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Isolation and physiological characterization of psychrophilic denitrifying bacteria from permanently cold Arctic fjord sediments (Svalbard, Norway)

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Summary

A large proportion of reactive nitrogen loss from polar sediments is mediated by denitrification, but microorganisms mediating denitrification in polar environments remain poorly characterized. A combined approach of most-probable-number (MPN) enumeration, cultivation and physiological characterization was used to describe psychrophilic denitrifying bacterial communities in sediments of three Arctic fjords in Svalbard (Norway). A MPN assay showed the presence of 10^3–10^6 cells of psychrophilic nitrate-respiring bacteria g^{-1} of sediment. Fifteen strains within the *Proteobacteria* were isolated using a systematic enrichment approach with organic acids as electron donors and nitrate as an electron acceptor. Isolates belonged to five genera, including *Shewanella*, *Pseudomonas*, *Psychromonas* (*Gammaproteobacteria*), *Arcobacter* (*Epsilonproteobacteria*) and *Herminiimonas* (*Betaproteobacteria*). All isolates were denitrifiers, except *Shewanella*, which exhibited the capacity for dissimilatory nitrate reduction to ammonium (DNRA). Growth from 0 to 40°C demonstrated that all genera except *Shewanella* were psychrophiles with optimal growth below 15°C, and adaptation to low temperature was demonstrated as a shift from primarily C16:0 saturated fatty acids to C16:1 monounsaturated fatty acids at lower temperatures. This study provides the first targeted enrichment and characterization of psychrophilic denitrifying bacteria from polar sediments, and two genera, *Arcobacter* and *Herminiimonas*, are isolated for the first time from permanently cold marine sediments.

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Introduction

Nitrogen is a major limiting nutrient of biological productivity in the coastal ocean (Rabalais, 2002; Howarth and Marino, 2006). The response of the nitrogen cycle to anthropogenic disturbances may be strongly influenced by the phylogenetic structure and physiological tolerances of microbial communities responsible for nitrogen loss in coastal marine ecosystems. Two microbially catalysed respiration processes, denitrification and anaerobic ammonium oxidation (ANAMMOX), convert dissolved inorganic nitrogen (NO_3^-, NO_2^-, NH_4^+) to gaseous N_2 and comprise the largest sink of reactive nitrogen from the coastal ocean on a global scale. Up to 50% of marine N removal is estimated to occur by denitrification and anammox in continental shelf sediments, with the remainder occurring in deep sea sediments and oxygen minimum zones (Codispoti, 2007). The relative contribution of sedimentary denitrification and anammox to N removal varies strongly with water column depth, but denitrification is generally considered the dominant pathway for N removal in shallow (< 100 m) shelf sediments (Dalsgaard *et al.*, 2005).

The Arctic Ocean is the shallowest of the world's ocean basins and is comprised of 50% continental shelf. Substantial denitrification and anammox rates have been measured on Arctic shelves, indicating that the Arctic basin has a substantial role in global N removal (Devol *et al.*, 1997; Rysgaard *et al.*, 2004; Gihring *et al.*, 2010). Future reductions in Arctic sea-ice cover may lead to altered fluxes of organic matter to sediments, resulting

Table 1. Description of the sampling stations, sediment characteristics and enumeration of nitrate-respiring bacteria using a most probable number (MPN) serial dilution assay.

Sample site (abbreviation)	Latitude/longitude	Depth	Sediment temperature	Sediment C : N	Denitrification rate (μmol N m^{-2} day^{-1})	MPN (cells g^{-1}) (95% confidence intervals)
Kongsfjörden (KF)	78°59.43′N 12°17.87′E	51 m	1.3°C	11[a]	34 (± 12)[a]	6.1×10^5 (1.5×10^5–2.5×10^6)
Smeerenbergfjörden (SM)	79°42.01′N 11°05.20′E	211 m	1.6°C	7.2[a]	289 (± 5)[a]	2.4×10^3 (56–1.0×10^3)
Ymerbukta (YM)	78°16.84′N 14°02.97′E	Intertidal	6.5°C	19.4	ND	3.0×10^6 (4.1×10^5–2.2×10^7)

a. Referenced from Gihring and colleagues (2010).
ND, not determined.

in major shifts in the biogeochemical cycling of nitrogen (Piepenburg, 2005; Arrigo *et al*., 2008). Thus, an understanding of the diversity and physiology of denitrifying bacteria from polar sediments is integral to understanding climate change related effects on nitrogen cycling in the Arctic.

Though sedimentary denitrification comprises an important N sink in marine ecosystems on a global scale and the majority of the seafloor is cold (< 5°C), few studies have addressed the physiological adaptation of denitrifiers to cold temperatures. Arctic shelf sediments are characterized by permanently cold conditions, but rates of microbial metabolism (e.g. hydrolysis, oxygen respiration and sulfate reduction) from Arctic sediments largely overlap with those of temperate sediments (Arnosti *et al*., 1998; Thamdrup and Fleischer, 1998; Kostka *et al*., 1999). This apparent lack of temperature limitation has been ascribed to the fact that microbes in these sediments are psychrophilic (see Morita, 1975). The permanently cold conditions in Arctic sediments may exert a strong selection for psychrophilic bacteria, but isolation of aerobic bacteria from Arctic sediments has yielded a mix of psychrophilic and psychrotolerant organisms (Groudieva *et al*., 2004; Helmke and Weyland, 2004; Srinivas *et al*., 2009). Denitrifying bacteria have been isolated from cold (≤ 4°C) marine waters from temperate environments under anaerobic conditions with nitrate as an electron acceptor (Brettar *et al*., 2001), but to date, no study has systematically investigated psychrophilic denitrifying bacteria in permanently cold sediments.

