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Isolation and gene quantification of heterotrophic N_2-fixing bacterioplankton in the Baltic Sea

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Summary

Cyanobacteria are regarded as the main N_2-fixing organisms in marine waters. However, recent clone libraries from various oceans show a wide distribution of the dinitrogenase reductase gene (*nifH*) originating from heterotrophic bacterioplankton. We isolated heterotrophic N_2-fixing bacteria from Baltic Sea bacterioplankton using low-nitrogen plates and semi-solid diazotroph medium (SSDM) tubes. Isolates were analysed for the nitrogenase (*nifH*) gene and active N_2 fixation by nested polymerase chain reaction (PCR) and acetylene reduction respectively. A primer-probe set targeting the *nifH* gene from a γ-proteobacterial isolate, 97% 16S rDNA similarity to *Pseudomonas stutzeri*, was designed for measuring *in situ* dynamics using quantitative real-time PCR. This *nifH* gene sequence was detected at two of 11 stations in a Baltic Proper transect at abundances of 3×10^4 and 0.8×10^3 copies per litre seawater respectively. Oxygen requirements of isolates were examined by cultivation in SSDM tubes where oxygen gradients were determined with microelectrodes. Growth, and thereby N_2 fixation, was observed as horizontal bands formed at oxygen levels of 0–6% air saturation. The apparent microaerophilic or facultative anaerobic nature of the isolates explains why the SSDM approach is the most appropriate isolation method. Our study illustrates how combined isolation, functional analyses and *in situ* quantification yielded insights into the oxygen requirements of heterotrophic N_2-fixing bacterioplankton isolates, which were confirmed to be present *in situ*.

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Introduction

Atmospheric nitrogen is transferred to aquatic and terrestrial ecosystems through N_2 fixation by free-living and symbiotic cyanobacteria as well as heterotrophic bacteria (Vitousek *et al*., 2002). Oceanic N_2 fixation is estimated to 100–200 Tg N per year (Karl *et al*., 2002), which is comparable to terrestrial N_2 fixation (Galloway, 1998). Marine waters are generally considered nitrogen limited (Capone, 2000) and therefore favourable environments for N_2-fixing organisms. To understand the dynamics of marine ecosystem productivity it is of importance to identify the N_2-fixing organisms and to determine the spatial and temporal distribution of their activity. The colonial cyanobacterium *Trichodesmium* has been regarded as the dominant diazotroph in the open ocean (Carpenter and Romans, 1991; Capone *et al*., 1997; Chen *et al*., 1998) together with heterocyst-forming cyanobacterial symbionts of diatoms (Villareal, 1990). However, recent studies show that unicellular cyanobacteria may equal or even exceed the N_2 fixation reported for the larger organisms (Zehr *et al*., 2001; Falcón *et al*., 2004; Montoya *et al*., 2004).

Molecular techniques provide new ways to examine the diversity and importance of prokaryotes in marine nitrogen cycling. For instance, the conserved *nifH* gene coding for the Fe protein subunit of the nitrogenase enzyme complex (Zehr and McReynolds, 1989) has been used to identify prokaryotes with a genetic potential for N_2 fixation. Heterotrophic bacteria also suffer from nitrogen shortage and their common occurrence in *nifH* gene clone libraries indicates that heterotrophic bacteria may contribute significantly to N_2 fixation in aquatic ecosystems. In particular, proteobacterial *nifH* phylotypes appear prevalent in samples from the Pacific and Atlantic Oceans (Zehr *et al*., 1998; Church *et al*., 2005a,b; Langlois *et al*., 2005), the Arabian Sea (Bird *et al*., 2005), and from lakes (Zani *et al*., 2000; Steward *et al*., 2004). The occurrence of *nifH* genes (Short *et al*., 2004; Steward *et al*., 2004; Langlois *et al*., 2005) or transcripts (Zani *et al*., 2000; Bird *et al*., 2005) in aquatic environments replete with fixed nitrogen suggests that complex mechanisms, in addition to nitrogen availability, drive the distribution and expression of *nifH*. Knowledge about diversity and distribution of *nifH*-containing phylotypes in the environment has increased dramatically (reviewed in Zehr *et al*., 2003a), but

information on the regulation and quantitative importance of *nifH*-containing heterotrophic bacteria in planktonic systems remains scarce.

Two recent studies used the high sensitivity of quantitative real-time PCR to examine the spatial distribution of *nifH*-containing bacterial phylotypes in Chesapeake Bay and in the North Pacific Ocean (Short *et al*., 2004; Church *et al*., 2005a). These studies introduced methodological means to study the distribution of potential N_2-fixing bacterial phylotypes; however, as *nifH* clones are usually not closely related to cultivated bacteria (Zehr *et al*., 2003a), functional couplings between such *in situ* dynamics and pure culture studies are speculative.

The Baltic Sea is a large nutrient-rich, semi-enclosed, non-tidal estuary with a salinity gradient from north to south of S = 2–8 (Larsson *et al*., 2001). In summer, plankton production in the Baltic Proper is nitrogen-limited (Granéli *et al*., 1990) and dominated by massive blooms of heterocyst-forming cyanobacteria of the genera *Aphanizomenon* and *Nodularia* (Stal *et al*., 2003). Larsson and colleagues (2001) found that several times more nitrogen was fixed by diazotrophs than incorporated into the biomass of heterocystous cyanobacteria in the Baltic Sea. They speculated that other N_2-fixing organisms could be responsible for this apparent discrepancy. Indeed, by including pico- and nano-plankton in their measurements, Wasmund and colleagues (2001) found a three to four times higher annual N_2 fixation, emphasizing the importance of smaller diazotrophs. The contribution by heterotrophic bacteria was not determined as coccoid cyanobacteria and heterotrophic bacteria could not be distinguished in their study.

In the present study, we successfully isolated heterotrophic N_2-fixing bacteria from Baltic Sea bacterioplankton using low-nitrogen plates and semi-solid agarose tubes containing diazotroph medium. Oxygen requirements for N_2-fixing bacteria were determined by means of microelectrode measurements and *in situ* abundance of a specific *nifH* gene sequence was quantified by real-time PCR.

