

ORIGINAL ARTICLE

Biogeography and phylogenetic diversity of a cluster of exclusively marine myxobacteria

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Myxobacteria are common in terrestrial habitats and well known for their formation of fruiting bodies and production of secondary metabolites. We studied a cluster of myxobacteria consisting only of sequences of marine origin (marine myxobacteria cluster, MMC) in sediments of the North Sea. Using a specific PCR, MMC sequences were detected in North Sea sediments down to 2.2 m depth, but not in the limnetic section of the Weser estuary and other freshwater habitats. In the water column, this cluster was only detected on aggregates up to a few meters above the sediment surface, but never in the fraction of free-living bacteria. A quantitative real-time PCR approach revealed that the MMC constituted up to 13% of total bacterial 16S rRNA genes in surface sediments of the North Sea. In a global survey, including sediments from the Mediterranean Sea, the Atlantic, Pacific and Indian Ocean and various climatic regions, the MMC was detected in most samples and to a water depth of 4300 m. Two fosmids of a library from sediment of the southern North Sea containing 16S rRNA genes affiliated with the MMC were sequenced. Both fosmids have a single unlinked 16S rRNA gene and no complete rRNA operon as found in most bacteria. No synteny to other myxobacterial genomes was found. The highest numbers of orthologues for both fosmids were assigned to *Sorangium cellulosum* and *Haliangium ochraceum*. Our results show that the MMC is an important and widely distributed but largely unknown component of marine sediment-associated bacterial communities.

The ISME Journal (2012) 6, 1260–1272; doi:10.1038/ismej.2011.190; published online 22 December 2011

Subject Category: microbial ecology and functional diversity of natural habitats

Keywords: 16S rRNA genes; fosmids; *Myxococcales*; North Sea

Introduction

Myxobacteria are unique, with a life style differing from all other prokaryotes. They are capable of excreting hydrolytic enzymes and decomposing various and complex biopolymers but can also lyse and degrade other prokaryotes and even eukaryotes (Shimkets *et al.*, 2006). Myxobacterial cells can spread and swarm on an excreted polymeric, mucus-like matrix. Most notably, under unfavourable nutrient conditions, many myxobacteria can aggregate to dense mushroom-like cell colonies and produce fruiting bodies and spores (Shimkets, 1990; Shimkets *et al.*, 2006). In this complex, density-dependent life style, signalling compounds have a key role (Goldman *et al.*, 2006), and myxobacteria

are well known for the production of various secondary metabolites and thus are prime targets for the search of new secondary metabolites (Gerth *et al.*, 2003; Wenzel and Müller, 2007; Weissman and Müller, 2010). They contain the largest genomes of all prokaryotes and the genome of *Sorangium cellulosum*, with >13 Mbp as the largest bacterial genome sequenced to date (Schneiker *et al.*, 2007). Myxobacteria form a phylogenetically coherent group and constitute the order *Myxococcales* in the class *Deltaproteobacteria*. They are subdivided into the three suborders *Cystobacterineae*, *Sorangineae* and *Nannocystineae* (Reichenbach, 2005; Garcia *et al.*, 2010).

Myxobacteria are widespread in terrestrial habitats. Historically and until recently, soil, dung and plant detritus were considered as the typical environments of these organisms (Dawid, 2000). Myxobacteria have been isolated also from limnetic habitats; however, they were considered as being washed in from the terrestrial surroundings and not as indigenous limnetic organisms (Reichenbach, 1999).

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Received 11 April 2011; revised 11 November 2011; accepted 11 November 2011; published online 22 December 2011

For a long time, it was assumed that myxobacteria are not able to dwell in saline and marine habitats. Some myxobacteria were isolated from marine environments but exhibited a growth optimum at rather low salinities and, therefore, were thought to be salt tolerant and brought in from the terrestrial surroundings, comparable to those in freshwater habitats (Rückert, 1984). During the recent past, several myxobacteria have been isolated from marine habitats and shown to be able to grow at sodium chloride concentrations around that of sea water, for example, *Haliangium ochraceum* and *H. tepidum* (Foudou *et al.*, 2002), *Plesiocystis pacifica* (Iizuka *et al.*, 2003a) and *Enhygromyxa salina* (Iizuka *et al.*, 2003b). All these species originate from coastal regions of Japan and affiliate to the suborder *Nannocystineae*. Halotolerant and halophilic myxobacteria appear to differ with respect to the formation of fruiting bodies, and the adaptation of their life cycle to the salty environment (Zhang *et al.*, 2005). Selected strains of *Myxococcus* form fruiting bodies also in sea water media (Wang *et al.*, 2007). Recently, Jiang *et al.* (2010) reported several clusters of myxobacterial sequences that were found in a few deep sea sediment samples and at one hydrothermal vent site. These myxobacteria are phylogenetically separated from their terrestrial counterparts, suggesting that distinct clusters of marine myxobacteria do exist. As nothing else than the phylogeny of these myxobacteria is known so far, further studies are needed to elucidate the ecology, physiology and genomics of these organisms.

During an investigation of the bacterial communities in the German Wadden Sea, southern North

Sea, by denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments, we persistently detected a phylotype affiliated to the *Myxococcales* in the surface sediment (Stevens *et al.*, 2005). This phylotype is related to several other uncultured phylotypes detected in various marine sediments, but only distantly to described species (<90% sequence similarity). Therefore, we comprehensively studied the occurrence of these myxobacteria in the North Sea and other marine systems. The study was complemented by analysis of a fosmid library from North Sea sediment. Two fosmids carrying 16S rRNA genes closely affiliated with other phylotypes of the new cluster were obtained and completely sequenced.

