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ORIGINAL ARTICLE

The Diversity of Bacterial Communities Associated with Atlantic Cod *Gadus morhua*

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Abstract The spatial and temporal changes in the bacterial communities associated with the Atlantic cod *Gadus morhua* were investigated using terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S recombinant DNA (rDNA). Epidermal mucous was sampled from 366 cod caught in three harvest locations (Baltic, Icelandic, and North Seas) over three seasons (spring 2002, autumn 2002, and spring 2003), and an automated method for the high-throughput processing of environmental samples was developed using a Qiagen BioRobot. The analysis revealed that a diverse consortium of bacteria were found on fish; γ-*proteobacteria* and *Cytophaga–Flavobacter–Bacteroides* (CFB) species were dominant. T-RFLP peak profiles suggested that operational taxonomic units (OTUs) related to *Photobacterium* sp., *Psychrobacter* sp., and *Bacteroides* sp. were common to all sites in all three seasons, but there were intersite variations in community composition. Cod caught from different seas had distinct reproducible bacterial assemblages. Whereas communities from fish caught in the Baltic and Icelandic Seas were relatively stable over the three seasons, those from fish from the North Sea changed significantly over time.

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Introduction

Atlantic Cod are a demersal cold-water fish distributed widely across the north Northern Atlantic, and as a migratory species, shoals may travel up to 200 miles or more to their breeding grounds. An omnivorous carnivore, cod may be found from the shoreline to the continental shelf at depths of up to 600 m, but their preferred depth range is 10 to 200 m. Females are prolific spawners, and in the spring, up to 6 million buoyant eggs may be released at offshore spawning sites. Nonetheless, as a result of over-fishing, this once plentiful species has become commercially rare, and it is now listed by the International Council for the Exploration of the Seas (ICES) as below safe biological limits (SBL).

Cod, in common with many marine organisms, secrete an exopolymer (EPS) matrix from goblet cells located within the epidermis, which hydrates rapidly on contact with water to form large quantities of a sticky mucous. This mucous reduces drag and protects the fish from environmental factors such as osmotic stress, UV light, and microbial pathogens [53]. Microbes are widespread in the sea and can colonize virtually any submerged surface [7], including marine organisms. Despite the presence of a number of antimicrobial factors within the epidermal mucous, fish are still colonized by bacteria, leading to the development of a biofilm and the formation of a microbial community. Recently, molecular ecology techniques have been used to investigate the bacterial flora associated with marine organisms including sponges [25, 52], molluscs [18, 39],

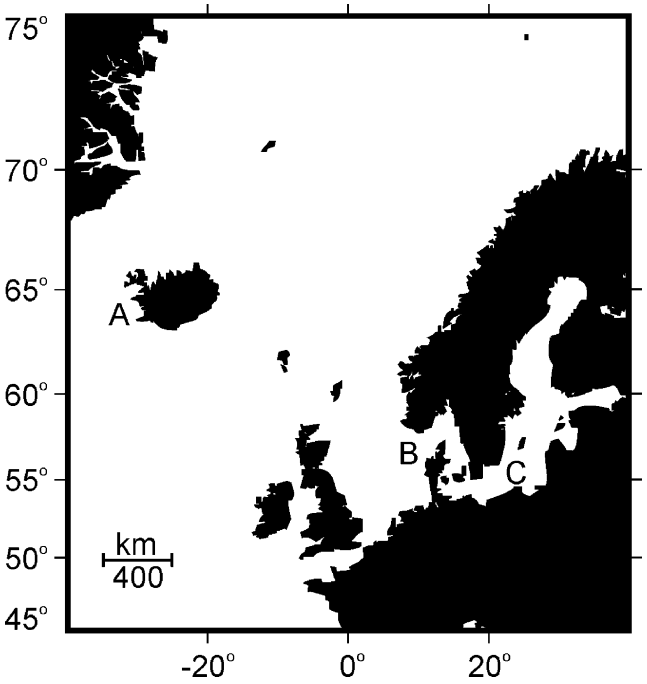

Figure 1 Sampling sites of Atlantic cod. *A* Icelandic Sea, *B* North Sea, and *C* Baltic Sea

nematodes [43], coral [30], and fish [6, 12, 51], and the factors affecting colonization of these organisms are still not fully understood. The association of bacteria with fish has, to the greater extent, been limited to the fields of aquaculture [2, 22, 38] and food microbiology [5, 11], and studies are usually directed toward defined culturable and pathogenic members sampled from fish reared in freshwater ponds and tanks [26]. Studies describing the bacterial populations present on the fish cuticle [9, 17, 27, 31, 36] have relied on the analysis of dilution plating of mucous suspensions, which biased the data in favor of bacterial species readily growing under these conditions. Furthermore, bacteria were only identified to the genus level, and because of this low resolution analysis, variability at the subgenus level could have been overlooked. As a result, very little is known about the composition and population dynamics of the natural bacterial flora of fish in nature.