Shallow sediments in the Arctic Ocean basin are active sites of denitrification, but the microbial communities mediating this process are understudied. Cultivation-independent methods have been used to study the community structure of denitrifying bacteria in coastal marine sediments from primarily temperate ecosystems (Braker *et al*., 2001; Mills *et al*., 2008), but horizontal gene transfer events of denitrification genes make it difficult to reconstruct phylogenies (Heylen *et al*., 2006a). Furthermore, primer coverage issues suggest that many organisms capable of denitrification are not identified in environmental surveys (e.g. Green *et al*., 2010). Therefore, cultivation of representative denitrifying bacteria is a crucial component to improving detection of environmentally relevant taxa by cultivation-independent approaches. A better understanding of the physiology of psychrophilic denitrifying bacteria is also a necessity to better predict the role of low temperature in controlling denitrification activity in polar sediments. In the present study, a primarily cultivation-based approach was used to investigate the ecology and physiology of psychrophilic denitrifying bacteria from Arctic fjord sediments. The main objectives of this study were to isolate and phylogenetically characterize psychrophilic denitrifying bacteria and examine the physiology of low temperature adaptation in representative genera of psychrophilic denitrifying isolates.

Results

Characterization of in situ *communities*

Cultivatable nitrate-respiring microorganisms were enumerated using an most-probable-number (MPN) serial dilution assay at each site. MPN counts were 2.4×10^3 cells g^{-1} wet sediment, 6.1×10^5 cells g^{-1} wet sediment and 3.0×10^6 cells g^{-1} sediment at Smeerenbergfjörden (SM), Kongsfjörden (KF) and Ymerbukta (YM) respectively (Table 1). Growth by nitrate respiration in the MPN tubes was inferred from higher turbidity as compared with control tubes (lactate only), as well as depletion of nitrate and transient accumulation of N_2O. Aerobic plates from the highest positive dilutions displayed either one or two distinct colony morphologies. Isolation of these colonies, followed by SSU rRNA gene sequencing and BLAST searches, indicated that the most enriched strain at KF and YM was closely related to *Psychromonas* spp., while *Shewanella* spp. and *Psychromonas* spp. were highly enriched at SM.

The sediment bacterial community composition was analysed by terminal restriction fragment length polymorphism (TRFLP) analysis of bacterial SSU rRNA genes amplified from extracted Genomic DNA (gDNA). The TRFLP profiles from the SM and KF sediments were

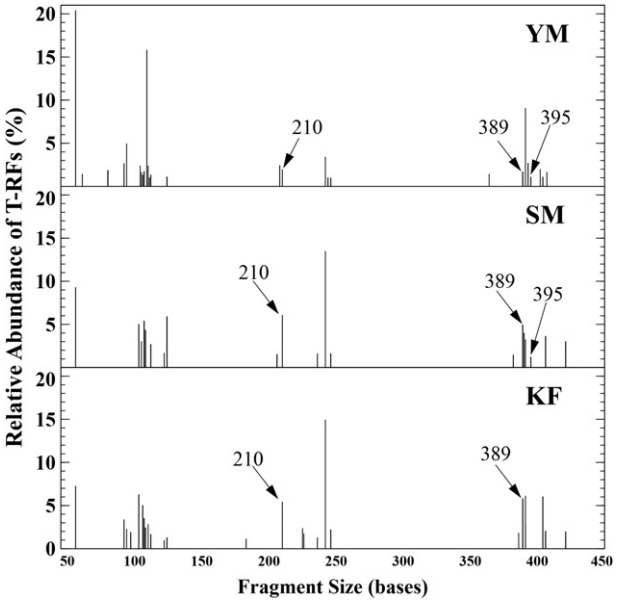

Fig. 1. Electropherograms of terminal restriction fragments of bacterial SSU rRNA amplicons generated for each of the sample sites showing the detection of phylogenetic groups affiliated with denitrifying bacteria isolated in this study. Peaks that were tentatively matched to isolated strains included *Shewanella* sp. (210), *Pseudomonas* sp. (389) and *Arcobacter* sp. (395). Sample site is indicated on each panel: YM, Ymerbukta; SM, Smeerenbergfjorden; KF, Kongsfjorden.

highly similar and indicative of a highly similar community structure for the two sediments (Fig. 1). For both sites, the most dominant peaks were seen at fragment sizes of 56, 103, 107, 210, 242 and 389 base pairs (bp). The TRFLP profile from the YM site differed from those of the SM and KF sediments, and was indicative of a distinct community structure. The most dominant peaks were at fragment sizes of 56, 109, 242 and 391 bp. An *in silico* digest of SSU rRNA gene sequences from the isolates obtained in this study (see following section) showed that peaks from all three sites at 210 and 389 bp matched the predicted fragment sizes from *Shewanella* and *Pseudomonas* respectively. A peak at 395 bp corresponding to *Arcobacter* was observed at sites SM and YM (Fig. 1).

Isolation and phylogenetic characterization

A systematic enrichment strategy was used to isolate putative denitrifying bacteria from one intertidal and two permanently cold sediments. The most rapid growth was observed in the serum vials amended with sediments from SM, followed by YM and KF. Visual observation of the plates indicated an abundance of slow growing, small colonies and fewer, fast growing, slightly pigmented colonies. More than 200 colonies were obtained from each enrichment, and by selecting isolates with different colony morphology and growth pattern, a total of 15 colonies were selected for further screening. The isolates belonged to the *Betaproteobacteria* (1 isolate; *Herminiimonas*), *Gammaproteobacteria* (8 isolates; *Pseudomonas*, *Psychromonas*, *Shewanella*) and the *Epsilonproteobacteria* (6 isolates; *Arcobacter*), and were 93–99% similar to previously described species based on BLAST comparison of SSU rRNA gene sequences (Table 2). A phylogenetic tree

Table 2. Phenotypic and genotypic characterization of denitrifying isolates.