Materials and methods

Study sites and sampling

Sediment and water samples in the North Sea were taken at 12 stations during a cruise with RV Heincke in September 2005, covering a transect from Bremerhaven, Germany, to 58°N close to the Norwegian coast (Figure 1a, Supplementary Table 1). For DNA extraction, 250 ml of seawater was prefiltered onto 5.0 µm polycarbonate filters (Nuclepore, Whatman Schleicher Schuell, Piscataway, NJ, USA) to obtain the fraction of particle-associated bacteria and subsequently onto 0.2 µm polycarbonate filters to obtain that of free-living bacteria. Sediment from the surface (0–0.5 cm horizon) was taken using a multiple corer. Samples were immediately frozen after sampling and stored at –20 °C until further

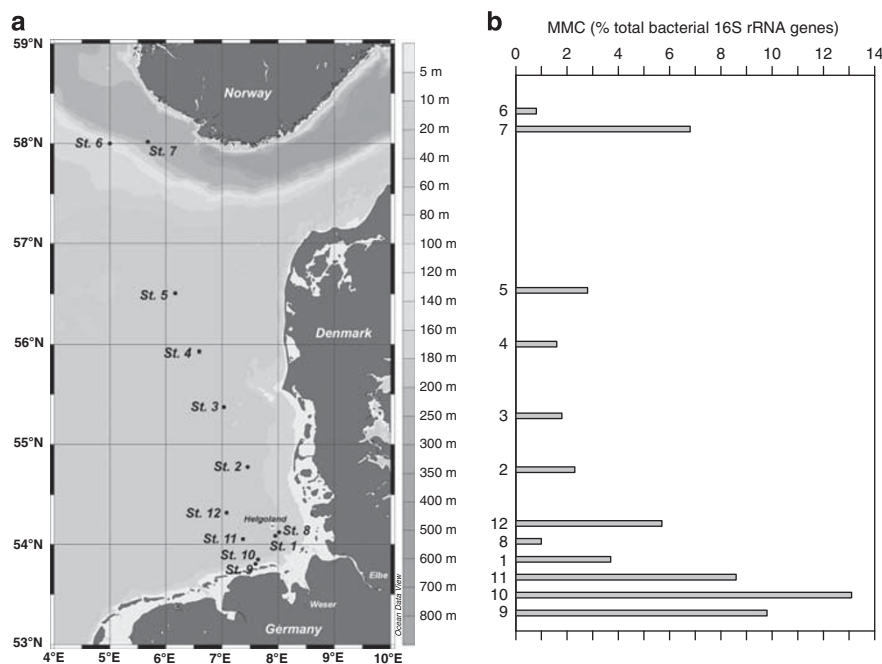


Figure 1 (a). Stations (St) in the North Sea visited during a cruise with RV Heincke in September 2005 and analysed for the presence of the MMC. (b). Abundance of 16S rRNA genes of the MMC (percent (%) of total bacterial 16S rRNA genes) in sediment surface samples of the various stations. Numbers on the y-axis refer to stations given in a.

processing. Further sediment samples (0–0.5 cm horizon) for the detection of myxobacteria were obtained from different water depths and oceanic locations, including the Atlantic, Pacific, Indian and Arctic Ocean, the Baltic, Mediterranean and Black Sea, and hypersaline waters (Supplementary Table 1). Samples for DNA extraction and subsequent construction of a fosmid library were taken on 23 March 2002 from surface sediment of an intertidal sand flat (Janssand) in the German Wadden Sea (53°43 N, 07°41 E). The cores were sectioned and immediately frozen at –20 °C. DNA taken for library construction was extracted from sediment of the 5–12 cm horizon (see below). A detailed description of all sampling procedures is given in the Supplementary Text 1.

Nucleic acid extraction

DNA for subsequent screening with a PCR specific for the marine myxobacteria cluster (MMC) was extracted following the protocol of Zhou and colleagues (1996), with modifications described by Giebel *et al.* (2009). DNA extraction was checked on a 1% agarose gel. Stock DNA was stored at –80 °C and subsamples at –20 °C until further analysis.

Design of the MMC PCR detection system

On the basis of available 16S rRNA gene sequences of the MMC, specific primers were designed using the ProbeDesign function of the ARB package (<http://www.arb-home.de/>). Two primer systems to detect the MMC were designed: MMC655f (5-AGT AATGGAGAGGGTGGC-3)/MMC841r (5-GGCACAG CAGAGGTCAAT-3) and MMC583f (5-AGGCGGACT CGCAAGTCG-3)/MMC734r (5-GTAAATGTCCAGGT GGC-3). Specificity of the primer sequences was checked *in silico* with the NCBI and RDP databases (<http://www.ncbi.nlm.nih.gov/>; <http://rdp.cme.msu.edu/>) and resulted in at least one mismatch to other organisms for MMC655f and MMC583f, and two mismatches for MMC734r. For the primer MMC841r, two sequences not included in the MMC showed no mismatch. As the first primer set covers more sequences of the MMC than the second, the first system was chosen for screening of environmental samples. The second MMC primer system was used for quantitative PCR because of higher specificity (see below). A cloned 16S rRNA gene fragment of a MMC bacterium was used as positive control and standard. Conditions for the MMC-specific PCR (primers MMC655f/MMC841r and MMC655f/1492r) and details on the development of the PCR conditions are given in the Supplementary Text 1. All DNA samples were prechecked with bacteria-specific primers 341f and 907r (Muyzer *et al.*, 1998) before specific PCR.

Cloning and sequencing of PCR products

To prove that positive screening results obtained from PCR with the primer pair MMC655f/MMC841r

and environmental samples derived from bacteria of the MMC, PCR products obtained with randomly selected samples and the combination of primer MMC655f and the bacteria-specific primer 1492r (Muyzer *et al.*, 1995) were cloned and sequenced using standard methods. For details, see Supplementary Text 1.

Quantitative PCR assays

To quantify the MMC, a real-time quantitative PCR assay was developed using the primer pair MMC583f/MMC734r (see above). To relate the abundance of MMC to total bacteria, a 390-bp fragment of the 16S rRNA gene was amplified with the primer pair 517f and 907r (specific for bacterial 16S rRNA genes, Muyzer *et al.*, 1998) following the protocol described by Süß *et al.* (2006). PCR-generated and purified 16S rRNA gene fragments of a 3.55-kb plasmid containing the 16S rRNA gene of a MMC phylotype obtained from the German Wadden Sea were applied as standards. Abundances of the MMC were determined as percent of total bacterial 16S rRNA genes. Details describing the conditions, technical details and evaluation of the MMC-specific PCR are given in the Supplementary Text 1.

