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Genomics and Ecophysiology of Heterotrophic Nitrogen-Fixing Bacteria Isolated from Estuarine Surface Water

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ABSTRACT 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_2H_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.

IMPORTANCE 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 intricate 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.

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The conversion of inert N_2 gas to ammonium (NH_4^+) by microorganisms, known as N_2 fixation, is fundamental for biological productivity in many environments. In the tropical and subtropical oligotrophic oceans and in eutrophic freshwater lakes, pelagic N_2 fixation may supply enough N to sustain a considerable fraction of the new production (1, 2). In these environments, cyanobacteria are generally considered the main N_2-fixing organisms (diazotrophs), yet the genetic potential to fix atmospheric N_2 is distributed among a diverse array of prokaryotes, including members of the *Proteobacteria*, *Firmicutes*, and *Archaea* (3). Functional genes involved in N_2 fixation, especially the nitrogenase reductase-encoding gene, *nifH*, have been used as phylogenetic markers to identify diazotrophs and to examine their distribution in various environments (4, 5). Subsequent surveys show that, in addition to cyanobacteria, heterotrophic putative diazotrophs are widespread in marine waters (reviewed in reference 6) and may even dominate *nifH* gene libraries (7). Moreover, significant N_2 fixation has been documented in waters where cyanobacteria are not believed to be present or active (e.g., see references 89 to 10). Nonetheless, the autecology and ecological importance of marine heterotrophic diazotrophs are still only poorly understood.

In order to estimate the potential contribution by hetero-

trophic organisms to marine N_2 fixation, cell-specific rates of N_2 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 N_2 fixation (12, 13). Such calculations are, however, potentially compromised by the lack of cell-specific N_2 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 N_2 fixation in relation to environmentally relevant stimuli have not yet been undertaken.

A multitude of constraints presumably regulate N_2 fixation by heterotrophic bacteria in pelagic marine systems. The reduction of N_2 to NH_4^+ is expensive in terms of reducing power and cellular energy, requiring at least 16 mol of ATP to reduce 1 mol of N_2 and, hence, demanding considerable amounts of organic carbon (C) compounds. In addition, the nitrogenase enzyme complex is usually highly sensitive towards O_2, and N_2 fixation is therefore supposedly tightly regulated by the presence of O_2, 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 N_2 fixation are currently poorly understood.

In this study, we report the comparative investigation of the genomic traits relating to N_2 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 het-

erotrophic diazotrophs. Furthermore, we report data on nitrogenase activity in these bacteria as a function of different concentrations of O_2, dissolved inorganic N (NH_4^+ and nitrate [NO_3^-]), and dissolved organic C. Lastly, we evaluate the applicability of relative *nifH* transcript abundances as a proxy for N_2 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 mono-, 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 "*Candidatus* Pelagibacter" sp. strain IMC9063 and *Fluviicola taffensis* DSM16823 (Table 1). In addition to N_2 fixation and ammonium assimilation, all three isolates seemed to be able to respire NO_3^- 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, N_2-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 macrozytogenes* (18), was recovered, but not a conventional molybdenum-iron (FeMo) nitrogenase-

TABLE 1 Comparison of selected genomic features for the Baltic Sea isolates and reference genomes of "*Candidatus* Pelagibacter" and *F. taffensis*

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
Aromatic compound metabolism subsystem (no. of defined features)					
Quinate degradation	1	1	4	1	1
Biphenyl degradation	3				
n-Phenylalkanoic acid degradation	13				
Benzoate degradation	9		4		
Chloroaromatic degradation	4	5	5		
Catechol branch of beta-ketoadipate pathway	8	9	8		2
Salicylate and gentisate catabolism	3	7	5	3	2
Homogentisate pathway of aromatic compound degradation	8	15	11		3
Salicylate ester degradation		2	1	1	
N-heterocyclic aromatic compound degradation		2			
Aromatic amine catabolism		5	7		
Protocatechuate branch of beta-ketoadipate pathway			14		
4-Hydroxyphenylacetic acid catabolic pathway			15		
Central metacleavage pathway of aromatic compound degradation			6		
Gentisate degradation				2	1
p-Hydroxybenzoate degradation		2	4		
Total	49	48	84	7	9
Nitrogen metabolism subsystem (no. of defined features)					
Nitrogen fixation	22	22	22		
Denitrification	22	12	9		
Nitrate and nitrite ammonification	26	9	20		
Ammonia assimilation	13	19	12	6	
Dissimilatory nitrite reduction	13				
Nitrosative stress	5	2	6		2
Cyanate hydrolysis	4	8			
Total	105	72	69	6	2

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 archeal and FeFe nitrogenase-containing cluster II (Fig. 1).