Genus (phylum) and isolate	Sample site	Electron donor	^{15}N-N_2 production	Acetylene block	NH4+ production	Closest isolate by BLAST (accession number)	BLAST % similarity
Herminiimonas (*Betaproteobacteria)*							
SP-B	SM	APB	ND	+	–	*H. fonticola* S-94 (AY676462)	97%
Arcobacter (*Epsilonproteobacteria*)							
KLS-1	KF	Lactate	+	ND	–	*A. nitrofigilis* CI (L14627)	94%
SAS-1	SM	Acetate	+	+	–	*A. nitrofigilis* CI (L14627)	93%
SL-3	SM	Lactate	+	ND	–	*A. nitrofigilis* CI (L14627)	95%
Y2S	YM	APB	+	ND	–	*A. venerupis*F67-11 (HE565359)	97%
YAPB-1	YM	APB	+	ND	–	*A. venerupis*F67-11 (HE565359)	97%
YAS-1	YM	Acetate	+	+	–	*A. venerupis*F67-11 (HE565359)	97%
Pseudomonas (*Gammaproteobacteria*)							
SL-1	SM	Lactate	+	+	–	*P. brenneri* (AF268968)	99%
SLB-2	SM	Lactate	+	ND	–	*P. frederiksbergensis* (AJ249382)	98%
UL-1	SM	Lactate	+	+	–	*P. migulae* (AF074383)	99%
Psychromonas (*Gammaproteobacteria*)							
SL-2	SM	Lactate	+	–	–	*P. ingrahamii* 37 (CP000510)	99%
Y2B	YM	APB	+	–	–	*P. boydii* (FJ822615)	99%
Shewanella (*Gammaproteobacteria*)							
KLB-1	KF	Lactate	–	–	–	*S. vesiculosa*(AM980877)	99%
SLB-1	SM	Lactate	–	–	+	*S. frigidimarina*(AJ300833)	99%
YLB-1	YM	Lactate	–	–	+	*S. vesiculosa*(AM980877)	99%

The closest validly described isolate by BLAST is given for identification purposes.
ND, not determined; APB, combination of acetate, propionate and butyrate.

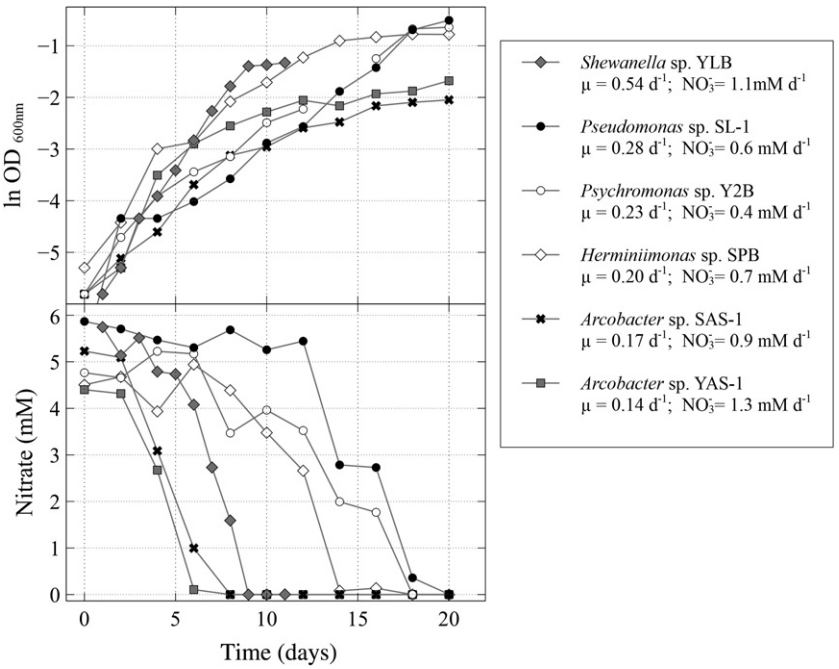

Fig. 2. Growth and nitrate utilization of selected psychrophilic denitrifiers at 5°C under denitrifying conditions (10 mM lactate, 5 mM NO_3^-). The average specific growth rate (μ) and nitrate utilization rate (mM day^{-1}) are given to the right of the figure.

of the isolates provided further confirmation of their taxonomic affiliation (Fig. S1).

Denitrification activity and optimal growth temperature

Denitrification capacity was confirmed in 12 of the 15 isolates by production of ^{15}N-labelled N_2, and near-stoichiometric conversion of nitrate to N_2O was observed in 10 isolates using the acetylene block method. (Table 2 and Table S2). The *Psychromonas* isolates did not show complete stoichiometric conversion of nitrate to N_2O with the acetylene block method but did produce ^{15}N-labelled N_2 (Table S2). The three isolates belonging to the genus *Shewanella* produced only small amounts of N_2O, and two of the *Shewanella* isolates were capable of dissimilatory nitrate reduction to ammonium.

Based on the phylogenetic analysis, six strains were selected (SL-1, Y2B, YAS-1, SAS-1, YLB, SP-B), for further physiological characterization (Fig. S1). The isolates were grown at 5°C in MSW media with 5 mM NO_3^- and 10 mM lactate, and the complete depletion of nitrate concomitant with exponential growth was observed (Fig. 2 and Fig. S2). Isolates from the *Gammaproteobacteria* had the highest specific growth rates (Fig. 2), with *Shewanella* sp. YLB-1 growing fastest (μ, 0.54 day^{-1}), followed by *Pseudomonas* sp. SL-1 (μ, 0.28 day^{-1}) and *Psychromonas* sp. Y2B (μ, 0.23 day^{-1}). Growth rates were lowest for the *Herminiimonas* sp. SPB isolate (0.20 day^{-1}) and both *Arcobacter* isolates (0.14–0.17 day^{-1}). The rate of nitrate utilization during exponential growth was highest in *Arcobacter* sp. SAS-1, *Shewanella* sp. YLB and *Arcobacter* sp. YAS-1.