Enrichment and isolation of myxobacteria

As no isolates of the MMC are available, we tried to enrich these organisms in a mesocosm experiment with sediment and water from the Wadden Sea (taken from an intertidal mud flat off the village of Neuharlingersiel on 22 August 2005). To enrich MMC bacteria on artificial surfaces, glass slides were used and prepared as follows: slides were coated with a ca. 1 mm thick layer of agar (1.5%) enriched with peptone (1%). One series of slides was transferred for 6 h in cultures of various bacterial strains (grown in marine broth (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) up to an $OD_{600} > 1$) to allow settlement of the cells (as prey organisms). Subsequently, the slides were mounted with a nylon lace to a rod above the mesocosm to keep the slides in the water column about 1 cm above the sediment surface. The slides were incubated in the mesocosm for 3 weeks. Every 2 days, samples were removed from the slides (with a sterile spatula) and DNA was extracted as described above. Presence of bacteria of the MMC in the biofilms was tested with the specific PCR approach.

Additionally, isolation of MMC bacteria from Wadden Sea sediment (taken from an intertidal mud flat off Neuharlingersiel on 30 September 2006) was tried by utilising the bacteriolytic properties of myxobacteria following the methods and using the media described by Reichenbach and Dworkin (1992). Myxobacteria were recognised by formation of fruiting bodies and swarming. Strain MX1 was

isolated and purified via several transfers on WCX (water cyxloheximide) agar plates containing 50% of seawater and streaks of dead or living *Escherichia coli* cells, followed by inoculations on CY (cassitone yeast) agar plates, also prepared with 50% of seawater. Strain MX2 was isolated and purified likewise but using WCX agar plates without seawater. Both strains were able to grow on CY agar plates or alternatively on VY/2 (vitamin B12, yeast/2 (half the amount of the normal concentration of VY medium)) agar plates at room temperature and at 30 °C, without and with the addition of 50% of seawater, respectively.

Further details for the enrichment and isolation procedures are given in Supplementary Text 1.

Fosmid library construction and screening for MMC 16S rRNA genes

A fosmid library with DNA from surface sediment of an intertidal sand flat of the German Wadden Sea (see above) was constructed as described by Mussmann *et al.* (2005) using the EpiFOS fosmid library production kit (Epicenter, Madison, WI, USA) according to the manufacturer's instructions. In total, 11 000 clones from the fosmid library were screened for MMC 16S rRNA genes using the primer pair MMC655f/MMC841r (see above).

Sequencing of the fosmids, ORF finding and sequence annotation

Fosmids were sequenced by a shotgun approach based on plasmid libraries with 1.5–3.5 kb inserts. Sequences were determined by using Big Dye 3.0 chemistry (Applied Biosystems, Foster City, CA, USA), M13 primers (Messing, 1983) and ABI3730XL capillary sequencers (Applied Biosystems) up to a 19-fold coverage. Resulting reads were assembled using the Phrap assembly tool (<http://www.phrap.org>). All manual editing steps were performed using the GAP4 software package v4.11 (Staden *et al.*, 2000). Prediction of protein-encoding sequences and open reading frames (ORFs) was initially accomplished with YACOP (Tech and Merkl, 2003), producing a combined set of genes predicted by the ORF-finding programs Glimmer (Delcher *et al.*, 1999), Critica (Badger and Olsen, 1999) and Z-curve (Guo *et al.*, 2003). All ORFs were manually curated and verified by comparison with the publicly available databases SwissProt, GenBank, ProDom, COG and Prosite using the annotation software ERGO (Overbeek *et al.*, 2003).

Comparative genomics and bioinformatics tools

The protein sequences encoded by the two fosmids were used for reciprocal BLAST comparisons as well as a global sequence alignment with the Needleman–Wunsch algorithm using the software tool BiBag (pers. comm. Antje Wollherr and Heiko Liesegang, University of Göttingen, Germany). Seven myxobacteria whole-genome protein data sets were taken as query organisms: *Anaeromyxobacter dehalogenans*

strain 2CP-C and strain 2CP-1, *Anaeromyxobacter* sp. K and sp. Fw109-5, *Myxococcus xantus* DK1622, *S. cellulosum* So ce 56 and *H. ochraceum* DSM14365. Orthologues were identified as reciprocal best BLAST hits with an *E*-value less than 1e-20, and a Needleman–Wunsch similarity-score more than 25%. Whole sequence alignments and visualisation were performed with the Genome Matcher software (Ohtsubo *et al.*, 2008) using reciprocal BLASTn comparison with a word size of 21 and *E*-value less than 0.01.

Sequencing of 16S rRNA genes and phylogenetic analysis

PCR products were sequenced using the DYEnamic Direct cycle sequencing kit (Amersham Life Science, Cleveland, OH, USA) and a Model 4200 automated DNA sequencer (LICOR, Lincoln, NE, USA) as described by Rink *et al.* (2007). Sequences were analysed by BLASTn search (<http://www.ncbi.nlm.nih.gov/blast>) and the ARB software package (<http://www.arb-home.de>, Ludwig *et al.*, 2004). A neighbour-joining tree showing the phylogenetic relationships of bacteria of the MMC within the *Myxococcales* based on 16S rRNA gene sequence similarity was calculated with sequences of at least 1300 bp length (Figure 2). Shorter sequences were added later with maximum parsimony, resulting in Supplementary Figure 1. The origin of all sequences was checked, indicating that they were all retrieved from marine samples. Further details on tree construction and detection of sequences affiliated with the MMC are given in Supplementary Text 1.

The nucleotide sequence data are available at GenBank under accession numbers HQ857564 to HQ857578 (16S rRNA genes), HQ191475 (fosmid MMCf1) and HQ191476 (fosmid MMCf2), GU323922 (*Myxococcus* sp. MX1) and GU323923 (*Myxococcus* sp. MX2).