The genetic information related to N$_2$ 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%), sup-

porting the notion that the N$_2$ 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 N$_2$ fixation subsystem, contributing to the rather large size of the N$_2$ fixation region in this genome. Despite their unknown function, these CDSs seem to be conserved among organisms fixing N$_2$ under microaerobic and aerobic conditions, and their transcription is increased under conditions favoring N$_2$ fixation, suggesting that they do play a role in aerobic N$_2$ fixation (38).

The N$_2$ fixation region of *R. palustris* BAL398 was in general similar to the composition of the FeMo N$_2$ 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 N$_2$ fixation region of *R. ornithinolytica* BAL286 constituted only 28.8 kb and comprised
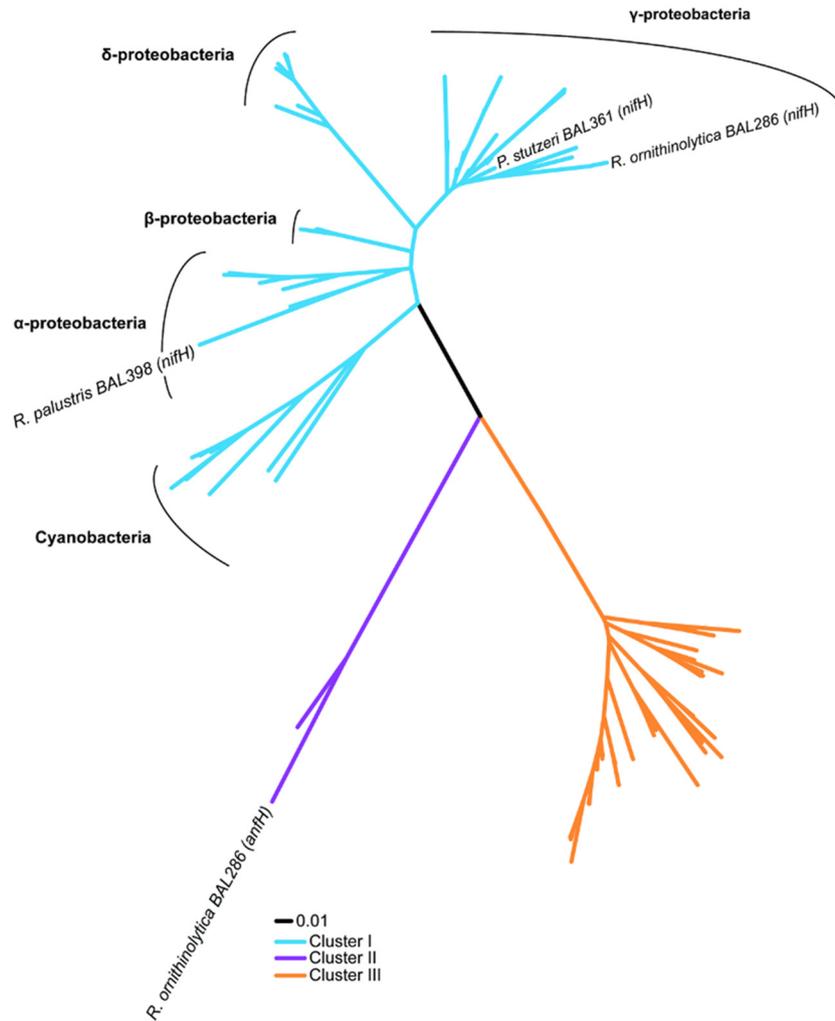
FIG 1 Amino acid sequence-based, unrooted, neighbor-joining tree showing the affiliations of the isolates with the canonical *nifH* clusters (37). Color-coded lines indicate cluster affiliations as shown in the key. *Pseudomonas stutzeri* BAL361 represents the gammaproteobacterial part of cluster I, *Rhodopseudomonas palustris* BAL398 represents the alphaproteobacterial part of cluster I, and *R. ornithinolytica* BAL286 represents the gammaproteobacterial part of cluster I, as well as cluster II.