All representative strains had optimal growth temperatures of 15°C or less, except *Shewanella* YLB-1, which had an optimal growth temperature of 18°C (Fig. 3). Strains of *Psychromonas*, *Herminiimonas*, and *Pseudomonas* exhibited growth rates at 0°C that were 50% of the optimal growth rate, whereas, *Arcobacter* and *Shewanella* had growth rates at 0°C that were 25% of the optimal growth rate. Growth was not observed in any of the strains above 30°C, and two strains, *Psychromonas* Y2B and *Herminiimonas* SP-B, did not grow above 25°C.

Fatty acid methyl ester profiles

Representative isolates were grown in MSW medium at 1.5°C, 5°C and 15°C under aerobic conditions to examine the acclimation of membrane fatty acid composition to low temperature (Table 3). At all growth temperatures, the primary FAMEs detected in all isolates were C16:0, 16:1ω7c and 18:1ω7c. These three fatty acids comprised greater than 95% of the total extracted fatty acids in strains Y2B (*Psychromonas*), SL-1 (*Pseudomonas*) and SPB (*Herminiimonas*). In addition to 16:1ω7c and 18:1ω7c, strains YLB-1 (*Shewanella*), YAS-1 (*Arcobacter*) and SAS1-1 (*Arcobacter*) also contained significant amounts of C14:0 (3–5%) and 14:1ω7c (1–8%). Both *Arcobacter* strains contained significant amounts of 16:1ω7t (5–10%) which was not present in any other strains. *Shewanella* strain YLB-1 had the most diverse fatty acid profile and was the only strain that contained branched fatty acids (20–28%), as well as eicoaspentaenoic acid (20:5ω3).

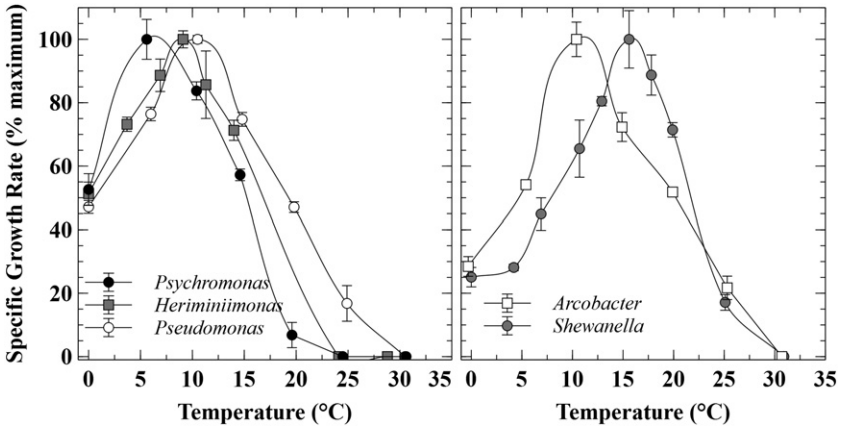

Fig. 3. Temperature response of growth of representative denitrifying isolates under denitrifying conditions (10 mM lactate, 5 mM NO_3^-). Error bars represent the standard deviation of triplicate measurements.

With decreasing growth temperatures, all strains except *Shewanella* YLB-1 exhibited a decrease in the relative abundance of the most abundant saturated fatty acid, C16:0. *Arcobacter* and *Herminiimonas* strains also exhibited a lower concentration of 18:1ω7c with lowered temperature. Concomitant with the relative decrease in saturated and long chain fatty acids at low temperature, increases in monounsaturated acids were observed that showed variation with respect to strain. *Psychromonas* Y2B and *Herminiimonas* SP-B exhibited an increase primarily in 16:1ω7c, while *Arcobacter* strains increased 14:1ω7c and 16:1ω7c. *Pseudomonas* SL-1 increased 16:1ω7c and 18:1ω7c in response to lowered temperature. *Shewanella* YLB-1 exhibited unique shifts in fatty acids with lowered growth temperature, including increases in C16:0 and 17:1ω8c and decreases in branched (i13:0–i15:0) fatty acids and 14:1ω7c.

Discussion

Denitrification is well recognized as a dominant pathway for the removal of reactive nitrogen in marine sediments, including polar sediments. However, no prior cultivation based studies have targeted denitrifying bacteria in permanently cold marine sediments. Previous enrichment studies from Arctic sediments have often been conducted under aerobic conditions, using complex cultivation media, short incubation times and incubation temperatures above *in situ* values (Srinivas *et al*., 2009; Kim *et al*., 2010; Yu *et al*., 2010). In this study, denitrifying bacteria were anaerobically enriched in a minimal medium with defined electron donors. Enrichments were carefully maintained at *in situ* temperatures and incubation times were lengthened (> 30 days) to mimic *in situ* conditions. This approach allowed for the isolation of taxa whose role in denitrification may have previously been overlooked.

Characterization of in situ *denitrifying communities*

Most-probable-number (MPN) enumeration indicated the presence of 2×10^3–3×10^6 cells of nitrate-respiring bacteria g^{-1} of sediment. Quantification of total bacterial abundance by direct counts in Svalbard surface sediments has shown the presence of 2×10^8–3×10^9 cells cm^{-3} of sediment, and site SM has been determined to have 2.1–4.7 $\times 10^9$ cells cm^{-3} (Sahm and Berninger, 1998; Ravenschlag *et al*., 2001). From these results, the relative abundance of denitrifying bacteria can be estimated to contribute between less than 0.01% to 1.5% of the total community. The relative abundance of denitrifying bacteria was similar (0.17%) for temperate estuarine sediments using a MPN-based approach, but the same study found up to two orders of magnitude more denitrifying bacteria using qPCR-based functional gene analysis (Michotey *et al*., 2000). Differences in denitrifying MPN cell numbers between sites did not directly correspond with reported denitrification rates. While site SM exhibited high rates of denitrification, it also had a lower number of cultivatable denitrifying bacteria than site KF. The choice of lactate as an electron donor for the MPN experiment may have biased the growth in SM sediments, and also, the use of only an organic electron donor may have limited the growth of autotrophic denitrifying bacteria. Site YM had the highest number of denitrifying cells (3.0×10^6), which may have been influenced by the input of macroalgal detritus in the intertidal zone. The C : N ratio of 19.4 ratio at site YM falls near the median value reported for macroalgae (Atkinson and Smith, 1983). It is clear from these results that further work is necessary to fully elucidate the population size and activity of denitrifying bacteria in marine sediments.