Results

Distribution of the MMC in the North Sea

During a cruise in September 2005 in the North Sea, samples were taken from the water column and the sediment surface. The samples covered a transect from the German Bight to 58°N close to the Norwegian coast (Figure 1a). A specific PCR was used to examine the samples for presence of the MMC. In the water column down to a depth of 3 m above the sediment surface, the MMC was detected only at two stations, once at station 4 (at 15 m depth) and at station 8 where the MMC was present in the whole-water column (see below). In water samples taken during previous cruises in the North Sea (2002 and 2003), the MMC was also detected but only sporadically (Supplementary Table 1). The MMC was detected in all sediment samples taken in 2005 (0 to 0.5 cm horizon), irrespective of the absolute water depth (27–260 m, Supplementary Table 1). Further, the MMC was detected in water samples

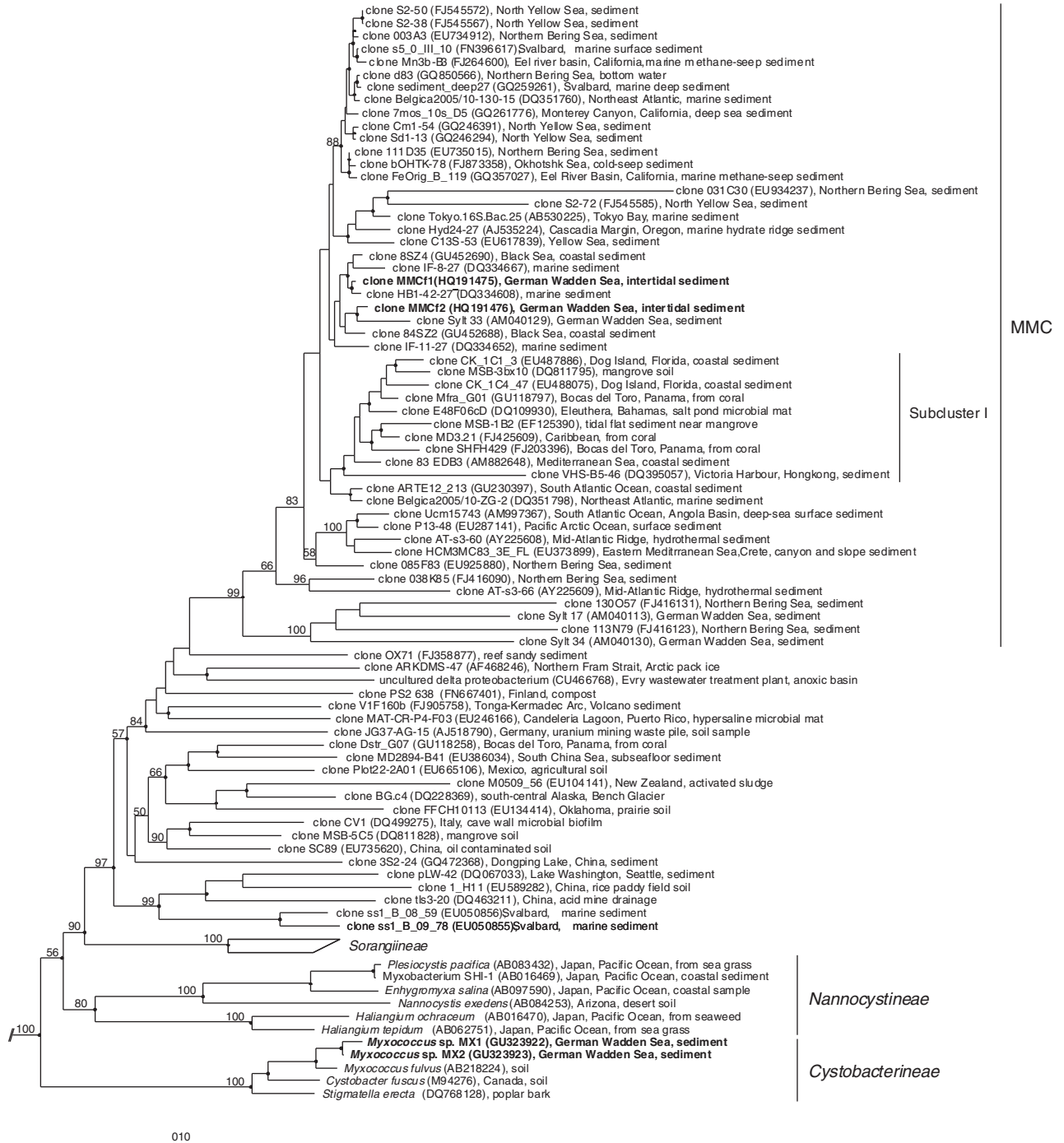


Figure 2 Neighbour-joining tree showing the phylogenetic relationships of bacteria of the MMC within the Myxococcales based on 16S rRNA gene sequence similarity. Sequences of at least 1300 bp were considered. Only bootstrap values > 50% (derived from 2000 replicates) at main nodes are shown. Filled circles indicate that nodes were also recovered reproducibly with maximum-likelihood. Selected members of the Cyanobacteria were used as outgroup (not shown) to define the root of the tree. GenBank sequence accession numbers are given in parentheses. In addition, the sampling site is indicated. The two clones of the fosmids sequenced in this study and the two new Myxococcus spp. are shown in bold. Bar, 0.10 substitutions per nucleotide position.

collected not more than 3 m above the sediment surface at stations 3, 5, 6 and 7. At station 8 with a depth of 29 m, close to the island of Helgoland, the MMC was consistently detected in a time series analysis of a tidal cycle in the particle fraction > 5 µm from 5 m depth to the bottom. These results

show that the MMC is present in sediments throughout the North Sea and also on resuspended particles in the nepheloid layer.

Abundance of the MMC in sediment samples taken in September 2005 was quantified by real-time quantitative PCR, yielding proportions of 0.8–13.1%

of bacterial 16S rRNA genes with highest values at stations 7 and 9–12 (Figure 1b). These results demonstrate that the MMC cluster is not only widely distributed but also a prominent component of the sediment-associated bacterial communities in the North Sea. A coherence of the abundance of MMC bacteria in samples taken on the North Sea transect in 2005 was found with the sediment type, that is, highest numbers of MMC bacteria were found in fine grained sand, compared with medium grained sand to gravel and fine grained sand to muddy sediment (Supplementary Figure 2).