Results

Isolation of bacteria using low-nitrogen plates

The low-nitrogen plates were used to isolate bacteria sampled on 16 occasions in a transect between Oskarshamn (mainland, N57°37′ E18°13′) and Visby (Island of Gotland, N57°16′ E16°30′) at the east coast of Sweden. Ninety-three bacterial strains were screened for the *nifH* gene by nested polymerase chain reaction (PCR). After the first PCR, 18 isolates yielded a PCR product with expected size (472 bp). Of these, eight isolates yielded a product of the correct size in the second PCR (359 bp). These PCR products were then cloned and sequenced to confirm their *nifH* identity. From each of the eight isolates, 5–12 inserts were sequenced and five isolates (BAL281–BAL285) seemingly contained sequences related to *nifH* genes (Table 1). For several of the isolates, clones obtained from a single isolate showed unexpected differences. Inserts from some clones did not yield any *nifH* BLAST hits while others grouped in different clusters (Table 1, Fig. 1). For example, clones obtained from BAL282 to BAL285 showed relatively high similarities (89–100%) with conventional and alternative nitrogenase genes from *Azomonas macrocytogenes* (*γ-proteobacteria*); though, these isolates belonged to *Actinobacteria* and *α-proteobacteria* (Table 1). To confirm the presence of a *nifH* gene in DNA from the isolated bacteria, specific primers against the *nifH* sequences obtained from the cloning experiment were designed and conventional PCR was run. The sequenced clones were used as positive controls. Only BAL281 was successfully amplified, hence, possessed the *nifH* gene. The *nifH* genes from BAL281 were most similar to *Pseudomonas stutzeri* (92%) and an environmental clone obtained from soil (95%) (Table 1). The phylogenetic identity of BAL281 was further determined by sequencing the 16S rRNA gene confirming a high similarity to *P. stutzeri* (97%).

Growth and isolation of bacteria using SSDM tubes

Growth of BAL281 was tested in nitrogen-free medium under aerobic conditions but no growth could be detected presumably due to inhibition of its nitrogenase by oxygen. Therefore, growth of BAL281 was tested in tubes with semi-solid diazotroph medium (SSDM) containing a vertical gradient in oxygen concentration. After 24 h, bacterial growth was seen as a faint band ~10 mm below the medium surface. After 3 days, a dense band could be seen (~1.2 mm thick; Fig. 2A). In tubes supplemented with nitrogen, growth was limited to the surface. The various control bacteria grew as expected: *Azotobacter vinelandii*, a N_2-fixing obligate aerobe, grew at the surface of medium with and without nitrogen; *Clostridium pasteurianum*, a N_2-fixing obligate anaerobe, grew and produced gas from > 2 cm below the medium surface with and without nitrogen; and *Escherichia coli* (not a diazotroph) did not grow in the tubes without nitrogen, but at the surface in medium supplemented with nitrogen.

The range of oxygen concentrations under which growth was observed was measured with an oxygen microelectrode. In medium without nitrogen, BAL281 grew exclusively at oxygen levels ranging from 0% to 6% air saturation (Fig. 2B). In contrast, in medium with nitrogen, it grew only at the medium surface at oxygen levels between 37% and 100% air saturation. In blank tubes (medium without bacteria) oxygen concentration

Table 1. Taxonomy of isolates analysed in this study. Number of clones sequenced for *nifH* and nearest relatives in GenBank with respect to *nifH* and 16S rRNA genes.

Isolates[a]	No. of clones sequenced	Nearest relative (*nifH* gene)[b]	% similarity (*nifH* gene)	Nearest relative (16S rRNA gene)	% similarity (16S rRNA gene)	Taxonomic affiliation
BAL281 (AY972868)	6	Uncultured bacterium clone g1-V-24 h-2, AY684103; (*Pseudomonas stutzeri*, AJ297529)	95 (92)	*Pseudomonas stutzeri*, AF152596	97	γ-*Proteobacteria*
BAL282 (AY972869)	12	*Azomonas macrocytogenes*, AY6434998				
		Uncultured bacterium clone g1-V-24h-2, AY684103; (*Pseudomonas stutzeri*, AJ297529)	95 (91)	*Sphingomonas paucimobilis*, AF039168	96	α-*Proteobacteria*
		Azomonas macrocytogenes (*anfH*), AY644348	99			
BAL283 (AY972870)	5	*Azomonas macrocytogenes*, AY644349	89	*Mycobacterium sacrum*, AY235429	99	*Actinobacteria*
BAL284 (AY972871)	10	*Azomonas macrocytogenes*, AY644349	92	*Brevundimonas diminuta*, D49422	99	α-*Proteobacteria*
		Azomonas macrocytogenes (*anfH*), AY644348	99			
BAL285 (AY972872)	11	*Azomonas macrocytogenes*, AY644349	92	*Microbacterium flavescens*, AB004716	99	*Actinobacteria*
		Azomonas macrocytogenes (*anfH*), AY644348	100			
BAL286[c] (AY972873)	5	*Klebsiella pneumoniae*, AF545638	85[d]	*Klebsiella ornithinolytica*, AJ251467	98	γ-*Proteobacteria*

a. Name followed by 16S rRNA gene accession number in GenBank.
b. If nearest relative was a clone, the nearest cultured relative is given in parentheses.
c. Isolated by SSDM technique.
d. Based on 263 bp.

decreased slowly with depth in the tube (100–75%, 1 cm down in the medium, data not shown) due to the slow diffusive equilibration of the medium with the overlaying air phase. While the oxygen gradient in the control tubes thus changed continuously but slowly, the bacterial oxygen consumption in tubes with bacteria stabilized the oxygen gradient, which therefore reached a steady state relatively fast.

The use of SSDM tubes for isolating marine N_2-fixing bacteria was tested more extensively using seawater sampled at a coastal site. Generally, colonies were distributed throughout the tubes, with slightly more colonies in the upper part of the tubes. Twenty-nine colonies, representing 17 different isolates after de-replication (see *Experimental procedures*), were purified and tested for acetylene reduction in addition to the previous isolate BAL281. Ethylene production could be demonstrated for three isolates, one of them being the BAL281 confirmed to possess the *nifH* gene. Interestingly, BAL281 showed significant ethylene production even when nitrogen was supplemented to the medium.

One of the other two isolates confirmed to reduce acetylene was further characterized by cloning/sequencing of the *nifH* gene and verification of the *nifH* gene in genomic DNA by PCR. The obtained isolate, named BAL286, showed 98% 16S rDNA similarity to *Raoultella ornithinolytica* (formerly *Klebsiella ornithinolytica*) (γ-*proteobacteria*) and carried a *nifH* gene related to the *anfH* gene of *Klebsiella pneumoniae* (85%, 263 bp; Table 1) and *A. vinelandii* (77%, 317 bp; Fig. 1). The five sequenced clones were almost identical (99%). BAL286 was non-motile and facultative anaerobic with maximum growth in the anoxic zone of SSDM tubes (Fig. 2B). When grown in tubes with nitrogen, BAL286 grew at an oxygen concentration of 0–4% air saturation.