Based on an *in silico* digest of SSU rRNA gene sequences from our isolates, three isolates (*Shewanella*, *Pseudomonas*, *Arcobacter*) were putatively detected in the TRFLP profiles from the fjord sediments. All of the

Table 3. Membrane fatty acid composition (% distribution) of representative psychrophilic denitrifying isolates cultivated at a range of temperatures.

Isolate	YLB-1 *Shewanella*			Y2B *Psychromonas*			YAS-1 *Arcobacter*			SAS-1 *Arcobacter*			SL-1 *Pseudomonas*			SP-B *Herminiimonas*		
Temperature (°C)	**1.5**	**5**	**15**	**1.5**	**5**	**15**	**1.5**	**5**	**15**	**1.5**	**5**	**15**	**1.5**	**5**	**15**	**1.5**	**5**	**15**
Saturated																		
C12:0	0.4	0.5	1.5				0.1	0.1	0.4	0.6		0.2						
C14:0	**3.5**	**2.7**	**3.7**	0.6	0.5	0.6	**4**	**4.2**	**4.7**	**4.6**	**5.3**	**4.4**	0.5	0.5	0.6	0.2	0.2	0.1
C15:0	**5.9**	**4.6**	**4.6**	0.1	0.3	0.1						0.1	0.2	0.4	0.1			
C16:0	**15.7**	**12.5**	**12.6**	**15.4**	**17.7**	**23**	**9.8**	**11.3**	**12.6**	**9.5**	**10.5**	**13.1**	**15.6**	**19.1**	**22.7**	**15.2**	**16.9**	**16.1**
C17:0	2.4	1.8	1.1		0.1									0.1				
Branched																		
i13:0	**5.9**	**6.1**	**9.9**															
i14:0	**0.7**	**3.3**	**3.6**															
i15:0	**9.2**	**12.7**	**12.5**															
Unsaturated																		
14:1ω7c	**0.8**	**1.9**	**4.9**	0.2	0.1	0.1	**7**	**5.5**	**5.1**	**8.1**	**6.6**	**4.2**	0.1	0.1	0.1			
15:1ω8c	1.6	1.1	1.3	0.1	0.1								0.1	0.1				
16:1ω9c	1.5	1.1	1														0.1	0.1
16:1ω7c	**25.8**	**27.6**	**25.6**	**67.2**	**62.7**	**58.9**	**56.9**	**57.1**	**48.6**	**52.2**	**58.9**	**50.6**	**65.7**	**61.4**	**60.7**	**72.3**	**66.8**	**60.5**
16:1ω7t	0.4						**7.3**	**4.5**	**9.6**	**8.1**	**ND**	**6.6**						
16:1ω5c		0.2	0.2				2.5	2.1	1.7	2.5	2.1	1.7					0.5	0.5
17:1ω8c	**9.9**	**8.1**	**5.6**	0.1	0.2	0.1						0.1	0.2	0.3	0.1	0.1		0.1
18:1ω7c	**4.4**	**5.3**	**4.4**	**15.8**	**16.6**	**15.9**	**11.7**	**14.5**	**16.1**	**14**	**15.4**	**18.1**	**17.3**	**16.8**	**14.4**	**10.3**	**11.8**	**17.6**
19:1ω6c	0.4	0.4	0.4				0.1	0.2	0.1	0.1	0.1	0.1				0.9	3	3.9
20:5	1.7	2	1.5															
Σ*X*:1	49.7	51.5	47.1	83.5	80.3	75.7	85.7	84.1	81.5	85.1	83.5	81.8	83.6	79.7	76.2	84.2	79.5	79.4

Bold numbers indicate fatty acids with a cumulative contribution of at least 90% of the total. Fatty acids that contributed less than 1% in all samples are not shown.
ND, not determined.

genera isolated in this study except *Herminiimonas* have been previously detected in polar marine sediments in SSU rRNA gene clone libraries. Bowman and colleagues (2003) observed a relative sequence abundance of 5–10% *Shewanella* and 2–5% *Psychromonas* in clone libraries from the top 1 cm of Antarctic coastal sediments. Members of *Shewanella* and *Pseudomonas* have also been detected in clone libraries from surficial sediments (0–5 cm) in the Beaufort Sea (Li *et al*., 2009). At Svalbard, *Pseudomonas* was detected in sediments at Hornsund fjord (Ravenschlag *et al*., 1999) and *Shewanella*, *Psychromonas* and *Arcobacter* were detected in sediments near site KF in Kongsfjorden (Tian *et al*., 2009). These studies provide further evidence for the widespread presence of the genera isolated in this study in the surficial layers of permanently cold sediments.

Distribution of psychrophily and denitrification within the genera isolated

Isolates from the genus *Arcobacter* have been obtained from a variety of marine environments, including hydrothermal vents, tissue from mussels and the water column off the coast of Europe and Africa (Eilers *et al*., 2000; Huber *et al*., 2003; Levican *et al*., 2012). Reduction of nitrate to nitrite is ubiquitous within the genus *Arcobacter*, and complete denitrification has been confirmed for *Arcobacter* isolates from activated sewage sludge (Heylen *et al*., 2006b). However, the *Arcobacter* isolates from this study are the first reported denitrifying isolates from the marine environment, as well as the first reported psychrophilic strains (Table S3). Some strains of *Arcobacter* are able to oxidize sulfide to produce filamentous sulfur (Wirsen *et al*., 2002), which may be coupled to denitrification under anaerobic conditions (Lavik *et al*., 2009). Substantial rates of sulfate reduction have been measured in surface sediments at sites SM and YM (Arnosti and Jorgensen, 2006; Sawicka *et al*., 2010), which may supply sulfide for autotrophic denitrification by *Arcobacter* species.