The aims of this study were to obtain more detailed information concerning the microbial assemblages associated with cod and to assess changes in diversity in relation to variations in space and time. We hypothesized that the bacterial composition within the epidermal mucous of cod would not differ greatly between geographically disparate fish populations and that we would expect to find a constitutive and ubiquitous "resident" microbial community. However, samples from spatially distinct sites may be dominated by populations that are only transiently present [42, 44], indicating a biogeographical trend in relation to community composition. An understanding of the interactions between microbes and host organisms is an important step in explaining the functioning of associated microbial communities and may illuminate differences in spatially and temporally distinct systems. In this study, we tested the hypothesis that the presence or absence of bacteria on fish will vary among locations, but will not vary among different seasons within a site.

Methods

Sample Collection

Atlantic cod *Gadus morhua* were sampled from scientific research vessels during benthic surveys of fish stocks from waters in the North-East Atlantic Ocean (Fig. 1) during spring 2002, autumn 2002, and spring 2003 (Table 1). Fish were selected from a wide variety of trawls at each site. Epidermal mucous samples were removed with a sterilized spatula from cod immediately following harvest and resuspended in 200 µl TE buffer [10 mM Tris–HCl and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0]. Samples were immediately snap-frozen in liquid nitrogen onboard and stored at −80 °C.

DNA Extraction and Polymerase Chain Reaction

Genomic DNA was extracted using a BioRobot 3000 (Qiagen, Carlsbad, CA) and a modified version of the QIAamp 96 DNA Swab BioRobot Kit protocol (Qiagen). The modifications were as follows: (i) 4 µl lysozyme (100 mg ml^{-1}) was initially added to frozen mucous samples which were then incubated at 37 °C for 30 min; (ii) the proteinase K digestion step was extended from 1 h to 2 h at 56 °C; and (iii) genomic DNA was eluted from QIAamp filters in 60 µl Tris buffer (10 mM Tris–HCl, pH 8.0). Eluted genomic DNA was stored at -20 °C.

Primers used for T-RFLP analysis in this study were specific for amplification of bacterial 16S ribosomal RNA (rRNA) genes and were a combination of a 5′ Cy5-labeled 63F primer (5′-CAGGCCTAACACATGCAAGTC-3′) and 1389R primer (5′- ACGGGCGGTGTGTACAAG-3′) [35,

Table 1 Numbers of cod caught and sampled during the study

	Spring 2002	Autumn 2002	Spring 2003	Total
Baltic Sea	32	45	47	124
Icelandic Sea	24	51	45	120
North Sea	20	54	48	122
Total	76	150	140	366

Table 2 Phylogenetic affiliations and frequency[a] of clonal phylotypes

Accession number and phylogenetic grouping (TRF sizes in bp)	Baltic sea			Icelandic sea			North sea			Nearest phylogenetic relative (accession number)[b]	% Identical 16S rRNA
	Spring 2002	Autumn 2002	Spring 2003	Spring 2002	Autumn 2002	Spring 2003	Spring 2002	Autumn 2002	Spring 2003		
α-Proteobacteria											
DQ263709 *Loktanella* (404)	0	0	0	29	20	0	0	0	0	*Loktanella salsilacus* (AJ582229)	100.0
γ-Proteobacteria											
DQ263710 *Pseudomonas* (456)	0	0	0	0	35	13	64	0	0	Marine γ-proteobacterium HTCC2246 (AY386337)	97.2
DQ263712 *Acinetobacter* (463)	9	31	36	25	19	13	0	2	0	*Acinetobacter* sp. LUH4547 (AJ301674)	98.9
DQ263713-DQ263717 *Photobacterium* (465, 467, 468, 482 and 483)	47	31	81	21	37	22	55	43	50	*Photobacterium phosphoreum* (AB179540)	99.3
DQ263711 *Pseudoalteromonas* (458)	6	0	0	4	21	22	5	41	52	*Pseudoalteromonas* sp. 'turbot' (AY227707)	99.9
DQ263707 and DQ263708 *Psychrobacter* (270 and 271)	69	53	60	83	83	82	82	13	69	*Psychrobacter okhotskensis* (AB094794)	99.7
CFB											
DQ263705 and DQ263706 *Bacteroides* (61 and 62)	41	38	55	46	31	31	41	65	19	*Bacteroides* sp. ASF519 (AF157056)	92.0
DQ263718 *Flavobacterium* (507)	6	0	0	0	0	0	5	0	0	*Flavobacterium frigoris* (AJ557887)	96.5
DQ263719 *Mesonia* (512)	0	0	0	0	0	13	0	0	0	Marine bacterium KMM3937 (AF536386)	99.9