29 CDSs. It was hence the most condensed N$_2$ 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 N$_2$ fixation clusters in the three organisms examined differ in structure, composition, and complexity, presumably reflecting different ecophysiologies of the organisms. Furthermore, it appears that the genetic clusters are in a state of flux, continuously being influenced by HGT.

Growth kinetics, N$_2$ fixation potential, and regulation. To ensure that C$_2$H$_4$ 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,
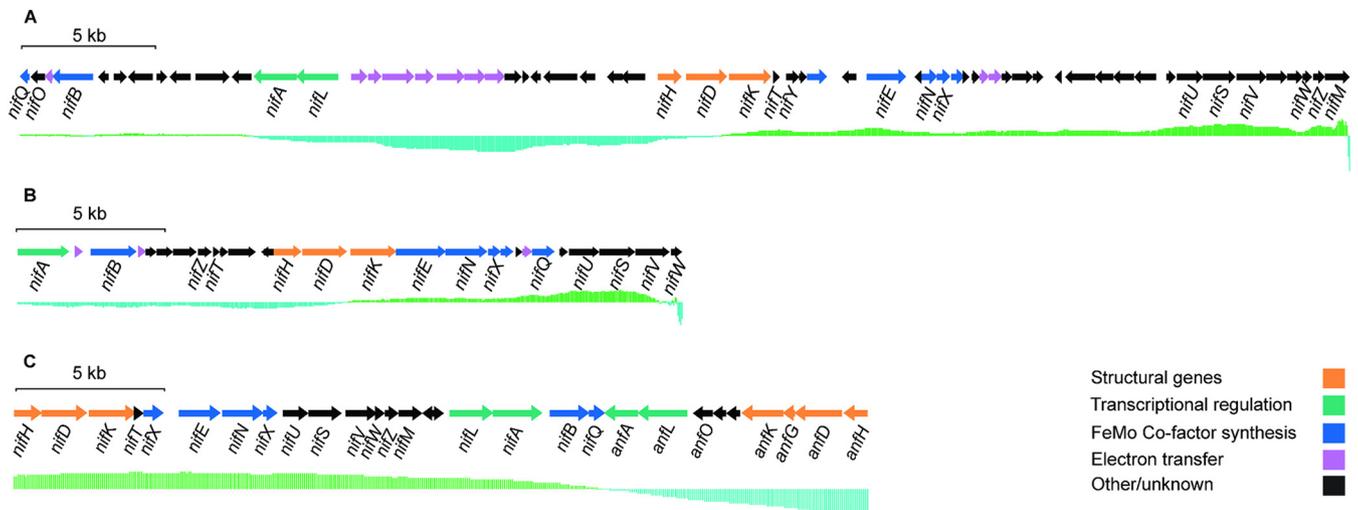
FIG 2 N$_2$ fixation gene clusters of *Pseudomonas stutzeri* BAL361 (A), *Rhodopseudomonas palustris* BAL398 (B), and *Raoultella ornithinolytica* BAL286 (C). Color-coded arrows indicate the locations of coding sequences (CDSs) and their orientations. The GC contents are depicted beneath the gene clusters (blue, GC content below cluster average; green, GC content above cluster average).

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 μmol 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 *Pseudomonas 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
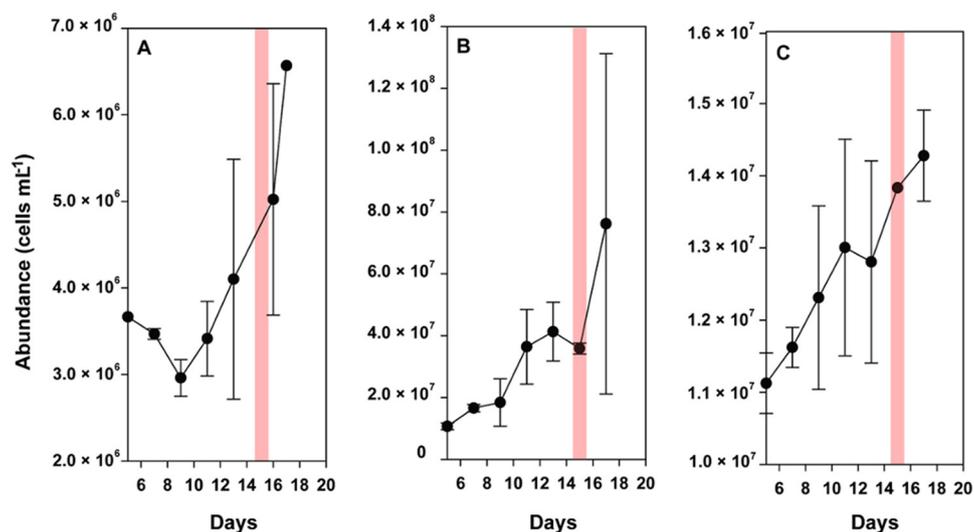