Three cores of sandy sediments in the German Wadden Sea (one core from the Gröninger Plate and two from the Neuharlingersiel Nacken, back-barrier area of Spiekeroog Island, southern North Sea) were examined for presence of the MMC (Supplementary Table 1). It was detected in all three cores to depths of ca. 2 m, indicating its presence not only in oxic but also in anoxic environments, as the sandy sediments in the Wadden Sea become readily anoxic below the surface (Wilms *et al.*, 2006). Analysis of the abundance of the MMC in cores from the Gröninger Plate and the Neuharlingersiel Nacken by quantitative PCR revealed that this cluster constituted 2.8 and 2.0% at the sediment surface, and 4.2 and 1.6% of bacterial 16S rRNA genes in the undermost horizons where the MMC was detected (ca. 2 m sediment depth; for details see Supplementary Table 1). These values are in the same range as those obtained for most sediment surface samples (Figure 1b).

Global distribution and phylogeny of the MMC

To assess the global distribution of the MMC, an analysis of a large set of predominantly sediment samples ($n=72$, see Supplementary Table 1) was performed. The MMC was detected in almost all samples from marine sediments covering subarctic (Spitzbergen/Svalbard) and temperate regions (eastern and western Atlantic coast, North Sea), the Mediterranean Sea (surface to 4300 m depth), subtropical (Baja California, Florida, Canary Islands, Red Sea, South African coast, Australia) and tropical regions (Caribbean Sea, Ceylon). Only in a few samples of sandy sediments or on macroalgae from various locations (Dominican Republic, Tarquinia (Italy), Red Sea, Majorca, Canary Islands, Khao Lak (Thailand)), at a hydrothermal vent in the Guaymas Basin, as well as in various freshwater habitats the MMC was not detected (Supplementary Table 1).

In total, 11 samples from sediments and cyanobacterial mats from different locations and salinities from 1–164 were also examined for presence of the MMC (Table 1). The results show that its presence is restricted to salinities from 6 to 60 ($P<0.05$, Chi square χ^2 -test with Yates correction), emphasising the occurrence of the MMC in marine and brackish but not in freshwater and hypersaline environments.

Table 1 Detection of the MMC in surface sediment samples at various locations as a function of salinity

Location ^a	Salinity	Presence of MMC
<i>Solar Lake, Egypt</i>		
Cyanobacterial mat 1	114	No
Cyanobacterial mat 2	114	No
<i>Baja California, Mexico</i>		
Natural cyanobacterial mat	60	Yes
Saltern pond 2	55	Yes
Saltern pond 4	92	No
Saltern pond 6	164	No
<i>Weser estuary, Germany (km)</i>		
Blexen (63)	25	Yes
Brake (41)	7	Yes
Berne (25)	7	No
Bremen (2)	6	Yes
River Elbe (Dresden, Germany)	1	No

Abbreviation: MMC, marine myxobacteria cluster.

^aFor latitude and longitude see Supplementary Table 1.

Phylogenetic analysis of MMC sequences obtained from our samples and closely related sequences found in public databases revealed a distinct cluster within the *Myxococcales* of exclusively marine, uncultured organisms. Described species in the three myxobacterial suborders are only distantly related to this cluster. Overall, we collected 50 MMC sequences with a length of >1300 bp (Figure 2) and additionally 134 sequences with a length of >450 bp (Supplementary Figure 1). The MMC includes the cluster C13 recently described by Jiang *et al.* (2010); however, the MMC encompasses more and deeper branching sequences. Robustness of the MMC was demonstrated by a bootstrap value of 99% and the same branching point obtained with a maximum likelihood tree. Sequences obtained with the MMC-specific PCR showed affiliation with the MMC, thus proving specificity of the primers. The deep branching sequence of clone H2 obtained with the specific PCR from North Sea sediment was considered to belong to the MMC, because it was affiliated to clone WHB21–22 (accession number AB426359, Supplementary Figure 1) within the MMC when a maximum likelihood tree was calculated.

Sequences affiliated with the MMC were detected worldwide by us and other authors, for example, in the Arctic Ocean, Greenland, the temperate Pacific coast of the United States of America and Japan, the mid-Atlantic ridge, Panama, Tasmania and Antarctica (Figure 3 and Supplementary Figure 1). Even though most sequences affiliated with the MMC were obtained from sediment samples, these bacteria inhabit also other surfaces, that is, corals, microbial mats and marine benthos.

Quantification of the MMC in some of the samples collected worldwide indicated a lower abundance compared with that found in North Sea samples.