[a] Frequency expressed as a percentage of the total number of fish sampled for a particular site and season having a certain phylotype.
[b] Frequency expressed as a percentage of the total number of fish sampled for a particular site and season having a certain phylotype in the GenBank database.

41]. A BioRobot protocol was designed to automate the polymerase chain reaction (PCR) setup by aliquoting genomic DNA into a predispensed PCR reaction mixture (in a 96-well PCR plate). The reaction mixture consisted of 1.25 mM $MgCl_2$, 200 μM dNTP, 2 U of *Taq* DNA polymerase in storage buffer A (Promega, Madison, WI), 300 nM Cy5-F63 primer, 150 nM R1389 primer, 400 ng μl^{-1} non-acetylated BSA [29]. Three microliters (2–10 ng) of sample genomic DNA (up to 94 samples) was added, along with a negative (distilled water) and positive (*Escherichia coli* ATCC strain 11303 genomic DNA) control. Amplification consisted of a cycle of 94 °C for 5 min, 27 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min, plus an extension step of 10 min at 72 °C.

Terminal Restriction Fragment Length Polymorphism

Amplicons were purified using the MinElute 96 UF PCR purification kit (Qiagen) and quantified using the Pico-Green double-stranded DNA (dsDNA) quantitation kit (Molecular Probes, Eugene, OR), as per the manufacturer's instructions. Following the BioRobot standardization of DNA concentrations across a 96-well PCR plate (such that each well contained 75 ng DNA before digestion), a

reaction mixture consisting of *MspI* (10 U sample^{-1}) was dispensed into each well, and volumes automatically made up to 20 μl using distilled water. Digests were incubated at 37 °C for 2 h. Digested amplicons were purified using a DyeEx 96 kit (Qiagen) as per the manufacturer's instructions, dried, and dissolved in 5-μl sample loading solution (SLS; Beckman Coulter, Fullerton, USA). An aliquot (1 μl) of each sample was added to 40 μl SLS and 0.3 μl size standard-600 (Beckman Coulter) and overlaid with mineral oil. The lengths of fluorescently labeled fragments were determined using a CEQ2000 XL DNA analysis system (Beckman Coulter).

Clone Library Construction

Sample genomic DNA were pooled for each site and PCRs performed in triplicate (using unlabeled F63 and 1389R primers). These triplicate PCR products were combined and the pooled duplicates for each site used as templates for cloning. Clone libraries were constructed in pDrive using a PCR cloning kit (Qiagen) following the manufacturer's recommendations. The clone libraries were subsequently introduced into *E. coli* DH5α [46]. From each library, 94 colonies containing inserts were randomly selected and grown at 37 °C overnight in Luria–Bertani media containing 50 μg ml^{-1} ampicillin [46]. An aliquot (1 μl) of each culture was amplified (using Cy5-labeled F63 and R1389 primers). Libraries were screened by digestion of inserts using *MspI* and *RsaI*, and analyzed by T-RFLP using a CEQ2000 XL DNA analysis system.

Sequencing and Phylogenetic Analysis

Plasmid inserts from representative clones were sequenced using T7 forward and SP6 reverse primers. The phylogenetic affiliation of 16S rDNA sequences of clones was determined by a preliminary comparison with those in the NCBI database using the Basic Local Alignment Search Tool (BLAST) algorithm [3], and sequences were examined for chimera formation using the CHECK_CHIMERA [34] program. Clonal sequences were aligned with a number of sequences from the BLAST analysis using the ClustalX software package [34, 49]. Phylogenetic trees were generated and bootstrap analyses (1,000 replicates) were performed using TREECON software [50] by the algorithm described by Jukes-Cantor and the neighbor joining method. Sequences were submitted to the GenBank database under accession numbers DQ263705 to DQ263719.

