer of 47. For R. ornithinolytica BAL286, a sequencing library was built using the Nextera XT DNA kit (Illumina, San Diego, CA) with 1 ng input DNA. This library was sequenced on the Illumina MiSeq platform as a 2 × 250-bp paired-end reads. This genome sequence was assembled using CLC Genomic Workbench 6.0.4 (CLC bio, Aarhus, Denmark) following standard quality trimming and adapter removal using the same software package. For P. stutzeri BAL361 and R. palustris BAL398, the N₂ fixation regions were reassembled and mapped against known reference genomes using the CLC platform. Genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and the online Rapid Annotation using Subsystem Technology (RAST) resource version 2.0 (63). The annotations of the N₂ fixation regions were revised manually using Artemis (64). The genomic features of the three sequenced isolates were compared to the genomes of two members of prevalent marine bacterial groups, i.e., (i) “Candidatus Pelagibacter” sp. IMC9063 (GenBank accession number CP002511), a SAR11 clade member related to prevalent phytophores recovered from Baltic Sea surface waters (65) and also exhibiting 99% 16S rRNA gene similarity to a sequence recovered from Baltic Sea surface water throughout 2003 (GenBank accession number DQ270281) (66), and (ii) the genome of Flavicola tassifera DSM16823 (GenBank accession number NC_015321), a member of the Bacteroidetes phylum exhibiting 92% 16S rRNA gene similarity to a sequence recovered from Baltic Sea surface waters during the summer and early autumn of 2003 (GenBank accession number DQ270281) (66).

Medium preparation and growth kinetics. To investigate the N₂ fixation potential of the isolates, various growth media were tested, including the liquid N-free medium previously applied to grow R. ornithinolytica BAL286 (18), diazotroph medium RBA (DSMZ), and the carbonate-buffered microoxic diazotroph medium used to isolate P. stutzeri BAL361 and R. palustris BAL398 (20). Only the latter medium supported the growth of all three isolates and was therefore used in the subsequent ecophysiological analyses.

The growth kinetics of the isolates were examined to ensure that acetylene reduction was assessed for growing cells. First, aliquots (25 ml) of medium were distributed into 50-ml borosilicate serum vials, which were capped with butyl rubber stoppers and crimp sealed. To ensure a low O₂ concentration (37 % O₂, 5 % CO₂, and 5 % H₂) and the liquid was allowed to equilibrate with the modified atmosphere for 2 h before the O₂ concentration was verified using a FireSting O₂ optical O₂ meter equipped with an OX50 fiber-optic O₂ sensor (Pyroscience, Aachen, Germany). The medium was then supplemented with a 0.2 µM-filtered glucose solution (20 mM liter⁻¹ final concentration). Second, for each isolate, cells were harvested from 2 ml of actively growing ZoBell cultures (4,000 × g for 5 min), washed twice in 1 ml phosphate-buffered saline (PBS), and suspended in 2 ml of the carbonate-buffered microoxic diazotroph medium. Serum vials containing 25 ml medium were then inoculated with 200 µl cell suspension and incubated for approximately 20 days in the dark at room temperature with shaking at 150 rpm. Third, growth in the vials was monitored by flow cytometry as described previously (67) using a FACSCanto II flow cytometer (BD Biosciences, NJ, United States).

In order to investigate the effects of lower glucose concentrations and higher O₂ concentrations on the bacterial growth, parallel incubations containing 0.2 µM glucose liter⁻¹ or ~200 µM O₂ liter⁻¹ were included. The effect of reactive N on the growth of the bacteria was examined by adding 60 µM NH₄Cl or NO₃⁻ liter⁻¹ (final concentration) to a subset of incubations. This was added on day 12 as in the acetylene reduction assay (see below).

Acetylene reduction assay. Nitrogenase activity was assessed using the acetylene reduction assay (68). Serum vials containing 25 ml carbonate-buffered microoxic diazotroph medium were inoculated and incubated as described above. After 14 days, the pressure was equilibrated and 10% of the headspace was replaced with laboratory-grade acetylene gas (C₂H₂; Air Liquide, Taastrup, Denmark). Incubations continued for 24 h after the C₂H₂ produced was quantified using a flame ionization detector (FID)-equipped CP9000 gas chromatograph (Chrompack, Bergen op Zoom, Netherlands). The base medium for these measurements contained 20 mmol glucose liter⁻¹ and 37 ± 4 µmol O₂ liter⁻¹, and was free of reactive N. To examine the regulation of N₂ fixation by different concentrations of C, O₂, and reactive N, one of these parameters was changed while the other two were kept constant. The effect of O₂ on N₂ fixation was investigated by regulating the O₂ concentrations of the base medium by adding 2, 4, or 8 ml of pure O₂ gas or by adding ferrous sulfate (FeSO₄) and dithiothreitol (DTT) in equivalent amounts (0.40, 0.60, 1.0, or 1.6 mmol liter⁻¹, final concentration of each compound) to triplicate vials. This produced O₂ concentrations in the medium ranging from 0 to 240 µmol O₂ liter⁻¹ at the time of C₂H₂ quantification. The effect of C was examined using base medium made with high-performance liquid chromatography (HPLC)-grade water (Sigma) instead of MilliQ. Triplicate vials contained one of eight concentrations increasing from 0.002 to 20,000 µmol glucose liter⁻¹ with log factor increments. Similarly, the effect of reactive N was examined using the following eight concentrations of either NO₃⁻ or NH₄Cl: 0, 0.1, 0.5, 1, 5, 20, 60, or 150 µmol liter⁻¹. The reactive N was added to triplicate vials on day 12 to ensure that the cultures did not reach stationary phase at the time of acetylene addition (day 14).

Nitrogenase reductase gene expression analyses. To couple nitrogenase activity to nitrogenase reductase gene expression, cell-specific nifH and anfH RNA transcript numbers in the different treatments were determined. Following C₂H₂ production measurements, 2 ml of culture from one of each of the different treatments was centrifuged at 8,000 × g for 5 min. The pellet was dissolved in 200 µl RNAalater (Ambion; Life Technologies) and kept at −80°C until extraction. RNA was extracted using the RNeasy minikit (Qiagen) with an additional DNase treatment applied after elution. Complete digestion of RNA was verified by PCR. cDNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems) and the reverse primer nifH3 (4). Primers were designed to target the nifH genes of the three isolates and the anfH gene of R. ornithinolytica BAL286 using Primer3 (version 0.4.0, online resource) and were checked for hairpins and dimers using NetPrimer (Premier Biosoft). Twenty-microliter qPCR mixtures were made containing 1X SYBR Select master mix (Life Technologies Europe BV), 300 nM of each primer, RT-PCR-grade water, and 2 µl template. The reactions were run on an Agilent Mx3005P qPCR thermal cycler using the following temperature settings: 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a melt curve analysis to check for unspecific PCR products. No PCR products other than the ones for the target genes were detected. Lastly, cell-specific gene expression was calculated based on the flow cytometry data obtained from the corresponding cultures.

Accession numbers. The genome sequences were deposited as whole-genome shotgun projects at GenBank under accession numbers JXXK00000000 (Pseudomonas stutzeri BAL361), JXXE00000000 (Rhodo-pseudomonas palustris BAL398), and JXFF00000000 (Raoella ornitholytica BAL286).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mbio.00929-15/-/DSSupplemental.
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