Quantification of the BAL281 nifH *gene in seawater*

The number of BAL281 *nifH* gene copies was quantified in samples from a transect between Oskarshamn (mainland) and Visby (Island of Gotland; Table 2). Samples were obtained the same day as BAL281 was originally isolated (21 May 2001). Station 2, at which BAL281 was originally isolated, gave a significant real-time PCR signal of $30 \pm 0.4 \times 10^3$ BAL281 *nifH* gene copies per litre (Table 2). Another station (8) yielded a signal of $0.8 \pm 3 \times 10^3$ copies per litre. In addition, we examined samples from Station 2 obtained in summer and autumn. No signals were detected in these samples. The amplification efficiency was on average 81 ± 15% and ranged from 51% to 94% (Table 2). Amplification efficiency showed no dependence on bacterial or cyanobacterial abundance. The standard curve ranged from 2.2 to 2.2×10^7 copies with an R^2 value for the regression of the

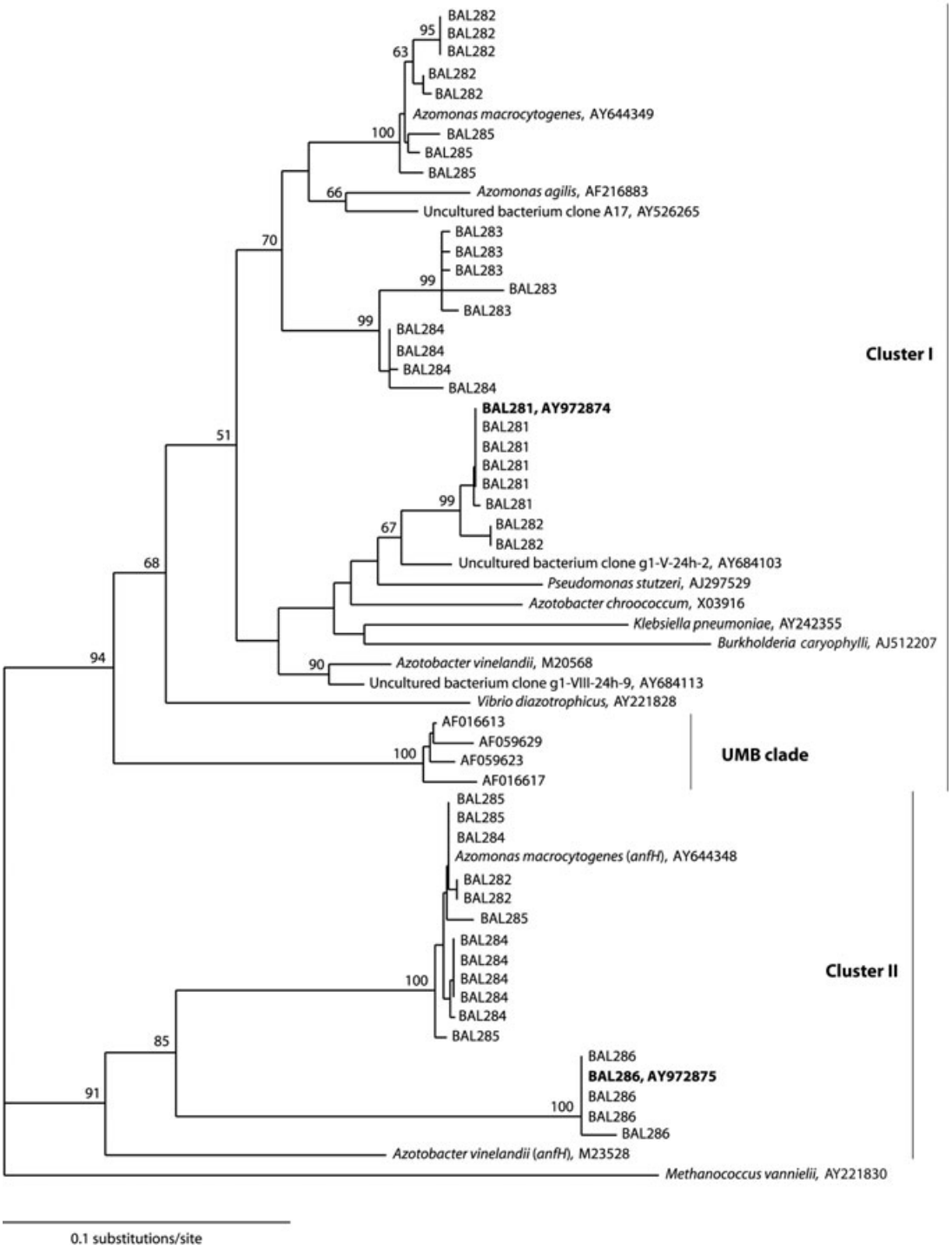

Fig. 1. Neighbour-joining phylogenetic tree of *nifH* sequences based on 317 bp. Isolate sequences deposited in GenBank are in bold type. Note that BAL281–285 were isolated from low-nitrogen plates, while BAL286 was isolated from SSDM tubes. Clusters are named according to Chien and Zinder (1996). UMB (Uncultured Marine Bacteria; Bird *et al*., 2005). Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values greater than 50% are shown.