Bacteria from the genus *Herminiimonas* have been previously reported to be psychrophilic; isolates were obtained from an Antarctic glacier (Garcia-Echauri *et al*., 2011), a deep (3042 m) Greenland glacial ice core (Loveland-Curtze *et al*., 2009), and Greenland sea ice brine (Møller *et al*., 2011). At least two other isolates of *Herminiimonas* have been shown to reduce nitrate, and the *Herminiimonas arsenicoxydans* genome contains the *nirK* gene (Muller *et al*., 2006; Lang *et al*., 2007). The isolate *Herminiimonas* SP-B from this study is the first confirmed denitrifying *Herminiiomonas* isolate from marine sediments, which broadens the potential functional role of *Herminiimonas* in marine ecosystems.

Members of the *Gammaproteobacteria* have been isolated and described from a wide range of oceanic regions, including deep-sea and polar sediments. Nearly all described species of the genus *Psychromonas* are psychrophilic, and isolates are readily obtained under aerobic conditions from sea-ice, marine water columns and sediments (Groudieva *et al*., 2003; Auman *et al*., 2006; Nogi *et al*., 2007). While nitrate reduction to nitrite is common within the genus, the only previous evidence for complete denitrification in *Psychromonas* is nitrite reduction by *Psychromonas hadalis* (Nogi *et al*., 2007) and the presence of nitrous oxide reductase genes in *Psychromonas ingrahamii* (Markowitz *et al*., 2012). Complete denitrification has already been confirmed for at least two *Shewanella* isolates from the marine environment (Brettar *et al*., 2002; Zhao *et al*., 2006). However, the marker gene for dissimilatory reduction to ammonium (*nrfA*) is more widespread within the genus (Simpson *et al*., 2010), which is consistent with the results from this study showing only DNRA capacity in the *Shewanella* isolates. Pseudomonads are ubiquitous in marine sediments, and the genus contains many denitrifying representatives (Zumft, 1997).

Adaptation of denitrifying bacteria to low temperatures

In the present study, low temperature adaption was confirmed in psychrophilic denitrifying bacteria by growth, nitrate depletion, and by a comparison of membrane lipid composition at low temperature. The optimal growth temperatures and high rates of growth at 0°C (25–50% of Topt) of the current isolates reflect the highly psychrophilic nature of our isolates. For all isolates except *Shewanella* YLB-1, we observed optimum temperatures for growth (Topt) that were amongst the lowest reported for the genera (Table S3). *Shewanella* was the only genus in the present study to perform DNRA rather than denitrification, and *Shewanella* YLB-1 also exhibited the highest optimal growth rate (18°C). Previous cultivation-based studies of estuarine sediments have demonstrated an increased importance of DNRA under warmer conditions, which may be explained by a higher affinity for nitrate at low temperature in denitrifying bacteria versus DNRA bacteria (King and Nedwell, 1984; Ogilvie *et al*., 1997).

A comparison of the three most abundant fatty acids (C16:0, C16:1, C18:1) from our isolates to literature values show the highest values of C16:1 unsaturated fatty acids in our isolates grown at 5°C (Table S3). Very few psychrophilic isolates have been grown at 5°C or less for FAME analysis, which precludes a fair comparison between our strains and previously isolated psychrophiles. We note the presence of a similar FAME profile for *P. ingrahamii* tested at 4°C (Auman *et al*., 2006). A decrease in the saturated fatty acid C16:0 and

an increase in C16 monounsaturated fatty acids with decreasing growth temperature was the main adaptation consistent amongst all taxa except *Shewanella* YLB-1. These results are consistent with previous studies that have demonstrated the importance of monounsaturated fatty acids for low temperature growth (Allen *et al*., 1999; Kiran *et al*., 2004). The genus *Shewanella*, in contrast, uses a strategy that involves regulating branched fatty acids and eicosapentaenoic acid in addition to monounsaturated fatty acids (Wang *et al*., 2009).

Conclusion

This study reports the first systematic enrichment of psychrophilic bacteria under denitrifying conditions in permanently cold marine sediments. The taxa isolated in this study are routinely detected by cultivation-independent techniques in surficial sediments, but only *Pseudomonas* and *Shewanella* species have been previously recognized in marine sediments for their ability to denitrify. The genera *Arcobacter* and *Herminiimonas* have not been previously isolated from permanently cold marine sediments, and there are no reports of psychrophilic marine *Arcobacter* strains. Further cultivation-independent studies are needed to confirm that the isolates from this study are the primary taxa that perform denitrification *in situ*. These results confirm the strongly psychrophilic nature of the present isolates and corroborate the hypothesis that denitrification activity in permanently cold sediments is maintained at relatively high levels due to the activity of psychrophilic bacteria.

Experimental procedures

Sample sites and sampling procedures

Sediment cores were collected in August 2008 from three fjord sites within the Svalbard archipelago (Table 1). At the time of collection, sediment surface temperatures ranged from 1.3–6.5°C. Sediments from Smeerenburgfjorden (SM) were black clayey and rich with organic matter, while the sediments from Ymerbukta (YM) and Kongsfjorden (KF) were black sandy and reddish-brown loamy respectively. Sediment cores were retrieved with a Haps corer, and subsamples from the upper 0–5 cm depth interval were collected aseptically into sterile conical tubes. Samples for cultivation were transported at 4°C and stored at 1.5°C until processed. Samples for molecular characterization were frozen immediately and stored at −80°C until further analysis.