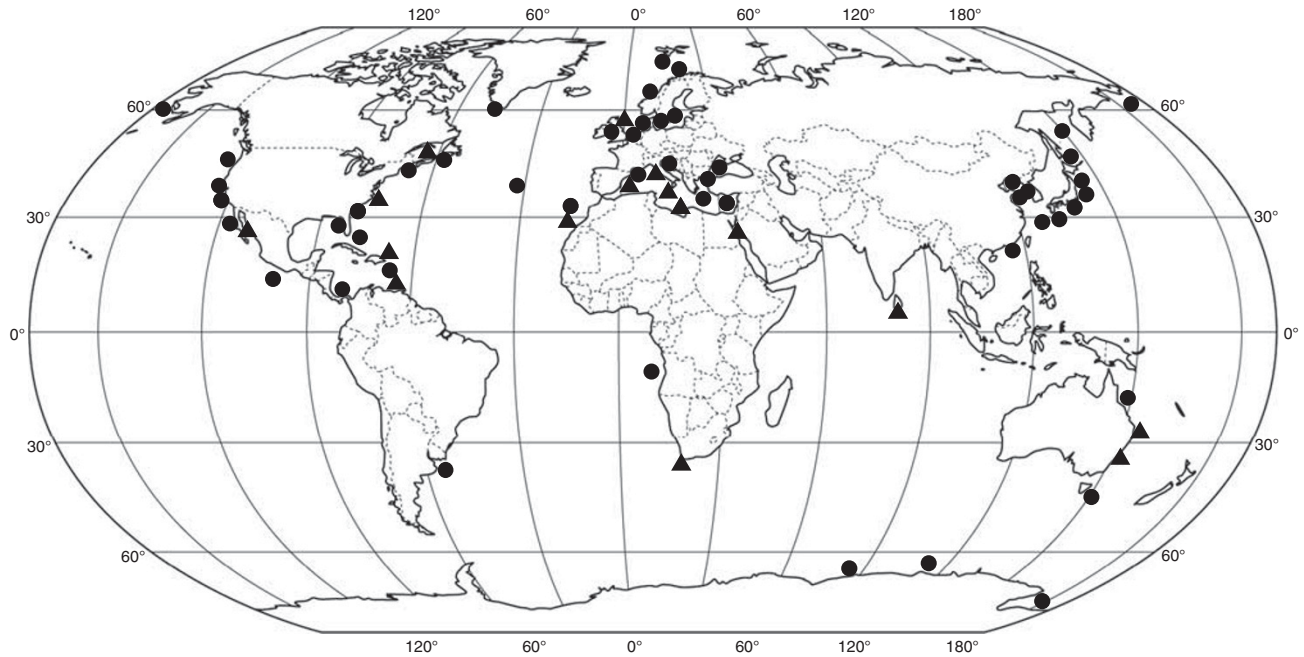


Figure 3 Map showing the world-wide distribution of the MMC. ▲, detection by the MMC specific PCR, ●, detection by sequencing and phylogenetic analysis of 16S rRNA genes obtained in this study and by other authors. The map was adopted from <http://www.smithlifescience.com> and subsequently modified.

In a sediment sample taken from the Baltic Sea (sample number 120, Supplementary Table 1), the MMC accounted for 0.71% of the total bacterial community. In other samples, that is, sediment from the eastern Mediterranean Sea (sample no. 167), sediment taken off the coast of North Carolina (sample no. 140) and from a cyanobacterial mat in Baja California (sample no. 186), abundances of the MMC were even lower (0.05%, 0.13% and 0.01%, respectively). This suggests that bacteria of the MMC prosper exceptionally well in North Sea habitats and/or generally in shallow marine sediments rich in organic material, such as intertidal and sublittoral mud flats.

One subcluster of the MMC contains 10 sequences >1300 bp, exclusively obtained from tropical and subtropical locations (Figure 2, subcluster I). Some sequences <1300 bp from other climatic regions, however, also affiliate to this cluster (Supplementary Figure 1, subcluster I). Most other clusters contain a mix of sequences from temperate and polar regions, but seem not to reflect adaptation of subgroups to specific marine environments or conditions.

Within the MMC, organisms appear to be strictly marine. In contrast, sequences between the MMC and the primarily terrestrial described suborders are from marine as well as from other, very diverse habitats, for example, soils, pack ice, a glacier, wastewater treatment plants, compost, a cave wall biofilm and an acid mine drainage (Figure 2).

Enrichment and isolation of myxobacteria

We were able to isolate two strains, *Myxococcus* sp. MX1 and *Myxococcus* sp. MX2, from Wadden Sea sediment taken on 30 September 2006. The new strains are affiliated to the *Cystobacterineae* (Figure 2), with *Myxococcus xanthus* strain ATCC 25232 as the closest described relative (16S rRNA gene similarity of 98% and 99% to strain MX1 and MX2, respectively) and show also close affiliation to *Myxococcus fulvus* ATCC 25199, the type species of the genus (16S rRNA gene similarity of 97% and 98% to strain MX1 and MX2, respectively). Our isolation effort with classical isolation techniques for myxobacteria, however, did not yield any organisms of the MMC.

We tried to enrich bacteria of the MMC in a mesocosm experiment with sediment and water from the Wadden Sea. The results indicated presence of bacteria of the MMC on artificial surfaces of agar-coated slides. After 2 days and until the end of the experiment, the MMC-specific PCR gave positive results for all samples removed from the slides, irrespective whether they were presettled with cultivated bacteria (as prey organisms) or not. Formation of biofilms on the agar-coated slides was visible by eye, and strong degradation of the agar became obvious after 2 weeks. Bacteria of the MMC, however, showed no growth after transfer of cells from the slides on agar plates with various media (that is, complex and minimal media with different concentrations of autoclaved seawater and natural sediment, dead marine bacterial strains as

prey organisms, cycloheximide to restrict development of fungi, incubation at *in situ* temperature in the dark).

Sequencing of fosmid, annotation and sequence comparison

Screening of a fosmid library constructed from a sediment sample of the German Wadden Sea revealed two fosmids containing 16S rRNA genes affiliated with the MMC (clone MMCf1 and clone MMCf2, Figure 2). The full sequences of both fosmids were determined. For the 37.1 kb-insert of fosmid MMCf1, 24 protein-encoding ORFs were predicted. The deduced amino-acid sequences of 20 ORFs showed significant similarities (*E*-value <1e-20) to proteins in the UniProtKB/TrEMBL database (Table 2). The second fosmid, MMCf2, contained an insert of 40.7 kb with 31 predicted protein-encoding ORFs, of which 23 showed significant similarities to proteins in the UniProtKB/TrEMBL database (Table 2). The sequences of both fosmids showed an overlap of ca. 32 kb (Figure 4). In the overlapping region, both fosmids highly resemble each other (89–94% DNA sequence identity). The differences indicate that both fosmids resulted from different strains. The G + C content is 62.3% for MMCf1 and 62.2% for MMCf2. Both fosmids possess a single 16S rRNA gene and no complete rRNA operon as found in most bacteria. Furthermore, no genes coding for transfer RNAs were found besides the 16S rRNA genes as usually found in the internal transcribed spacer regions between 16S and 23S rRNA genes.