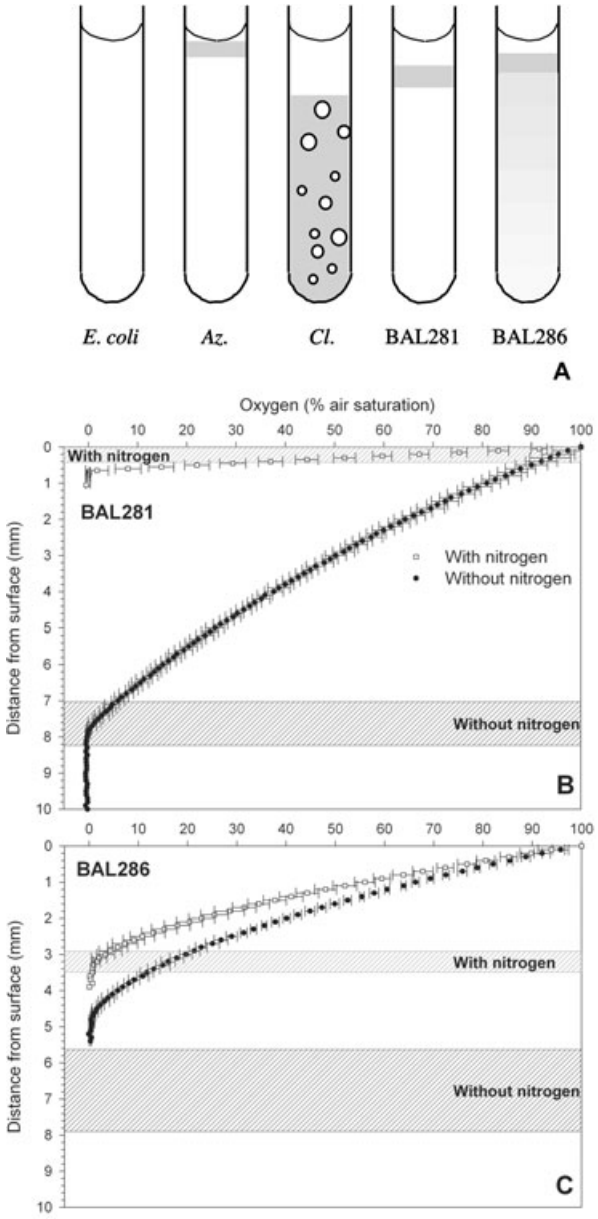

Fig. 2. Growth in SSDM tubes.
A. Drawing, not to scale, of growth in tubes. Grey areas indicate bacterial growth. *Escherichia coli* (*E. coli*) no growth, *A. vinelandii* (*Az.*) growth close to the surface, *C. pasteurianum* (*Cl.*) growth and gas production from 20 mm below the surface, BAL281 sharp band 7.0 mm below the surface, and BAL286 sharp band 5.6 mm below the surface and some growth all the way down.
B and C. Depth profiles of oxygen concentration in SSDM tubes for BAL281 (B) and BAL286 (C) (triplicate tubes ± SD). Grey areas indicate bacterial growth with and without supplemented nitrogen.

resulting threshold values (C_t) versus gene copies of 0.994.

Contamination of nested PCR

A disturbing result was that *nifH* gene sequences were obtained from nested PCR products from four isolates, where presence of *nifH* in genomic DNA could not be confirmed. In order to examine whether the nested PCR generated false positives, an additional nested PCR was performed on the four 'false' isolates and BAL281 to test whether these were consistently amplified (Fig. 3, top, Day 1). In the first PCR, three isolates (BAL281, 282, 284) produced a PCR product of correct size (472 bp). The second PCR yielded products of sufficient density and correct size (359 bp) from BAL281 and 285. The negative control that was transferred from the first to the second PCR yielded a faint, unspecific product. This result differed from our previous nested PCR where all five isolates yielded PCR products of correct size (in both PCR runs; data not shown). To test the reproducibility of these results, the nested PCR was run again the next day with the same reagents. Again the results were different from previous results (Fig. 3, bottom). The first PCR of Day 2 was similar to the one from Day 1. The second PCR, though, yielded strong bands for BAL281, 282, 284, 285. Additionally, the negative control that was transferred from the first to the second PCR yielded a strong band of the correct size.

Discussion

Pioneering efforts on cultivation and analyses of single-cell cyanobacteria have yielded important insights into their ecology (e.g. Falcón *et al.*, 2005), although conceptual bridges between pure-culture studies and natural assemblages of marine N_2-fixing heterotrophic bacteria remain absent. In the present study, the isolation of N_2-fixing heterotrophic bacterioplankton was coupled with *in situ* quantification of a specific nitrogenase gene sequence in samples from the Baltic Sea. The study illustrates that the combination of cultivation, physiological analyses and molecular quantification of specific nitrogenase genes *in situ* may be a fruitful and manageable endeavour by which future insights to the role and ecology of marine N_2-fixing heterotrophic bacterioplankton may be gained.

The capability of N_2 fixation is widely distributed among bacteria and includes anaerobic, facultative anaerobic and aerobic genera (Paerl and Zehr, 2000). However, few studies have successfully isolated heterotrophic N_2-fixing bacteria from seawater (Werner *et al.*, 1974; Wynn-Williams and Rhodes, 1974; Guerinot and Colwell, 1985; Tibbles and Rawlings, 1994). In the present study, we succeeded to isolate N_2-fixing heterotrophic bacterioplankton from the Baltic Sea using two cultivation techniques.

Screening of isolates from low-nitrogen plates

Colony-forming bacteria form a high proportion of the bacterioplankton in the Baltic Sea relative to less produc-

Table 2. Real-time PCR quantification of the BAL281 *nifH* gene sequence in seawater samples from a transect between Oskarshamn (St. 1, mainland) and Visby (St. 11, Island of Gotland) on 21 May 2001 ($n = 3$, ± SD).

St.	Location	*nifH* copies ($\times 10^3$ l^{-1})	PCR efficiency (%)	Bacteria ($\times 10^9$ l^{-1})	Cyanobacteria ($\times 10^8$ l^{-1})
1	N57°16′2 E16°30′7	BDL	89	1.36	0.58
2	N57°15′7 E16°40′7	30 ± 3.8	86	1.05	0.61
2(S)	N57°15′8 E16°41′9	BDL	66	1.48	1.22
2(A)	N57°17′6 E16°46′6	BDL	74	1.04	1.97
3	N57°18′6 E16°49′0	BDL	91	1.40	0.58
4	N57°22′6 E16°57′3	BDL	94	1.11	0.33
5	N57°25′7 E17°05′7	BDL	90	0.56	0.34
6	N57°27′5 E17°15′9	BDL	93	0.65	0.20
7	N57°29′5 E17°27′5	BDL	92	0.48	0.17
8	N57°31′6 E17°38′3	0.8 ± 0.3	90	0.46	0.23
9	N57°33′5 E17°49′5	BDL	82	0.44	0.23
10	N57°35′4 E18°00′0	BDL	51	0.67	0.20
11	N57°37′5 E18°13′3	BDL	52	0.72	0.14

BDL, below detection limit of 500 *nifH* gene copies per litre seawater; S, summer (11 July 2001); A, autumn (22 October 2001).

tive waters (Simu *et al*., 2005). However, only one (BAL281) out of 93 strains isolated from low-nitrogen plates carried the *nifH* gene and was capable of acetylene reduction. Similarly, Hill and Postgate (1969) examined putative N_2-fixing bacteria isolated on nitrogen-free plates from soil and seawater and found that most of their strains were incapable of acetylene reduction. They speculated that these bacteria were nitrogen scavengers taking up low levels of reduced nitrogen from the atmosphere, which may also be the case in our study.