FIG 3 Growth of *Pseudomonas stutzeri* BAL361 (A), *Rhodopseudomonas palustris* BAL398 (B), and *Raoultella ornithinolytica* BAL286 (C) in carbonate-buffered microoxic diazotroph medium under standard conditions (20 mmol glucose liter^{-1}, $37 \pm 4 \mu$mol O$_2$ liter^{-1}). Cell counts were obtained from each of the three cultures daily and are depicted from day 5 to 17 as means of values obtained within 48-h periods. Error bars represent standard deviations of the means. Pink bars indicate the 24-h period when cells were exposed to acetylene in the acetylene reduction assay.
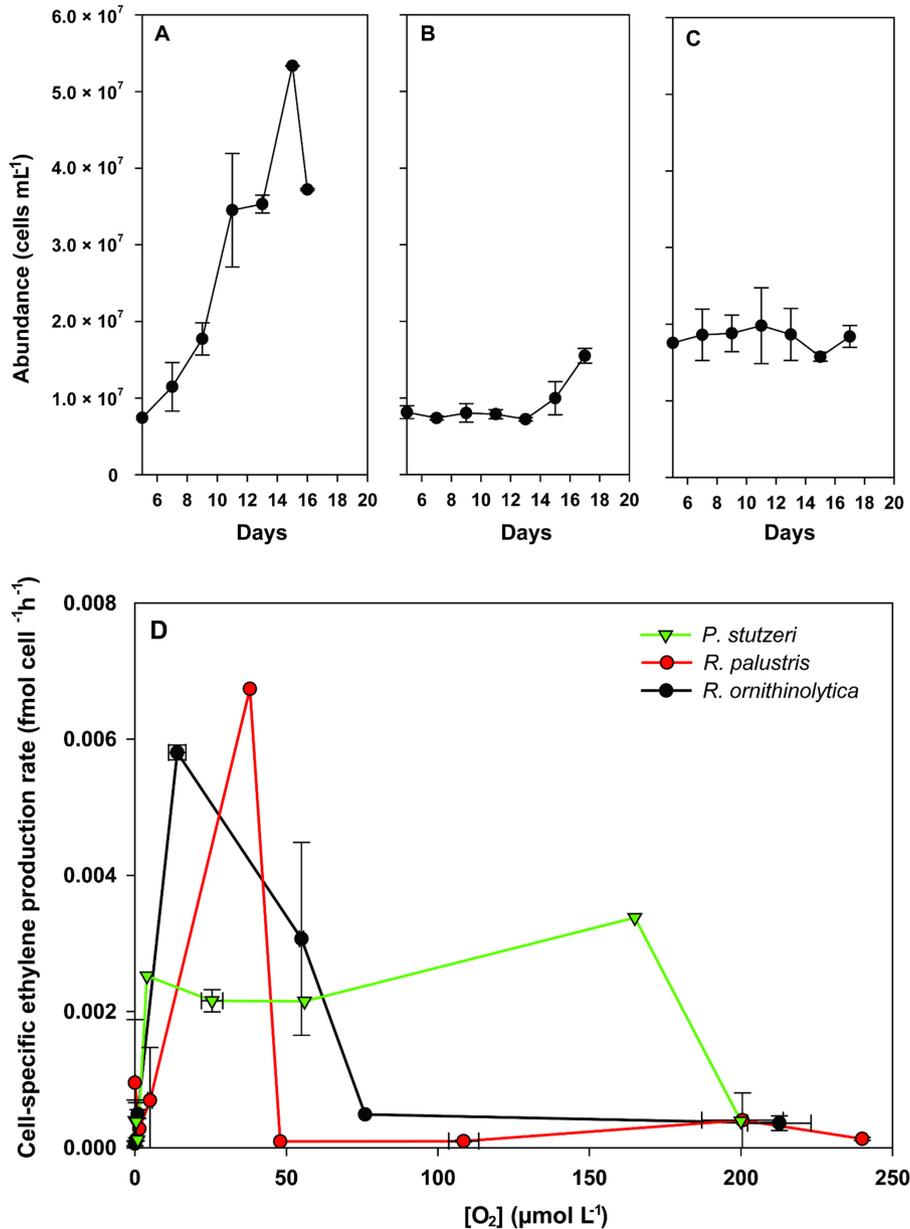
FIG 4 Growth of *Pseudomonas stutzeri* BAL361 (A), *Rhodopseudomonas palustris* BAL398 (B), and *Raoultella ornithinolytica* BAL286 (C) in an oxygenated version of the growth medium ($\approx$200 μmol O_2 liter^{-1} at the time of inoculation). Cell counts were obtained from each of the three cultures daily and are depicted from day 5 to 17 as means of values obtained within 48-h periods. Error bars represent standard deviations of the means. (D) Cell-specific ethylene (C_2H_4) production rates as a function of the O_2 concentrations measured in the cultures at the time of C_2H_4 quantification. Horizontal and vertical error bars, where present, indicate the standard deviations of the mean O_2 concentration values measured at the time of C_2H_4 quantification and of the mean C_2H_4 production values, respectively.