Statistical Analysis

Independent duplicate T-RFLP profiles for each sample were combined, and the fragment data transformed into a binary (presence or absence) matrix using T-ALIGN software [47]. Samples were grouped by discriminant function analyses of data (using a jack-knifed classification matrix) with Systat v11 (Systat Software, Inc., San Jose, CA).

Results

Phylogenetic Diversity of 16S rRNA Genes from Baltic Sea Fish Mucous Samples

T-RFLP analysis of prokaryotic 16S rRNA genes present in mucous samples from cod caught in the Baltic Sea revealed a total diversity of 55 OTUs over the three seasons sampled. The community composition of the mucous layer was relatively stable during the sampling period with 67% of the OTUs recorded being present in all three seasons; an analysis of variance (ANOVA) confirmed that the diversity remained unchanged over time ($p>0.05$). Following T-RFLP screening of the Baltic Sea clone library and the subsequent sequence analysis of representatives, a number of OTUs were described by clonal sequences. The phylotypes consisted solely of members of the γ-*proteobacteria* and *Cytophaga–Flavobacterium–Bacteroides* (CFB) groups (Table 2) with the 16S rDNA clone library being dominated (63%) by a single clone that displayed over 99% sequence identity to that of *Photobacterium* spp., a γ-proteobacterium. All clonal sequences appear most closely related to 16S rDNA sequences from bacterial species previously isolated from marine environments. Over the three seasons, T-RFLP data showed that phylotypes putatively identified as *Bacteroides* spp., *Psychrobacter* spp., and *Photobacterium* spp. were consistently the most frequently sampled from Baltic Sea fish, with between 31 and 81% of cod harvested being associated with these species, and the number of fish colonized by these organisms was significantly higher in the spring seasons (Table 2). Another member of the γ-proteobacterial group, *Acinetobacter* spp., was found on increasing numbers of fish over the course of the sampling period, whereas phylotypes representing *Flavobacterium* spp. and *Pseudoalteromonas* spp. were only observed at low distribution levels during spring 2002.

Table 3 Average number of OTUs per fish with the standard deviation in brackets after each term

	Baltic	Iceland	North	Average
Spring 2002	5.6 (1.9)	5.2 (2.4)	5.9 (3.1)	5.5 (2.4)
Autumn 2002	6.6 (3.4)	7.5 (3.6)	5.9 (2.4)	6.7 (3.2)
Spring 2003	8.2 (3.0)	6.0 (3.0)	5.5 (2.0)	6.8 (3.0)
Average	7.0 (3.2)	6.7 (3.3)	5.7 (2.4)	

The averages for each season are shown in the final column, and the averages per site are shown in the final row.

Table 4 Two-way analysis of variance (ANOVA) of the number of OTUs per fish using season and sites as factors (the number of OTUs were square-root-transformed before analysis)

Source	Sum-of-squares	*df*	Mean square	*F* ratio	*p* value
Season	3.141	2	1.571	5.232	0.006
Site	2.050	2	1.025	3.414	0.034
Season × site	4.158	4	1.039	3.462	0.009
Error	107.179	357	0.300		

Phylogenetic Diversity of 16S rRNA Genes from Icelandic Sea Fish Mucous Samples

The total bacterial diversity of fish caught in the Icelandic Sea numbered 46 OTUs, with 42, 46, and 41 OTUs recorded in the spring 2002, autumn 2002, and spring 2003 sampling seasons, respectively. The stability of the resident microbial population, however, was higher than in the Baltic Sea, with 41 OTUs being present in every sample season. However, ANOVA results indicated that there were no significant differences in the richness of terminal restriction fragments (TRFs) between the seasons ($p>0.05$), and therefore, any variability in diversity arises from differences in the number of OTUs from fish to fish rather than the differences in the OTUs present from season to season. Fourteen OTUs were represented by clonal sequences, with *Psychrobacter* spp. comprising the most abundant sequence (45%) in the Icelandic clone library, followed by *Photobacterium* spp. (6%). The T-RFLP profile data was in agreement with the clonal library, indicating that the highest diversity of bacteria belonged to the *γ-proteobacteria* group (Table 2). *Psychrobacter* spp. dominated communities within the epidermal mucous and formed the greater part of the bacterial assemblages (as evidenced by T-RFLP fluorescence intensity data and the abundance of clones present in the libraries) on more than 82% of fish sampled in Icelandic waters. In comparison, *Photobacterium* spp. were found on lower numbers of fish than in either the Baltic Sea or North Sea. Members of the CFB group were found at similar levels in all seasons: They were consistently present as a significant portion of the mucous community in 31–46% of the fish caught. Uniquely, an α-proteobacterium with 100% 16S rRNA gene sequence identity with *Loktanella salsilacus* was found on a great number of fish in the first two seasons in Icelandic waters only.