Isolation of heterotrophic N_2-fixing bacteria with SSDM tubes

Cultivation in tubes with SSDM makes use of a gradient in oxygen concentration; a principle also used in, for

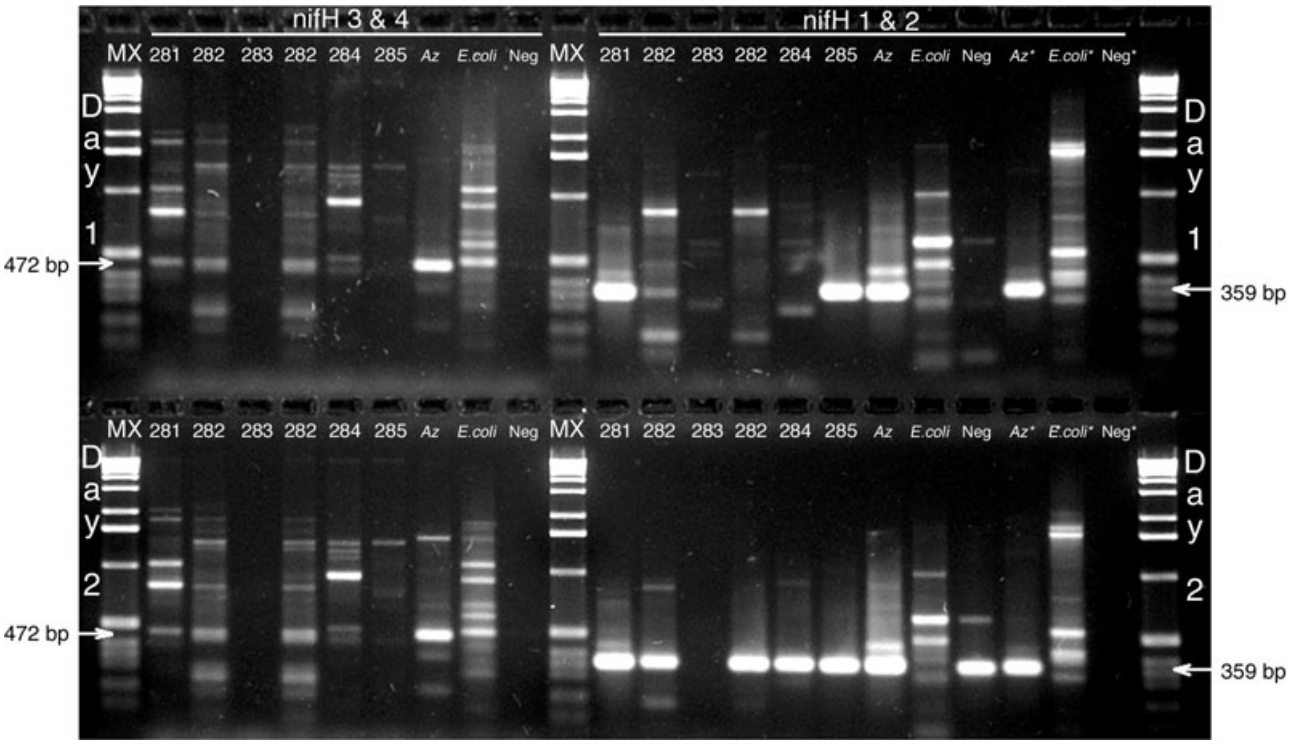

Fig. 3. Agarose gel with nested PCR products with primers *nifH* 3 and 4 (left) and primers *nifH* 1 and 2 (right). Full nested PCR was run on two successive days (day 1 top, day 2 bottom). Isolate names are given on top of lanes (BAL282 was run twice). *Azotobacter vinelandii* (Az) was used as positive and *E. coli* as negative control. Neg indicates the no-template control and asterisks indicate PCR products from second (*nifH* 1 and 2) PCR only.

example, oxygen-sulfur gradient cultures (Nelson *et al*., 1986). The successful cultivation of two isolates capable of acetylene reduction suggested that the SSDM approach is a more efficient means of isolating N_2-fixing bacteria than the low-nitrogen plates. When subsequently grown in these tubes, isolates capable of acetylene reduction formed horizontal bands (Fig. 2). In comparison, *E. coli* and the four isolates, in which the *nifH* gene could not be confirmed, never showed signs of growth.

The lack of acetylene reduction for 15 out of 17 strains isolated using the SSDM tubes (data not shown) suggests that we may have inadvertently isolated nitrogen scavenging bacteria or bacteria growing on trace nitrogen supplied to the medium with the cell concentrate. Alternatively, the acetylene reduction assay did not detect nitrogenase activity. For instance, alternative (Mo-independent) nitrogenases produce both ethylene and ethane when reducing acetylene and are less efficient than Mo-dependent nitrogenases (reviewed in Eady, 1996); hence, such activity would presumably be harder to detect in the acetylene reduction assay. Wynn-Williams and Rhodes (1974) found that the acetylene-reduction capacity for their 21 isolates were inexplicably variable, even under closely standardized condition, possibly due to small but crucial changes in oxygen concentrations (Wynn-Williams and Rhodes, 1974). In our case lack of acetylene reduction could also be due to energetic limitation as oxygen and/or substrate limitations in the gradient cultures may have affected nitrogenase activity.

Guerinot and Colwell (1985) successfully isolated marine heterotrophic N_2-fixing bacteria by adding filters containing bacteria directly into SSDM. In the present SSDM approach the dispersion of cells throughout the medium may allow for a more efficient use of the vertical oxygen gradient as compared with the filter approach by Guerinot and Colwell (1985). Clearly, the SSDM method used here requires further optimization to exclusively select for N_2 fixers. However, as compared with cultivation on low-nitrogen plates, the SSDM approaches for isolation of single strains of N_2-fixing heterotrophic bacteria used by us and by Guerinot and Colwell (1985) have a large potential and show the importance and strength of incorporating key aspects of the natural environment when studying N_2-fixing bacteria in the laboratory.

N_2 fixation and phylogeny of BAL281 and BAL286

BAL281 was most similar to the γ-*proteobacterium P. stutzeri* on the 16S gene sequence (97%) and on the *nifH* gene sequence (92%). Strains from the genus *Pseudomonas* are heterotrophic Gram-negative rods and have often been isolated from the Baltic Sea (e.g. Hagström *et al*., 2000). *NifH* genes similar to *P. stutzeri* have been found in a lake (Steward *et al*., 2004) and in isolates from soil (Rösch *et al*., 2002). γ-*Proteobacteria* carrying the *nifH* gene seem to be widespread and, for instance, sequences within the UMB cluster ('Uncultured Marine Bacteria'; Bird *et al*., 2005; Fig. 1) have been found in the Atlantic and Pacific Oceans (Zehr *et al*., 1998) and the Arabian Sea (Bird *et al*., 2005). However, the isolates obtained here were not closely related to this cluster (< 78% similarity on *nifH*). BAL281 consistently reduced acetylene, even when nitrogen was supplemented to the medium. Generally, reduced nitrogen inhibits nitrogenase expression (e.g. in *Vibrio*; Urdaci *et al*., 1988); however, it may be that nitrogen exhaustion occurred locally in the tubes, as the oxygen microprofiles indicated an intense bacterial activity when nitrogen was added. Interestingly, a few studies have found *nifH* transcripts in aquatic environments replete with reduced nitrogen (Zani *et al*., 2000; Bird *et al*., 2005) suggesting that complex mechanisms regulate nitrogenase expression.