Bidirectional BLAST comparisons of protein sequences of both fosmids with each other and against seven myxobacteria whole-genome protein datasets were carried out. The two fosmids share 20 orthologues (Supplementary Figure 3), 16 of these genes show a similarity score over 90% in the Needleman–Wunsch alignment. For MMCf1, the highest number of orthologues with 14 out of 23 (61%) was identified in two organisms, *S. cellulorum* and *H. ochraceum*. Moreover, the ORFs MMCf1_21–22 had the same synteny in the two organisms. This was also observed for MMCf1_23–24 in *H. ochraceum* and for the two subunits of the highly conserved glutamate synthase (*gltAB*) in all seven datasets and many genomes of other bacteria from various taxa. A comparison of the genomic loci flanking the glutamate synthase genes revealed no specific genomic locus or any evidence for syntenic gene organisation in other myxobacteria. Besides highly similar ORFs coding for a protein kinase (MMCf1_9) the following ORFs were found in all seven species (Supplementary Figure 3): a nucleoside triphosphatase (MMCf1_12), a conserved hypothetical protein, YyaL-like (MMCf1_13), a putative aldo/keto reductase (MMCf2_9). Nevertheless, the gene order of the two fosmids remains unique among the myxobacteria. The highest number

of orthologues for MMCf2 with 15 out of 30 (50%) was assigned to *S. cellulorum*, closely followed by *H. ochraceum* with 14 (47%) orthologous genes. Ten ORFs (MMCf1_3/10/17 and MMCf2_3/5/7/10/13/20/27) have no detectable orthologues in any of the references. Interestingly, for nine of these genes (all except MMCf2_10) even no significant BLAST hit in the public databases could be found (Table 2), implying that they are unique for the two fosmids.

The ORFs MMCf1_13 and MMCf2_23 are highly similar to the *Desulfobacterium autotrophicum* ‘hypothetical protein HRM2_15540’. Orthologues are also present in all completed myxobacteria genomes (Supplementary Figure 3), as well as in other bacteria, archaea and eukaryota and share several domains, for example, a domain of unknown function DUF255 (Pfam03190), a thioredoxin fold (IPR012335) and a six-hairpin glycoside transferase domain (IPR012341). The orthologues of eukaryotic origin are annotated as ‘spermatogenesis-associated proteins’ (for example, UniProtKB/Swiss-Prot entry Q80YT5) for which, according to the Gene Ontology project (GO:0007275, Ashburner *et al.*, 2000), the molecular function is described as ‘multicellular organismal development’, compatible with the myxobacterial life cycle.

In general, genes on the MMCf1 and MMCf2 fosmids could not be assigned to one certain pathway, but seem to be involved in various metabolic routes. Some of the genes (MMCf1_2 and MMCf2_12) might be involved in methylamine metabolism (*mauG*) or the synthesis of polyamines. The ORFs MMCf1_23 and MMCf1_24 encode a saccharopine dehydrogenase and a carboxynorspermidine decarboxylase. This clustered pair of genes is also present in other bacterial lineages and most probably part of an alternative route for the synthesis of both sym-norspermidine and spermidine (Lee *et al.*, 2009). Other genes (MMCf1_6/7 and MMCf2_16/17) are likely part of amino-acid metabolism (glutamate synthase, *gltAB*).

Discussion

Similar to Gram-positive bacteria, myxobacteria have been considered for a long time as typical terrestrial organisms. Strains of both groups sporadically isolated from marine habitats were thought not to be indigenous but introduced from terrestrial habitats (Zobell and Upham, 1944; Goodfellow and Haynes, 1984; Reichenbach, 1999; Dawid, 2000). Today, it is widely accepted that distinct marine clusters of Gram-positive bacteria exist, which are only distantly related to clusters comprising also Gram-positive bacteria from freshwater and soil (for example, Rappé *et al.*, 1999; Mincer *et al.*, 2002). Here, we demonstrated that bacteria of the MMC are distributed worldwide in marine sediments and also present on several other marine surfaces but are