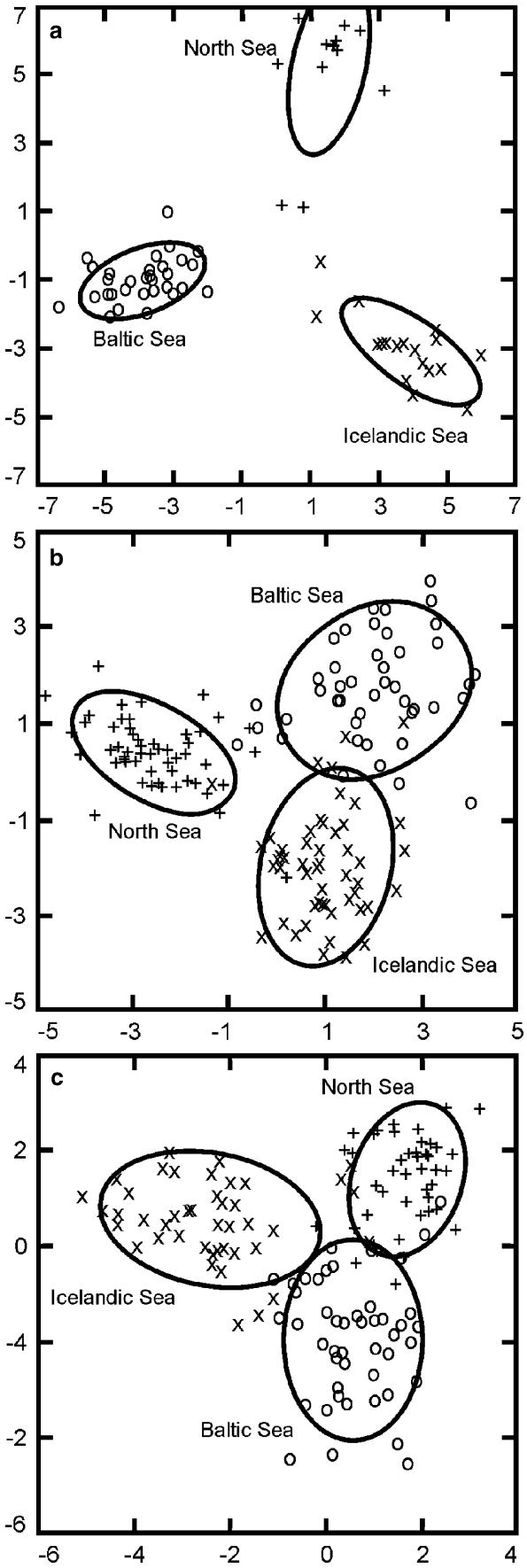

Figure 2 Discriminant function analyses using the grouped T-RFLP profile for the data obtained from sampling during **a** spring 2002, **b** autumn 2002, and **c** spring 2003. Bacterial communities associated with cod were geographically distinct (**a**–**c**). *Plus symbol* North Sea, *circle* Baltic Sea, and *ex symbol* Icelandic Sea

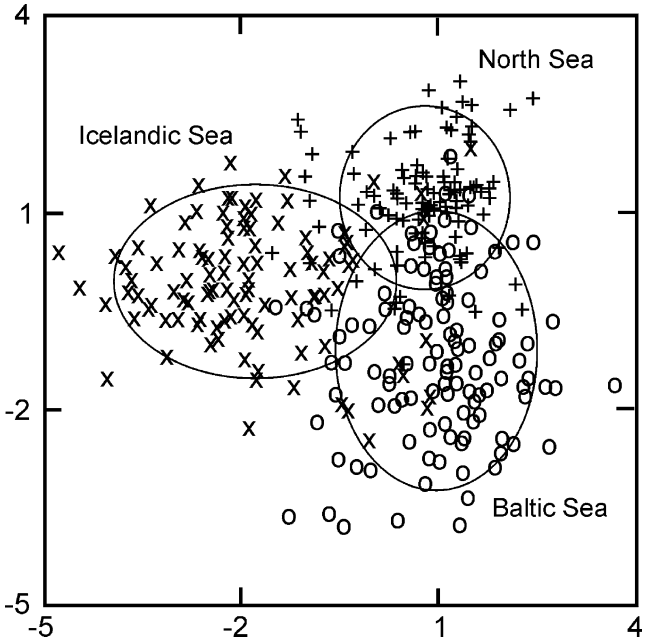

Figure 3 Discriminant function analyses combining the data from the three sampling seasons. *Plus symbol* North Sea, *circle* Baltic Sea, and *ex symbol* Icelandic Sea