The 16S rRNA gene sequence of BAL286 was 98% similar to the γ-*proteobacterium*, *R. ornithinolytica* (formerly *K. ornithinolytica* within the heterogeneous *Klebsiella* genus; Drancourt *et al*., 2001). *Klebsiella* are generally facultative anaerobic Gram-negative rods capable of N_2 fixation and have been isolated from marine environments (Werner *et al*., 1974; Jones and Rhodes-Roberts, 1980). The five *nifH* gene sequence of BAL286 was most similar to an *anfH* gene (the gene coding for an alternative nitrogenase) from *K. pneumoniae.* BAL286 showed acetylene reduction only when nitrogen was absent from the medium, which is consistent with previous analyses of *Klebsiella* (Collins and Brill, 1985).

Abundance of the BAL281 nifH *gene in samples from the Baltic Sea*

The distribution of the N_2-fixing BAL281 was examined in seawater samples using specific real-time PCR. The BAL281 *nifH* gene was detected in two samples collected in a transect between Oskarshamn and Visby in the Baltic Proper. At one station the abundance was close to the detection limit of our assay and therefore associated with some uncertainty. High abundance ($3 \pm 0.4 \times 10^4$ *nifH* gene copies per litre) was only found in the sample from which the bacterium was originally isolated and only on one occasion. A similar abundance of specific *nifH* phylotypes have been found in other marine waters using quantitative PCR (Short *et al*., 2004; Church *et al*., 2005a). Variability in real-time PCR amplification efficiency (Table 2) might have contributed to the observed patchiness; however, marked changes in bacterial community compositions have previously been observed on micrometer (Long and Azam, 2001) to kilometer (Ghiglione *et al*., 2005) scales, indicating that patchiness in bacterial distribution may be a common phenomenon.

Given that our samples were obtained from the seawater cooling system of a ship, we cannot unambiguously exclude that the BAL281 isolate and the extracted RNA originate from biofilms in the system.

Oxygen requirements for N_2 fixation

The poor isolation of N_2-fixing bacteria on aerobic low-nitrogen plates, and the exclusive growth in narrow horizontal bands at low oxygen concentrations in the SSDM tubes, indicate that Baltic bacterioplankton may require low oxygen (0–6% air saturation, BAL281) or anoxic (BAL286) conditions for N_2 fixation. Similarly, inhibition of nitrogenase activity has been observed in various *Vibrio* strains at 0.1–2% oxygen (Guerinot and Patriquin, 1981; Urdaci *et al*., 1988). The widespread ability to grow under anoxic conditions among marine bacterioplankton (Riemann and Azam, 2002; Alonso and Pernthaler, 2005) suggests that oxygen conditions suitable for N_2 fixation are available in the planktonic environment. Paerl and Prufert (1987) found that N_2 fixation within planktonic cyanobacterial aggregates was associated with low-oxygen microzones. Isolation of strictly anaerobic bacteria (Bianchi *et al*., 1992), products of anaerobic processes associated with marine snow (Shank and Reeder, 1993), and anoxic microzones in non-sinking faecal pellets (Alldredge and Cohen, 1987) suggest that various types of marine snow may be important habitats for N_2-fixing heterotrophic bacteria. While anoxia in freely suspended aggregates may be ephemeral (Ploug *et al*., 1997), more permanent anoxic conditions may develop as particles sediment and are mineralized on the sediment surface. In shallow waters (< 30 m), as in the present study, re-introduction of such particles into the water column by vertical mixing could be a source of bacteria capable of anaerobic metabolism (McCandliss *et al*., 2002; Alonso and Pernthaler, 2005).

Uncertainties of the nested nifH *PCR*

The screening of the 93 plate isolates by nested PCR yielded eight positives; however, further analyses (cloning/sequencing of *nifH*, primer construction, growth in SSDM tubes) showed that seven were false positives even though precautions were taken to avoid PCR contamination. This is worrisome as the approach developed by Zehr and co-authors (Zehr and McReynolds, 1989; Zani *et al*., 2000) has been widely used to investigate diversity and dynamics of putative N_2-fixing bacteria in aquatic environments (reviewed in Zehr *et al*., 2003a). Recently, it was found that PCR reagents might contain trace amounts of *nifH*-containing genomic DNA producing unwanted amplification products (Zehr *et al*., 2003b; Goto *et al*., 2005). Our results are in agreement with this. For instance, *nifH* sequences in nested PCR amplicons from the α-proteobacterial and Actinobacterial isolates BAL282–BAL285 were all closely related to nitrogenase genes in a *γ-proteobacterium* (Table 1) and could not be confirmed in genomic DNA by conventional PCR.

The consistency of the nested PCR protocol for detecting *nifH* genes in isolates was tested by running two nested PCRs on two occasions using the same batches of PCR reagents. The false positives yielded different results in the different runs (except for BAL285), while true positives capable of acetylene reduction (BAL281 and *A. vinelandii*) consistently yielded PCR products of correct size. Also, contamination of the negative control appeared inconsistent. These incoherent results emphasize the methodological difficulties associated with the extreme sensitivity of nested PCR. Polymerase chain reaction using specific *nifH* gene primers showed that further confirmation of the presence of *nifH* is necessary after positive amplification in order to eliminate potential false positives.

In conclusion, an essential prerequisite for making links or extrapolate from cultivation-dependent approaches to the natural environment is that experiments are conducted under conditions mimicking key aspects of the natural microenvironment of the bacteria, e.g. with respect to oxygen conditions. Future development of the approach presented here, focusing on isolations and pure-culture studies along with *in situ* DNA and expression analyses as well as activity measurements on bacteria growing in gradients, has the potential to contribute to the eventual goal of understanding the importance of heterotrophic bacteria to N_2 fixation in the sea.

Experimental procedures

Sampling

Sampling was performed in the Baltic Sea, which is a large relatively eutrophic estuary. Samples were collected from the cooling water (inlet at ~4 m depth) of the ferry M/S *Thjelvar* (141 m long, 23 m wide), on 16 occasions between 21 May 2001 and 22 October 2001 along a transect in the southern part of the Baltic Sea consisting of 11 sampling sites between Oskarshamn (mainland, N57°37′ E18°13′) and Visby (Island of Gotland, N57°16′ E16°30′) at the east coast of Sweden. Seawater samples for nucleic acids extraction (50 ml) were filtered onto 25 mm diameter, 0.2 μm Supor®-200 polyethersulfone membrane filters (PALL Corporation) and immediately frozen in liquid nitrogen. For bacterial enumeration, 50 ml of samples were fixed with 0.2 μm filtered formaldehyde (4% final) and stored at 4°C. Bacteria were enumerated by epifluorescence microscopy after DAPI staining (Porter and Feig, 1980) and cyanobacteria were viewed through autoflourescence.