time of 3.6 days compared to 15 days under low initial O_2 concentrations (Fig. 3A versus 4A). ARA showed that ethylene (C_2H_4) was produced at 4 to 165 μmol O_2 liter^{-1}, with the highest cell-specific C_2H_4 production rates, 0.0034 fmol C_2H_4 cell^{-1} h^{-1}, observed at 165 μmol O_2 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 O_2 diffusion and facilitate N_2 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 O_2 liter^{-1} (Fig. 4B and C); however, low-O_2 conditions stimulated N_2 fixation in these isolates (Fig. 4D). C_2H_4 production was low ($\approx$0.001 fmol C_2H_4 cell^{-1} h^{-1}) in the 0 to 5 μmol O_2 liter^{-1} range but increased at 38 μmol O_2 liter^{-1} to 0.0067 fmol C_2H_4 cell^{-1} h^{-1} in *R. palustris* BAL398. The facultative anaerobic *R. ornithinolytica* BAL286 displayed the same overall pattern, reaching a peak C_2H_4 production
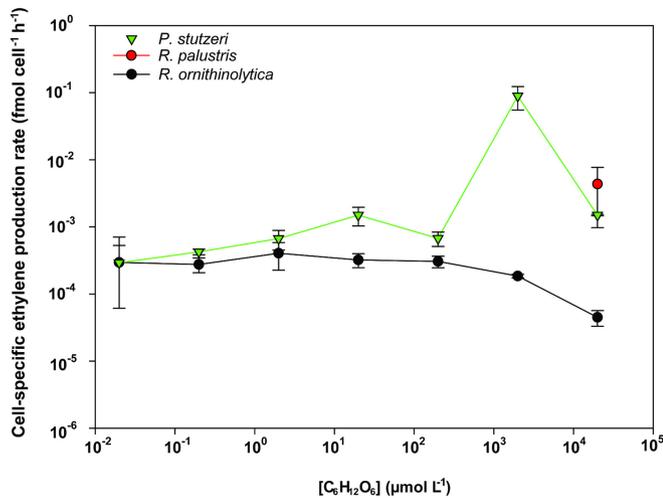
FIG 5 Cell-specific ethylene (C_2H_4) production rates measured in triplicate serum vials as a function of eight glucose concentrations. Bulk C_2H_4 production did increase in *R. ornithinolytica* BAL286 cultures as a function of increasing glucose concentrations (not shown), but the higher number of cells meant a decrease in the cell-specific rates. Error bars indicate standard deviations of values from triplicate serum vials. Note the double logarithmic scale.

rate of 0.0058 fmol C_2H_4 cell^{-1} h^{-1} in the presence of 14 μmol O_2 liter^{-1}, supporting observations that N_2 fixation in enterobacterial diazotrophs is stimulated by the presence of low concentrations of O_2 (45, 46). Hence, these diazotrophs are likely faced with the challenge of balancing the detrimental effects of O_2 on the nitrogenase complex with the need to produce sufficient energy to fuel it.