Phylogenetic Diversity of 16S rRNA Genes from North Sea Fish Mucous Samples

Cod populations from the North Sea had the lowest diversity of bacteria associated with the epidermal mucous, with 44 OTUs assessed over the course of the study. Diversity peaked in spring 2003 at 41 OTUs (with 8 and 38 OTUs in spring 2002 and autumn 2002, respectively). Of the three sampling areas, bacterial communities from fish caught in the North Sea showed the greatest changes in diversity over time, with only eight OTUs found to be common to all seasons. No significant differences in the richness of TRFs were recorded between the spring 2002 and autumn 2002 samples ($p>0.05$) or between the autumn 2002 and spring 2003 samples ($p>0.05$); however, there was a significant difference between the spring 2002 and spring 2003 samples ($p=0.046$). Sequence analysis of the North Sea clone library revealed six phylotypes, and members of the γ-proteobacterial group were seen to predominate, accounting for 56% of the clone library. *Psychrobacter* spp. and *Photobacterium* spp. were the most abundant clonal phylotypes, comprising 34 and 21% of the library, respectively. This was also reflected by the T-RFLP profile data (Table 2); both phylotypes were also found to be present on more fish sampled from the North Sea than other phylotypes. In contrast with the other seas, levels of the phylotype related to *Acinetobacter* spp. were much lower, present on only 2% of fish in the autumn 2002 season (Table 2). The two dominant phylotypes (*Psychrobacter* spp. and *Photobacterium* spp.) were associated with greater numbers of fish in the spring seasons, whereas *Bacteroides* spp. were conversely found distributed on peak levels of fish in the autumn 2002 season.

Comparison of Site Bacterial Assemblages

The results of T-RFLP analysis of cod bacterial assemblages indicated that the average richness of TRFs on individual fish ranged from 5.2±2.4 to 8.2±3.0 operational taxonomic units (OTU; Table 3), whereas overall, the richness of TRFs present per fish changed across the sampling basins and through time (Table 4). The total diversity of recorded OTUs across fish within the Baltic and Icelandic Seas varied little between seasons. The temporal stability of the communities varied from site to site, with communities from the Baltic and Icelandic Seas showing great stability, with the majority of phylotypes (67 and 89%, respectively) found to be present on fish throughout the course of the study, whereas those from North Sea were far more changeable. In the Baltic Sea, this is most likely a result of the relative isolation of the Baltic Sea Basin, when compared with the North Sea, where increased mixing among water masses would occur.

The T-RFLP profile data for clonal sequences (Table 2) showed that, whereas the numbers of fish colonized by a particular phylotype might differ from site to site, a number of phylotypes (*Psychrobacter* spp., *Photobacterium* spp., and *Bacteroides* spp.) were common to bacterial populations present at all sites. Of these, the presence of *Bacteroides* spp. was the most stable in terms of both space and time, recorded on consistent levels of 41±14% of fish sampled throughout the study. In contrast, the relationship between the frequency of colonization by the dominant phylotypes (*Psychrobacter* spp. and *Photobacterium* spp.) and sample site varied substantially: On average, *Psychrobacter* spp. was present on 66±23% of fish sampled, whereas *Photobacterium* spp. was seen to have colonized only 43±19% of fish in the North-East Atlantic, yet there were significant differences in the numbers of fish colonized by the different phylotypes from site to site; for example, in the Icelandic Sea, *Psychrobacter* spp. and *Photobacterium* spp. were associated with the highest (83±1%) and lowest (27±9%) mean levels of cod, respectively, during the study.

The presence or absence of certain phylotypes was, however, more site-specific. Phylotypes related to *Pseudoalteromonas* spp. were (with the exception of a very low level detected [2%] in the Baltic Sea spring 2002 sample set) only found on cod caught in Icelandic and North Seas, and this was reflected by the total absence of this species in the Baltic Sea clone library. Similarly, *Acinetobacter* spp. was not recorded in either the North Sea T-RFLP profile data or

clone library, whereas *Loktanella salsilacus* was unique to both Icelandic Sea profiles and the clone library.