Bacterial isolation on agar plates

For bacterial isolation, 100 μl of seawater was spread on low-nitrogen agar plates [4 M glucose, 8 mM KH_2PO_4,

0.64% agarose in 0.2 μm filtered Baltic summer seawater ($NO_3^- \leq 0.05$ μM), autoclaved at 121°C for 20 min] and grown in the dark at 15°C. An average of five isolates with different morphology from each sampling station were transferred and streaked on ZoBell agar plates (ZoBell, 1963). From each sampling date, six isolates with different morphology were further clean-streaked, resulting in 93 isolates from the 16 sampling occasions.

Growth in liquid media

Isolates were analysed for the ability to grow in nitrogen-free diazotroph medium [Medium 441, Deutche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)] with changes as follows: (i) yeast extract and vitamin solution were excluded, (ii) HPLC grade water (Sigma) was used, (iii) in solution A 270 ml of the water was exchanged with artificial seawater (17.70 g of NaCl, 1.50 g of Na_2SO_4, 0.08 g of $NaHCO_3$, 0.20 g of KCl, 0.04 g of KBr, 1.23 g of $MgCl_2{\cdot}2H_2O$, 0.40 g of $CaCl_2{\cdot}2H_2O$, 8.0 mg of H_3BO_3, 1 l of HPLC water), (iv) the trace element solution used consisted of 2.86 g of H_3BO_3, 1.81 g of $MnCl_2{\cdot}4H_2O$, 0.222 g of $ZnSO_4{\cdot}7H_2O$, 0.039 g of $Na_2MoO_4{\cdot}2H_2O$, 0.079 g of $CuSO_4{\cdot}5H_2O$ and 40.4 mg of $Cl_2Co{\cdot}6H_2O$ in 1 l of HPLC grade water, and (v) salinity was adjusted to S = 7 with HPLC grade water. Growth was monitored with a spectrophotometer, OD_{600} nm (Biowave CO8000, WPA) and with DAPI staining (Porter and Feig, 1980).

Oxygen sensitivity of N_2 fixation

Overnight culture (2 ml) (ZoBell medium) was centrifuged (4000 *g*, 5 min, 4°C) and washed twice with diazotroph medium. Semi-solid diazotroph medium was prepared from 100 ml of diazotroph medium (described above), 150 ml of HPLC grade water (Aldrich 27073-3) and 0.625 g of low-melt agarose (Sigma A9414). Semi-solid diazotroph medium was autoclaved and 12 ml was aseptically transferred to acid washed, sodium glass-tubes (VWR 109986-17). Tubes with medium were cooled to 35°C and 100 μl of the bacteria suspension was added, vortexed and left to solidify. Over time an oxygen concentration gradient was established in the tubes ranging from full air saturation at the medium surface to completely anaerobic conditions in deeper zones of the tube, governed by the slow diffusion of oxygen into the semi-solid medium and by bacterial respiration. Reference tubes containing medium with added nitrogen (0.5 mM NH_4Cl, final) served as controls. *Azotobacter vinelandii* (DSMZ 2289) and *C. pasteurianum* (DSMZ 525) served as positive, and *E. coil* (DSMZ 498) as negative control (see above). The tubes were incubated at 20°C for 3 days.

Oxygen profiles

Growth in the SSDM tubes was seen as a single horizontal sharp band located at various depths in the tubes. To determine the oxygen levels in these bands, oxygen profiles were measured with a Clark-type pO_2 microsensor with a guard cathode (Revsbech, 1989) connected to a picoammeter (PA2000, Unisense A/S, Århus, Denmark). The microsensors had a stirring sensitivity of < 1–2%. Linear calibration of the microsensor signal was performed from readings in the uppermost agarose layer (100% air saturation) and in deeper anoxic layers (0% oxygen) within the test tubes. Additional zero readings were obtained in medium made anoxic by addition of sodium dithionite. The microsensor was mounted on a computer-controlled motorized (Oriel, Stratford, USA) micromanipulator (Märzhäuser GmbH, Wetzlar, Germany). The measured signals were transferred to a computerized data acquisition system, which also controlled the micromanipulator (Profix, Unisense A/S, Denmark). The microsensor tip diameter was ~10 μm and vertical microprofiles of oxygen concentration were measured in the tubes in steps of 100 μm.

Cultivation of N_2-fixing bacteria in SSDM

To examine whether N_2-fixing bacteria could be isolated using SSDM tubes, seawater was collected from 3 m depth (total depth 10 m) at a station near the University of Kalmar in the strait between mainland and the Island of Öland (5 April 2005, N56°37 295, E16°21 846). Bacteria from 2 l of seawater were concentrated by filtration onto 0.2 μm polycarbonate filters (47 mm diameter), washed with 3 × 1 ml of diazotroph medium, and resuspended in 4 ml of diazotroph medium. Bacterial suspension (100 μl) was transferred to SSDM tubes (as above) and incubated in 20°C. After 10 days, colonies were isolated, purified and de-replicated on the basis of plate colony morphology and restriction enzyme cleavage (HaeIII and NdeII according to manufactures description, Roche) of the whole 16S rRNA gene.

Acetylene reduction assay

N_2 fixation by isolates was determined by acetylene reduction (Capone, 1993). Washed overnight cultures were mixed with SSDM (with and without added nitrogen; 0.5 mM NH_4Cl, final) in 20 ml acid-washed glass serum bottles, as described above for SSDM tubes. After the medium had solidified, the bottles were sealed, acetylene injected (~10% of headspace), and the samples were incubated at 20°C in the dark for 4 days. *Escherichia coli* and *A. vinelandii* served as reference bacteria. Ethylene production was measured using a gas-chromatograph (Autosystem 9000, Applied Biosystems) equipped with a flame ionization detector and a Porpack N glass column (Supelco) at 65°C, using N_2 as carrier gas.

DNA extraction, screening for nifH *genes and 16S rDNA sequencing*

Strains isolated from agar plates (93) were grown in ZoBell broth (5 g of peptone, 1 g of yeast extract, 15 g of agar, 800 ml of filtered seawater, 200 ml of Milli-Q water, autoclaved at 121°C for 20 min) overnight and DNA was extracted with the DNeasy Tissue kit (Qiagen) following the protocol for Gram-negative bacteria. All isolates were screened for the *nifH* gene by nested PCR using degenerate primers *nifH* 3 and 4 and 1 and 2 (Zehr and McReynolds, 1989; Zani *et al*., 2000). *Azotobacter vinelandii* was used as positive and

Table 3. Oligonucleotide sequences and reaction conditions.