Increasing C concentrations were found to stimulate N_2 fixation in *P. stutzeri* BAL361. C_2H_4 production increased steadily from <0.001 fmol C_2H_4 cell^{-1} h^{-1} in cultures supplemented with 0.020 μmol glucose liter^{-1} to 0.089 fmol C_2H_4 cell^{-1} h^{-1} in cultures with 2,000 μmol glucose liter^{-1} (Fig. 5). A decrease in C_2H_4 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 O_2 concentration to a level impairing N_2 fixation. Hence, interactions between factors controlling N_2 fixation may complicate the interpretations made from culture-based estimates of N_2 fixation. C_2H_4 production was only observed at the highest C concentration (20,000 μmol glucose liter^{-1}) for *R. palustris* BAL398. Here, cell-specific C_2H_4 production rates reached 0.0044 fmol C_2H_4 cell^{-1} h^{-1}, suggesting that this organism required more energy to sustain N_2 fixation. *R. palustris* is known to produce substantial amounts of H_2 when fixing N_2 (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 C_2H_4 production in response to increasing glucose concentrations, and yet the cell-specific rates dropped, suggesting that other factors controlling N_2 fixation interfered in these treatments.

Due to the high energy consumption by the N_2 fixation reaction, it is generally believed that N_2 fixation shuts down in response to available reactive N. All three isolates decreased N_2 fix-
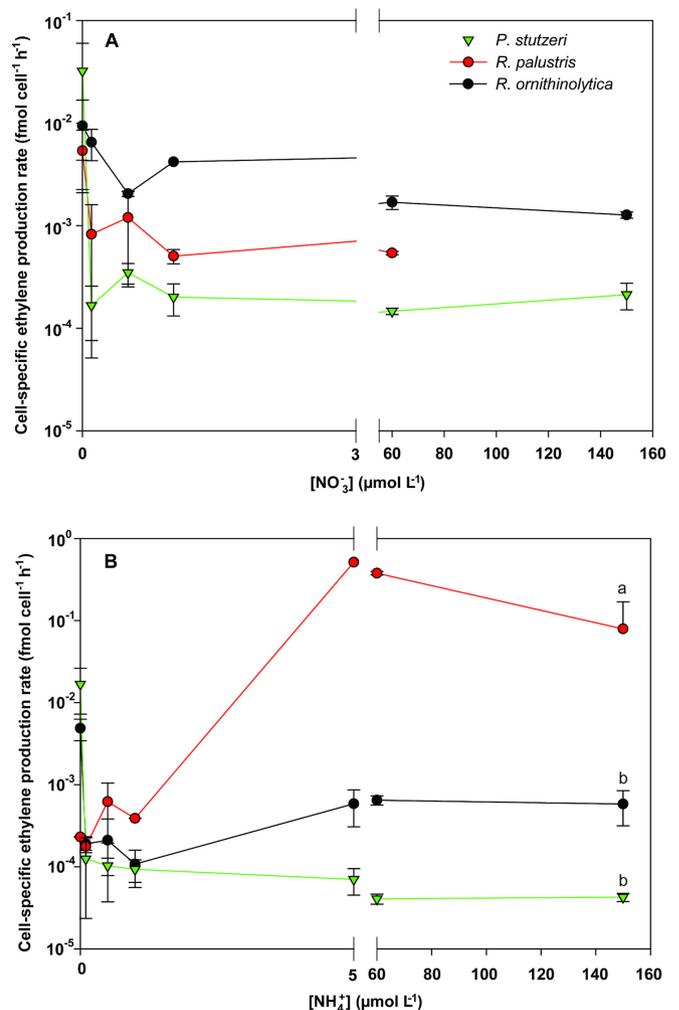