Discriminant analyses (using the grouped T-RFLP profile data for each season) separated samples into site-specific clusters, demonstrating that for each sampling time point, bacterial communities associated with cod were geographically distinct (Fig. 2a–c). The pooling of bacterial profile data for each site (such that the sampling timescale was not considered) revealed that, despite there being an overlap between sites, there still existed defined, site-specific clustering (Fig. 3). This shows that cod harbors a relatively diverse bacterial population that is distinct for each of the three basins examined.

Discussion

Phylogenetic analysis revealed that there was substantial diversity among sequences, and when compared with database sequences, they were most similar to isolates and clones retrieved from marine samples (Table 2). Sequences corresponding to the γ-*proteobacteria* dominated the clonal libraries for each sampling site and had high similarity (97–100%) to 16S rDNA sequences of *Photobacterium* sp., *Psychrobacter* sp., *Acinetobacter* sp., and *Alteromonas* sp. In contrast, α-*proteobacteria* dominate free-living water marine bacteroplankton communities [8, 19, 21]. Indeed, only a single member of the α-proteobacterial group, with a 100% 16S rRNA gene identity to *Loktanella salsilacus*, was identified in our study, and it comprised 4% of the Icelandic Sea clone library only. γ-*Proteobacteria* dominate libraries of bacteria isolated from marine systems [23]. The nutrient-rich conditions within the mucous may have enriched for γ-*proteobacteria*, which would be why the data presented in this paper agrees more closely with that regarding cultured marine taxa. Clones clustering with 16S rDNA sequences from the CFB group showed the lowest level of similarity to the published 16S rDNA sequence data, with two clones showing only a 92% similarity to the nearest relative (*Bacteroides* sp. ASF519; Table 2), and it is possible that these clonal sequences (accession numbers DQ263705 and DQ263706) might represent novel genera.

More variability existed in the North Sea from year to year as compared to the other two basins sampled, and it was the only temporal difference to reveal a significant difference in the richness of TRFs. However, as only three basins were sampled for 2 years, it is premature to generalize as to why this difference would have been detected only in the North Sea, but it does indicate that annual differences in the species composition of the bacteria warrants further study. In each of the three sampling sites, the fish-associated bacterial communities comprised a resident population (that consist of phylotypes that were present throughout the study) and a transient one (whose members varied from season to season). Similar community fractions have been reported in free-living marine bacterial communities [4], where over the course of a 3-year study in Arctic waters, the majority of DGGE bands were present but additional bands were recorded from year to year. Similarly, observations of communities associated with nematodes [43] showed that they consisted of a few bacterial populations (related to members of the *Cytophaga* and sulfate-reducing bacteria) that were constitutive, whereas a greater number were transient. In this study, the dominant phylotypes (including OTUs corresponding to *Photobacterium* sp., *Psychrobacter* sp., and *Bacteroides* sp.) were also the resident ones, and for the most part, the levels of these were unaffected by sample season, remaining relatively unchanged throughout the study. Seasonal variation in dissolved organic matter (DOM) levels might affect the composition of free-living marine bacterial communities [4], and several studies support this theory [42, 55], reporting that members of the CFB group dominate marine consortia after an algal bloom. Seasonal variations in the numbers of culturable bacterial flora of flatfish and cod have also been linked with surface blooms [31] but only after a considerable delay, allowing for a gradual settling of algal detritus to greater depths. In this study, members of the CFB group occurred at a relatively constant level in all sites during the study, and so, it seems that populations may have been unaffected by seasonal algal levels. Differences in water temperature with the seasons would also represent a significant environmental factor affecting communities; cod, however, are demersal fish, swimming near the sea bottom at depths of up to 600 m, where water temperatures would not change as dramatically with season as would surface waters. This study confirms the ubiquity of the CFB previously reported for marine samples [20, 28, 42].

Discriminant analyses clustered T-RFLPs by sample location (Fig. 2a–c), suggesting that the biogeography of bacterial communities differs significantly with water mass location. Much work has been published as to the ubiquity of certain groups (e.g., the SAR11 cluster [19, 37]) in oceans on a global scale. In this study, members of γ-*proteobacteria* consistently dominated cod epidermal communities throughout the North-East Atlantic, in correspondence with previous studies of the bacterial flora of fish indigenous to this area [17, 31]. However, within the γ-proteobacterial group, there were site-specific differences in the numbers of cod colonized by either *Photobacterium* sp. or *Psychrobacter* sp. (Table 2). These results are in agreement with previous studies based upon culturable bacteria [42, 44], which showed that samples from spatially distinct sites were frequently dominated by different species.