Enrichment and isolation of denitrifying bacteria

A bicarbonate buffered minimal saltwater medium (MSW) was prepared and dispensed according to Widdel and Bak (1992), with the modifications of omitting sulfate, resazurin, selenite and tungstate. The medium contained the following components per litre: NaCl (20 g), NH_4Cl (0.250 g), KH_2PO_4 (0.200 g), KCl (0.5 g), $MgCl_2{\cdot}6H_2O$ (3.0 g) and $CaCl_2{\cdot}2H_2O$ (0.150 g) $NaHCO_3$ (2.5 g), trace element solution (TES; 1 ml), vitamin B_{12} (1 ml), vitamin mix (1 ml) and thiamine (1 ml). The composition of vitamin solutions and trace element solution are given in Table S1. The medium was autoclaved and poured under strictly anoxic conditions with a $N_2 : CO_2$ (80:20) headspace, resulting in a final pH of 7.0. All enrichments and physiological screening of the isolates was conducted in this medium with modifications to the electron donor and NO_3^- concentration as indicated.

Enrichment experiments were conducted with 1 mM NO_3^- as the electron acceptor and with either acetate (10 mM), lactate (10 mM), or a APB (acetate, propionate, butyrate, 10 mM each) as the source of carbon and energy. Enrichments were inoculated with 10% (w/v) sediment from each sample site and incubated in the dark at 1.5°C. Enrichments were transferred to fresh medium every 10 days using a 10% inoculum (v/v). All isolates were obtained from the fourth transfer of the enrichments. After the second transfer, the concentration of NO_3^- was raised from 1 mM to 5 mM in order to prevent growth limitation and cell lysis by nitrate depletion.

For isolation and purification, the MSW medium was supplemented with 10 mM Hepes (Fisher Scientific) and 1.8% molecular grade agar (Sigma-Aldrich) as a buffering and solidifying agent respectively. Streak plates were prepared and incubated at 1.5°C under aerobic conditions. Morphologically distinct colonies were picked using sterile toothpicks and purified by multiple re-streakings onto fresh plates. The purity of each culture was reconfirmed by PCR amplification and sequencing of the small subunit (SSU) ribosomal RNA (rRNA) gene. Culture stocks were preserved at −80°C in 20% glycerol.

Purified isolates were screened for nitrate depletion and gaseous nitrogen production under denitrifying conditions in anaerobic MSW medium amended with ^{15}N-enriched NO_3^- (98 atom %; Cambridge Isotope Laboratories, Andover, MA). Cultures and uninoculated controls were prepared in 10-ml Hungate tubes. At the initial time-point (immediately after inoculation) and after maximum cell density was achieved, growth was terminated in duplicate cultures by the addition of 1% (w/v) $HgCl_2$. The production of N_2 was determined by the accumulation of excess ^{15}N-N_2 using a membrane inlet mass spectrometer configured and calibrated according to An and colleagues (2001). Nitrate depletion was confirmed using a colorimetric method (Cataldo *et al*., 1975).

Isolates were also screened for stoichiometric conversion of NO_3^- to N_2O using the acetylene block method (cf. Mahne and Tiedje, 1995). Isolates were grown in 180 ml

serum bottles with anaerobic minimal MSW medium with 10 mM lactate and 5 mM NO_3^-. For each isolate, duplicate bottles were amended with a 10% acetylene headspace and duplicate bottles were not amended with acetylene. Gas samples for N_2O analysis were extracted from the headspace through the rubber septa cap using a 100 μl gas-tight syringe and were immediately analysed by gas chromatography using a Shimadzu GC-8A gas chromatograph equipped with a Porapak-Q column and an electron-capture detector.

Most probable number enumeration

Psychrophilic nitrate-respiring bacterial populations from Arctic fjords were enumerated by the three-tube MPN assay using 10-fold serial dilutions of fjord sediments in MSW growth medium. Tubes were incubated at ambient sediment temperature (1.5°C) for two months. Lactate was chosen as the electron donor for the MPN experiments, based on the vigorous growth and taxonomic coverage in initial lactate-amended enrichments. Growth was monitored by culture turbidity, depletion of added nitrate, and accumulation of N_2O in the vial headspace as compared with nitrate-free controls. The MPN index was determined from statistical tables published by the American Public Health Association (1960).

Bacterial community profiling by TRFLP

Genomic DNA (gDNA) from frozen sediment grabs was extracted in triplicate using a Mo-Bio Power Soil™ DNA kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The SSU rRNA gene was amplified by PCR using the 27F and 1492R general bacterial primers (Lane, 1991). The forward primer (27F) was fluorescently labelled with 6-carboxy fluorescine (FAM) for TRFLP profiling. All PCR reaction volumes were 50 μl and contained the following concentrations of reagents: 1 μl DNA, 1× PCR buffer, 200 μM dNTP mixture, 0.25 μM primer, 0.05 U μl^{-1} Taq polymerase (EconoTaq Plus, Lucigen Corporation, Middleton, WI), with 10–50 ng input gDNA. Thermocycling was performed with a 95°C incubation for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 2 min, with a final extension step at 72°C for 10 min. PCR products were column purified using the UltraClean™ PCR clean-up kit (Mo Bio Laboratories). A single enzyme digestion of PCR products was performed using the restriction enzyme *Bsh1236I* (5′-CG/CG-3′) (Fermentas, Glen Burnie, MD). Digestion reaction products were read by an ABI 310 genetic analyser at the Florida State University sequencing facility (Tallahassee, USA). Processing of TRFLP profiles was performed using Gene Mapper software (Applied Biosystems, Foster City, CA). Terminal restriction fragments less than 50 base pairs in length and peaks that contributed less than 1% of the total electropherogram area were excluded from the analysis.