FIG 6 Cell-specific ethylene (C_2H_4) production rates measured in triplicate serum vials as a function of eight different concentrations of NO_3^- (A) and NH_4^+ (B). (A) C_2H_4 production rates dropped 1 to 2 orders of magnitude for all three isolates when exposed to even low NO_3^- levels. (B) C_2H_4 production rates dropped for *R. ornithinolytica* BAL286 and *P. stutzeri* BAL361, while they increased significantly for *R. palustris* BAL398 compared to the rates for the other isolates when exposed to NH_4^+ ($P = 0.01$). Error bars represent the standard deviations of values from triplicate serum vials, and lowercase letters indicate Tukey's honestly significant difference (HSD) groupings. Note the logarithmic scale on the *y* axis.

ation in response to increasing NO_3^- concentrations, with the C_2H_4 production rates dropping 1 to 2 orders of magnitude (Fig. 6A). N_2 fixation decreased significantly in response to increasing NH_4^+ concentrations in *P. stutzeri* BAL361 and *R. ornithinolytica* BAL286 cultures as well, but in *R. palustris* BAL398 cultures, N_2 fixation increased dramatically upon the addition of 5 μmol NH_4^+ liter^{-1} or more (Fig. 6B), reaching the highest cell-specific C_2H_4 production rates measured in any of the ARAs (0.515 fmol C_2H_4 cell^{-1} h^{-1}). The cell-specific C_2H_4 production rates were not significantly different between *P. stutzeri* BAL361 and *R. ornithinolytica* BAL286 in the presence of NH_4^+ ($P = 0.90$), whereas the C_2H_4 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 CO_2 and N_2 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 N_2 fixation as an electron sink when grown at suboxic conditions on glucose and NH_4^+. This falls in line with the many spurious findings of marine N_2 fixation at NH_4^+ concentrations of up to 200 μmol liter^{-1} (8, 50) and highlights that regulation of N_2 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 C_2H_4 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 C_2H_4 production rates were never significant (Spearman's rank-order correlation test, $\alpha = 0.05$) (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 N_2 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 NH_4^+), N_2 fixation was highest in cultures where O_2 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 C_2H_4 production to N_2 fixation in these cultures using the theoretical conversion factor of 3 (54), the heterotrophic isolates fixed N_2 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 N_2 fixation *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 4.7×10^4 and 7.9×10^4 cells liter^{-1}, respectively (20). Applying the measured rates, cells at these densities could contribute with N_2 fixation rates of 0.790 to 56.9 pmol N liter^{-1} day^{-1}.

Converting the C_2H_4 production rates obtained from *R. palustris* BAL398 cultures subjected to NH_4^+ additions to N_2 fixation rates as described above, significantly higher N_2 fixation rates are obtained (0.17 fmol N cell^{-1} h^{-1}). Hence, under conditions low in O_2 and replete in C and NH_4^+, for instance in association with fecal pellets or aggregates (55), the observed abundances of *R. palustris* BAL398 (4.7×10^4 cells liter^{-1} [20]) could account for N_2 fixation rates of 192 pmol N liter^{-1} day^{-1}. Interestingly, Guerinot and Colwell (56) reported that N_2 fixation by diazotrophic isolates in seawater incubations was only quantifiable when particulate material was present, supporting the idea that these may be hot spots for N_2 fixation despite being replete in reactive N. In addition, actively N_2-fixing photosynthetic bacteria have been found to be associated with copepods in the Caribbean Sea (57) and, recently, N_2-fixing heterotrophs in the particulate size fraction of >10 μm were shown to be more abundant in oxygenated water than in suboxic waters (19).

The rates measured for diazotrophic isolates are low relative to the peak rates of estuarine surface waters (47 to 83 nmol liter^{-1} day^{-1} [24]) but are comparable to the rates obtained from deeper estuarine waters (0.44 nmol liter^{-1} day^{-1} [8]) and the lower end rates measured in Pacific or Atlantic surface waters (0.24 to 35 nmol liter^{-1} day^{-1} [58, 59]). The rates obtained for individual isolates under optimal laboratory conditions are likely much higher than the rates would be per cell *in situ*; however, it should be kept in mind that a bulk *in situ* rate presumably represents the commensal activity of multiple diazotrophic species in the sample. With that in mind, our reported rates for diazotrophic isolates indicate that, under the right conditions, heterotrophic diazotrophs have the potential to contribute significantly to *in situ* rates.