Hansen and Olafsen [24] proposed that bacteria present in the surrounding water mass are involved in the initial (epidermal and enteric) colonization of larval fish and

dominate subsequent communities in adult fish. Results from a study [43] investigating the indigenous microflora of sediment-associated nematodes support this theory. When Atlantic cod spawn, their eggs are released across an area that might stretch for many miles, and upon hatching, the planktonic larvae may be dispersed widely by ocean currents. Microbial assemblages detected on adult cod would, therefore, reflect the bacterial composition within the water mass surrounding the fish in the egg and larval stages. Any differences in the bacterial community composition of cod sampled from the same area would be a result of fish from separate spawning locations (colonized initially by different bacteria), shoaling together in adult life. The resistance of these initial colonized communities to change and the extent to which the bacterial composition of the water might contribute is not known. These differences in bacterial communities from fish sampled from a single population are not easily explained, and as previously stated [43], there exist inexplicable incongruities between the bacterial populations associated with very similar environments. A comparison of the microflora of farmed and wild salmon [26] reported that differences between bacterial communities associated with fish might reflect a response by the community to local conditions but the timescale of this response is not yet known. The size and age of each fish did not have a significant effect on microbial community composition (data not shown).

It can be assumed that the bacteria abundant within the mucosal biofilm are invariably well adapted to an otherwise hostile environment; mucous is the first line of defense in fish and is made up of a number of antimicrobial factors, including lysosymes, lectins, proteases, and agglutinens [53]. If we assume no PCR (or other) bias, the dominant phylotypes detected in this study using T-RFLP are found to be existing in significant abundance, and must be assumed highly active. Much has been published with regards to the success with which attached or biofilm-associated communities thrive in contrast with their free-living counterparts; organisms embedded within a mucosal matrix are protected from protozoal grazing [54] and UV [33] benefit from the adsorption of nutrients to mucous [32] and permit the development of microconsortia and retention of extracellular enzymes [16]. The oligotrophic nature of seawater and scarcity of nutrients mean that bacterial association with attached surfaces (such as marine aggregates and organisms) represents a good growth strategy and explains the composition of communities within fish epidermal mucous. Both γ-proteobacterial and CFB groups are comprised of copiotrophic organisms and have previously been reported to dominate attached marine communities [1, 8, 13]. The *α-proteobacteria*, on the other hand, which are adapted to low nutrient concentrations, predominate in free-living communities within the oligotrophic surface waters and represent a smaller fraction of attached communities. Organisms related to the *γ-proteobacteria* (similar to those identified in this study) have been found to make up a significant proportion of particle-attached communities and to dominate these communities at depths greater than 50 m [1], similar to the depths at which the dermersal cod exists. Bacteria related to *Psychrobacter* sp., *Acinetobacter* sp., and *Photobacterium* sp. have all been previously isolated from fish [5, 6, 26]; *Photobacterium* sp. (and closely related *Vibrio* sp.) have received much attention due to their luminous symbioses with marine organisms [14, 15, 45] and chemotactic response to fish mucous [40]. This species has also been cited as a important agent in the spoilage of both salmon and cod in cold storage [11].

The CFB group exists in higher abundance in attached states rather than as free-living bacterioplankton [10], and members of this group have been found to be associated with fish [38, 48]. In this study, relatively high levels of CFB bacteria on all fish sampled were found. This correlates well with their affinity for nutrient-rich environments, and the phenotypic property of the group for the degradation of HMW compounds and biopolymers [8, 28]. Because mucous consists primarily of hydrated long-chain polysaccharides [53], it is very likely that CFB bacteria are well adapted to metabolize this abundant source of dissolved organic matter (DOM). In a review of the group, Kirchman states that the levels of DOM in oceans is too low to support the high abundance of CFB bacteria that are found in a free-living state and suggests that it is the release of these bacteria from associated communities that contributes to these unusually high numbers [28]. The CFB bacteria were found to be a dominant group in the mucous of almost all cod sampled in this study. It is, therefore, likely that these fish-associated communities serve as a significant reservoir for the group in aquatic systems.

Very little is known with regards to the structure and function of the microbial communities associated with fish and other marine organisms. The work in this study confirmed that a relative few phylogenetic clusters dominated the assemblages in the epidermal mucous of Atlantic cod and that these comprised both resident and transient organisms. The data obtained in this study suggest that there may be a stable periodicity to bacterial community structure but the degree to which temporal and spatial scales affect this composition cannot be elucidated without analyzing additional data over an extended sampling period.

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