Set name	Type	5′ to 3′ sequence (optimal concentration)	Temp./$MgCl_2$	Reference
nifH 1 and 2	Forward primer	*nifH*1F (2 μM) TGYGAYCCNAARGCNGA	54°C/2.5 mM	Zehr and McReynolds (1989)
	Reverse primer	*nifH*2R (2 μM) ANDGCCATCATYTCNCC		
nifH 3 and 4	Forward primer	*nifH*4F (2 μM) TTYTAYGGNAARGGNGG	54°C/4.0 mM	Zani and colleagues (2000)
	Reverse primer	*nifH*3R (2 μM) ATRTTRTTNGCNGCRTA		
BAL281	Forward primer	BAL281F (900 nM) CCGGAGCAGACGATGTAGATTT	54°C/5.5 mM	This study
	Reverse primer	BAL281R (50 nM) TCACCGCGATCAACTTCCTC		
	Probe used in real-time PCR	BAL281P (150 nM) 6-FAM-AAGTCGAGGTCGTCGTCATAGGCGC-TAMRA		
BAL286	Forward primer	BAL286F (200 nM) ACCGCAAGAAACGCTAATGG	55°C/1.5 mM	This study
	Reverse primer	BAL286R (200 nM) TCGAGGTCATCGGTATAGGCTT		

E. coli as negative control. Each 20 μl of PCR reaction consisted of 40 pmol of each primer (PAGE and HPLC purified, New England Biolabs; Table 3), 80 μM dNTPs, 4 mM $MgCl_2$, 20 ng of template, 2 μl of buffer and 0.4 U of Taq DNA polymerase (Roche). In the negative PCR control the template was replaced by water (Sigma W4502). Samples were amplified with a GeneAmp PCR System 2400 (Applied Biosystems) for 30 cycles (1 min at 94°C, 1 min at 54°C and 1 min at 72°C) with an initial denaturing step at 94°C for 2 min and a final extension step at 72°C for 7 min. The PCR products were analysed on a 1.8% agarose gel. Products with correct size (~472 bp) were further amplified using *nifH* 1 and 2 primers (Table 3). One microlitre of PCR product was transferred to a new 50 μl PCR mix (as above with the exception of 2.5 mM $MgCl_2$ and 5 μl of buffer). Mixing of reagents was done in a sterile flow bench, DNA was added in a PCR/UV workstation in a separate room (DNA/RNA UV-cleaner UVC/T, Talron Biotech) and single tubes (not strips) were used. All pipettes, tips and water were UV treated (20 min). Nested PCR products with the correct size (359 bp) were excised, gel-purified (QIAquick gel extraction kit, Qiagen) and cloned into the pGEM-T easy vector according to the manufacturer's instructions (Promega). A number of plasmid-containing clones were purified (QIAprep spin miniprep kit, Qiagen) and sequenced with the DYEnamic™ ET terminator cycle sequencing kit (Amersham Biosciences) using vector primer SP6 and an ABI PRISM 377 sequencer (Applied Biosystems) as described by the manufacturer. Bidirectional sequencing of the entire 16S rRNA gene from five positive isolates was performed using six different primers (27F, 530F, 926F, 1492 R, 907R and 519R, where numbers represent position of the 3′ end of the primer relative to *E. coli* numbering and letters indicate forward or reverse orientation). The sequences were aligned using the MegAline software (DNASTAR) and the CLUSTAL W method. Trees were constructed with the neighbour-joining algorithm in CLUSTAL X (Thompson *et al*., 1997) and examined with the TREEVIEWPPC software (Page, 1996).

Primer construction and nifH *gene verification*

Primers specific for *nifH* from positive clones were designed with the software Primer Express (Applied Biosystems). Each 50 μl of PCR reaction consisted of 10 pmol of each primer, 200 μM dNTP, 1.5 mM $MgCl_2$, 20 ng of template, 5 μl of buffer and 1 U of Taq DNA polymerase (Roche). The sample (isolate genomic DNA) and positive reference (*nifH* clone from each isolate) were amplified for 30 cycles (30 s at 94°C, 30 s at 55°C and 45 s at 72°C) with an initial denaturing step at 94°C for 2 min and a final extension step at 72°C for 7 min. Primers for BAL281 and BAL286 are shown in Table 3.

Real-time PCR on total community water

To determine the distribution of isolate BAL281 in Baltic seawater, samples from a transect (Visby to Oskarshamn, 11 samples, 21 May 2001) were analysed for the BAL281 *nifH* gene. Additional samples (11 July and 22 October 2001) from the location where BAL281 was originally isolated were also analysed (N57°15′7 E16°40′7). DNA was extracted from filters by SDS – enzymatic lysis followed by ethanol precipitation using tRNA as co-precipitant according to Boström and colleagues (2004) with the exception that 25 μg of tRNA was used per filter. Extracts from five filters were pooled, precipitated and resuspended in a final volume of 50 μl. A blank filter (no bacteria, extracted as the filters with seawater) served as a negative control.

For real-time PCR, the specific BAL281 *nifH* primers were combined with a specific probe designed using the software Primer Express (Applied Biosystems; Table 3). The high specificity of the BAL281 *nifH* primer probe set was confirmed in several test runs using five different clones from the present study (43–87% similarity to the *nifH* target sequence) and *A. vinelandii* genomic DNA as negative controls. No unspecific amplification was detected. Samples were amplified with a ABI PRISM®7700 Sequence Detector using a PCR mixture (25 μl) consisting of 5.5 mM $MgCl_2$, 1 mM dNTP,

900 nM forward BAL281 *nifH* primer and 50 nM reverse BAL281 *nifH* primer (151 bp product), 150 nM BAL281 *nifH* probe, 1 μl of DNA, 0.25 U of AmpErase UNG enzyme, 2.5 μl of TaqMan buffer and 0.625 U of AmpliTaq Gold (Applied Biosystems). The sample was amplified for 2 min at 50°C and for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 54°C. A linearized plasmid (pGEMT-easy, Promega) with a BAL281 *nifH* gene insert was used as template for the standard curve ranging from 2.2 to 2.2×10^7 copies per sample. Given the volume seawater filtered and the amount of sample DNA used in the PCR, the lower boundary of the standard curve corresponds to a sample detection limit of ~500 *nifH* gene copies per litre seawater.

Amplification efficiency was measured for each sample by amplifying a mix of sample (1 μl) and standard (2.2×10^5 copies, 1 μl) as described above. Efficiency was calculated according to Short and colleagues (2004) from the formula $X_n = X_0 \times (1 + E_x)^n$ where X_n is number of molecules at cycle n, X_0 is the initial molecule number, E_x is the amplification efficiency and n is the number of cycles (C_t). X_n was first calculated in the amplification with only standard (using $E_x = 1$). Using this number (X_n), the efficiency was then calculated relative to the X_n obtained from the amplified mix of standard and sample. All samples, standards, no-template control and efficiency control were made in triplicate PCR reactions. A blank filter (with no bacteria), handled exactly as sample filters, produced a weak real-time PCR signal, which was subtracted from all samples.

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