Phylogenetic analyses of Isolates

Genomic DNA (gDNA) of the recovered isolates was extracted using the Mo Bio UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories) according to the manufacturer's instructions. SSU rRNA genes were amplified from gDNA of each isolate using the primers 27F/1492R without fluorescent label, as described above. Low quality data were trimmed from the sequences using the software package Sequencher (Gene Codes, Ann Arbor, MI) prior to generating the composite sequences. Partial gene sequences were submitted to GenBank under the accession numbers JX865376–JX865390. The basic local alignment search tool (BLAST; Altschul *et al*., 1997) was used to identify closely related sequences.

SSU rRNA gene sequences of isolates recovered in this study, and those of the most similar sequences were aligned using the software package Greengenes (DeSantis *et al*., 2006). This alignment was imported into the phylogenetic software package MEGA (Tamura *et al*., 2011) and into the software package MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) for phylogenetic tree construction. Neighbour-joining phylogenetic trees were constructed with aligned sequences using the maximum composite likelihood substitution model with complete deletion of gapped positions. The robustness of inferred tree topologies was evaluated by 1000 bootstrap resamplings of the data. For maximum likelihood trees, the Tamura-Nei substitution model was employed, with complete deletion of gapped positions, and 1000 bootstrap resamplings of the data. Additionally, Bayesian analyses were performed on the aligned sequence data by running five simultaneous chains (four heated, one cold) for six million generations, sampling every 1000 generations. The selected model was the general time reversible (GTR) using empirical base frequencies and estimating the shape of the gamma distribution and proportion of invariant sites from the data. A resulting 50% majority-rule consensus tree (after discarding the burn-in of 25% of the generations) was determined to calculate the posterior probabilities for each node. In all cases, the split-differential at 6 million generations was below 0.01.

Fatty acid methyl ester analysis

The response of membrane-derived fatty acid composition to shifts in temperature was determined for a representative isolate of each genus under aerobic conditions at 1.5°C, 5°C and 15°C using the MSW medium supplemented with low levels of peptone (0.1%), yeast extract

(0.1%) and beef extract (0.05%) as a carbon source. Freeze-dried cells (60 to 90 mg) were extracted using a modified Bligh and Dyer procedure (methanol-chloroform-water,10:5:4). The solid cellular residue was recovered by centrifugation and the solvent phase partitioned by addition of chloroform and water to a final ratio of 10:10:9. The lower chloroform layer containing the total lipid extract (TLE) was removed and dried under N_2. Fatty acid methyl esters (FAME) were prepared by treatment of the TLE by transesterification with freshly prepared 0.1 N methanolic NaOH for 60 min at 37°C (White *et al*., 1979). FAME were identified by GC-MS as described by Jahnke and colleagues (2004). The double-bond positions of FAME were determined by preparing dimethyl disulfide adducts by heating at 35°C for 35 min (Yamamoto *et al*., 1991).

Nitrate utilization and optimum growth temperatures

The growth rate and nitrate utilization potential were determined in batch culture for representative isolates of each identified genus. A 5% (v/v) inoculum from mid-log phase cultures was added to MSW media amended with 10 mM lactate and 5 mM NO_3^- for all isolates. Cultures were incubated in triplicate at 5°C in 160 ml serum bottles, and nitrate-free controls were used to test for fermentative growth. Growth was monitored as optical density at 600 nm using a Shimadzu UV-Vis spectrophotometer. Nitrate + nitrite and nitrite were determined by chemiluminescence detection after reduction with vanadium (Braman and Hendrix, 1989) or iodide (Garside, 1982).

Optimum growth temperatures were determined for representative isolates in a temperature gradient block incubator. The incubator consisted of a 2 m long insulated aluminum block with 30 rows of 3 parallel wells for culture tubes, and was heated at one end and cooled at the other in order to maintain a stable temperature gradient. Isolates were grown under denitrifying conditions in MSW with 10 mM lactate and 5 mM NO_3^- at 7–10 temperatures between 0°C and 30°C. Optical density at 600 nm was monitored twice daily in a Spectronic 21 spectrophotometer by placing an entire Balch tube into the instrument. Specific growth rates (μ) were calculated as the slope of the linear portion of the plot of the natural log (ln) of OD versus time.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Phylogenetic tree reflecting the relationships of SSU rRNA gene sequences from select isolates. The tree topology was obtained from a boot-strapped neighbour-joining analysis. Nodes for which bootstrap values equaled or exceed 70% are indicated by a numerical value. The bootstrap value derived from maximum likelihood analysis is also indicated (NJ/ML). Nodes supported by Bayesian analysis, with posterior probability values greater than 95%,

are indicated with black circles. Nodes with posterior probability values greater than 70% are indicated with white circles. The scale bar indicates 0.02 substitutions per nucleotide position. Isolates selected for further analysis are highlighted in gray.

Fig. S2. Full Results from growth experiments of representative isolates (see Fig. 2). Optical density for nitrate-amended versus lactate only controls indicates higher growth yields when nitrate is used as an electron acceptor. Dissolved nitrogen species are plotted in the bottom of each panel. Error bars represent ± 1 standard deviation of samples analysed in triplicate. Lactate only controls were measured in duplicate.

Table S1. Composition of concentrated vitamin solutions and trace element solution (TES) used in preparation of minimal sweater media (MSW) based on Widdel and Bak (1992).

Table S2. Results from screening of isolates by ^{15}N label addition and acetylene block. Where NH_4^+ increased, the change in concentration is reported. The N mass balance is not reported in the ^{15}N-label screening results due to decreased aqueous concentrations after headspace equilibration.

Table S3. Summary comparison of phenotypic features for representative isolates of this study in comparison to previously described isolates of closely affiliated species. Isolates described in the literature as marine, psychrotolerant, or psychrophilic were chosen for comparison. Values reported for C16:1 and C18:1 are the sums of all monounsaturated fatty acids with chain lengths of 16 and 18 respectively. (Topt, optimal growth temperature; FAME Temp, growth temperature for fatty acid analysis)