Collectively, our study documents that cultivable diazotrophs from temperate estuarine waters exhibit great metabolic versatility and that the genomic N_2 fixation-related features are conserved and yet distinct between organisms, possibly reflecting the physiology of the organism. Furthermore, our data indicate that the gene clusters encoding the nitrogenase complexes are influenced by HGT events and are in a constant state of flux, facilitating adaptation to changing environmental conditions through the introduction of alternative nitrogenases and shuffling of the genetic contexts. The high level of cell-specific N_2 fixation observed under some conditions in this cultivation-based study suggests that heterotrophic bacteria may, at least occasionally, contribute significantly to overall N_2 fixation in temperate estuarine waters, given their high *in situ* abundances (e.g., see references 20, 24, and 29). Regulation of N_2 fixation by O_2, C, or reactive N was not straightforward and in some cases counterintuitive. Hence, making predictions about the occurrence and scale of N_2 fixation in different environments based on such parameters is difficult. Due to these discrete mechanisms of regulation in response to different environmental factors, coupling genomic features and ecophysiological characteristics in multiple and diverse representative isolates seems to be necessary in order to make better community-level generalizations about N_2 fixation.

MATERIALS AND METHODS

The bacteria being investigated were isolated from surface waters in the Baltic Sea. *P. stutzeri* BAL361 (*nifH* nucleotide accession number KC140355) and *R. palustris* BAL398 (*nifH* nucleotide accession number KC140365) were isolated from carbonate-buffered, microoxic diazotroph enrichment cultures devoid of N that were inoculated with surface water (3 m; total depth, 459 m) from the Landsort Deep (58°36′N, 18°14′E) in March 2009 (20). *R. ornithinolytica* BAL286 (*nifH* nucleotide accession number AY972875) was isolated from a depth of 3 m (total depth, 10 m) at a station in the strait between mainland Sweden and the island of Öland (56°37′N, 16°21′E) in April 2005 using semisolid 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 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 N$_2$ 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 N$_2$ 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 phylotypes 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 DQ270271) (66), and (ii) the genome of Fluviicola taffensis 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 N$_2$ 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 O$_2$ concentration (37 ± 4 μmol O$_2$ liter^{-1}) in the medium prior to inoculation, the headspaces were replaced three consecutive times with an atmosphere containing 90% N$_2$, 5% CO$_2$, and 5% H$_2$ and the liquid was allowed to equilibrate with the modified atmosphere for 2 h before the O$_2$ concentrations were verified using a FireStingO$_2$ optical O$_2$ meter equipped with an OXR50 fiber-optic O$_2$ sensor (Pyroscience, Aachen, Germany). The medium was then supplemented with a 0.2 μm-filtered glucose solution (20 mmol 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 O$_2$ concentrations on the bacterial growth, parallel incubations containing 0.2 μmol glucose liter^{-1} or ≈200 μmol O$_2$ liter^{-1} were included. The effect of reactive N on the growth of the bacteria was examined by adding 60 μmol NH$_4^+$ or NO$_3^-$ 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 (C$_2$H$_2$; Air Liquide, Taastrup, Denmark). Incubations continued for 24 h before the C$_2$H$_4$ 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 O$_2$ liter^{-1}, and was free of reactive N. To examine the regulation of N$_2$ fixation by different concentrations of C, O$_2$, and reactive N, one of these parameters was changed while the other two were kept constant. The effect of O$_2$ on N$_2$ fixation was investigated by regulating the O$_2$ concentrations of the base medium by adding 2, 4, or 8 ml of pure O$_2$ gas or by adding ferrous sulfate (FeSO$_4$) 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 O$_2$ concentrations in the medium ranging from 0 to 240 μmol O$_2$ liter^{-1} at the time of C$_2$H$_4$ 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 NO$_3^-$ or NH$_4^+$: 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 C$_2$H$_4$ 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 RNAlater (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 DNA 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 1× 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 JXXD00000000 (Pseudomonas stutzeri BAL361), JXXE00000000 (Rhodopseudomonas palustris BAL398), and JXXF00000000 (Raoultella ornithinolytica BAL286).

SUPPLEMENTAL MATERIAL

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

Figure S1, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.

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