Table 2 Annotation of genes of fosmids MMCf1 and MMCf2

MMcf1 ORF no. (aa)	MMcf2 ORF no. (aa)	Predicted protein	MMcf1 closest homologue Uniprot BLASTp E-value <1e-20	MMcf2 Closest homolog Uniprot BLASTp E-value <1e-20
	1 (212)	Peroxidase (EC 1.11.1.7)		<i>Haliangium ochraceum</i> DSM 14365 3e-88
	2 (143)	Putative thioesterase superfamily protein		<i>Myxococcus xanthus</i> DK 1622 2e-23
	3 (91)	Hypothetical protein		—
	4 (131)	Hypothetical protein		—
	5 (134)	Hypothetical protein, polyketide cyclase/dehydrase-like		—
	6 (185)	Transcriptional regulatory protein		<i>P. pacifica</i> SIR-1 1e-28
	7 (88)	Conserved hypothetical protein DUF1876 family		—
	8 (243)	Conserved hypothetical protein, metallo-hydrolase/oxidoreductase superfamily protein-like		<i>Geodermatophilus obscurus</i> DSM 43160 1e-85
	9 (318)	Putative aldo/keto reductase, containing potassium channel β -chain regulatory domain		Uncultured bacterium Bio6 e-110
	10 (705)	Putative extracellular matrix protein, <i>Dictyostelium discoideum</i> (slime mold) prestalk protein-like		<i>Dictyostelium minutum</i> 4e-26
1	11	16S rRNA gene		
2 (470)	12 (470)	mauG: methylamine utilisation protein mauG	<i>Alcanivorax</i> sp. DG881 2e-119	<i>Alcanivorax</i> sp. DG881 1e-113
3 (148)	13 (152)	Putative MarR transcriptional regulator	—	—
4 (293)	14 (303)	C-5 sterol desaturase (EC 1.3.-.-)	<i>Flavobacteriales</i> bacterium ALC-1 8e-87	<i>Flavobacteriales</i> bacterium ALC-1 2e-87
5 (1475)	15 (1670)	Conserved hypothetical protein associated with cell-cell interaction, containing Myxococcus cysteine-rich repeat	<i>H. ochraceum</i> DSM 14365 3e-22	<i>H. ochraceum</i> DSM14365 2e-31
6 (490)	16 (490)	gltB: glutamate synthase (NADPH) small chain (EC 1.4.1.13)	<i>Blastopirellula marina</i> DSM 3645 0.0	<i>Blastopirellula marina</i> DSM 3645 0.0
7 (1532)	17 (1532)	gltA: glutamate synthase (NADPH) large chain (EC 1.4.1.13)	<i>Blastopirellula marina</i> DSM 3645 0.0	<i>Blastopirellula marina</i> DSM 3645 0.0
8 (176)	18 (176)	AsnC/Lrp-family transcriptional regulatory protein	<i>M. xanthus</i> DK 1622 2e-28	<i>Sorangium cellulosum</i> 4e-28
9 (412)	19 (412)	Protein kinase (EC 2.7.-.-)	<i>S. cellulosum</i> 1e-76	<i>S. cellulosum</i> 4e-77
10 (255)	20 (255)	Serine protease, trypsin family, histidine active site	—	—
11 (429)	21 (402)	Predicted DD-peptidase family peptidoglycan-binding protein	<i>H. ochraceum</i> DSM 14365 9e-45	<i>P. pacifica</i> SIR-1 2e-29
12 (207)	22 (207)	Ham1-family nucleoside triphosphatase (EC 3.6.1.15)	<i>Pseudovibrio</i> sp. JE062 8e-34	<i>Pseudovibrio</i> sp. JE062 4e-41
13 (800)	23 (800)	Conserved hypothetical protein, YyaL-like	<i>Desulfobacterium autotrophicum</i> 0.0	<i>D. autotrophicum</i> 0.0
14 (320)	24 (320)	Conserved hypothetical protein	<i>H. ochraceum</i> DSM 1436 2e-41	<i>H. ochraceum</i> DSM 14365 7e-42
15 (327)	25 (327)	Mandelate racemase/muconate lactonising enzyme	<i>Sulfitobacter</i> sp. NAS-14.1 8e-78	<i>Oceanicola batsensis</i> strain HTCC2597 3e-78
16 (262)	26 (262)	Hypothetical protein hydrolase-like	<i>Planctomyces maris</i> DSM 8797 1e-33	<i>Planctomyces maris</i> DSM 8797 1e-31
17 (401)	27 (401)	Hypothetical protein	—	—
18 (161)	28 (161)	Flavin reductase-like, FMN binding	<i>Chloroflexus aurantiacus</i> DSM 637 1e-28	<i>Chloroflexus aurantiacus</i> DSM 637 2e-28
19 (228)	29 (228)	Phosphoglycerate mutase family protein	<i>Thermus thermophilus</i> HB8 1e-27	<i>T. thermophilus</i> HB8 2e-28
20 (341)	30 (341)	Predicted cytochrome c551 peroxidase (EC 1.11.1.5)	<i>Parabacteroides johnsonii</i> 2e-86	<i>Parabacteroides johnsonii</i> 8e-87
21 (633)	31 (633)	Putative activator of 2-hydroxyglutaryl-CoA dehydratase	<i>S. cellulosum</i> 3e-164	<i>S. cellulosum</i> 3e-162
22 (1183)		Putative CoA-substrate-specific enzyme activase	<i>S. cellulosum</i> 0.0	
23 (395)		Saccharopine dehydrogenase-like oxidoreductase	<i>H. ochraceum</i> DSM 14365 3e-178	
24 (202 truncated)		Predicted carboxynorspermidine decarboxylase (truncated)	<i>S. fumaroxidans</i> DSM 10017 7e-54	

Abbreviations: FMN, flavin mononucleotide; MMC, marine myxobacteria cluster; NADPH, nicotinamide adenine dinucleotide phosphate; ORF, open reading frame.

absent in non-marine habitats. Thus, our study confirms the existence of distinct marine myxobacterial clusters (Jiang *et al.*, 2010) and shows that they evolved separately from the known myxobacterial suborders. Furthermore, it extends our knowledge

about the biogeography and diversity of these organisms and allowed a first insight into their genomic features. Phylogenetic analysis revealed that sequences that classify the phylogenetic tree between the MMC and the primarily terrestrial

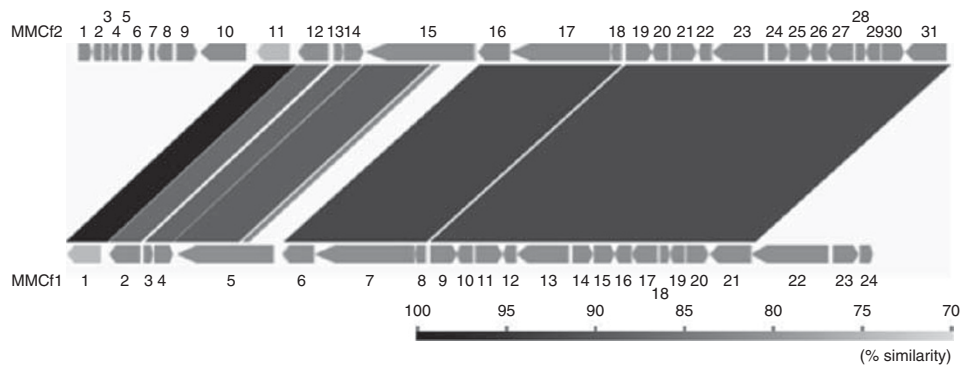


Figure 4 Alignment of fosmids MMCf1 and MMCf2. Protein coding ORFs are depicted in light-grey, the 16S rRNA genes in dark-grey. Alignment of the two fosmids is generated by the GenomeMatcher software. The identity score of BLASTn of each block is given in grey scale; white: no similarity; black: highest similarity.

clusters of the known suborders were obtained from marine but also from other, very diverse non-saline habitats (Figure 2). This overlap might reflect a transition and adaptation from a terrestrial to a marine lifestyle.

The MMC is only sporadically present in the water column, and if so, then mostly on aggregates close to the sediment surface. Thus resuspension of sediment particles by tidal currents (Jago *et al.*, 2002) is a possible reason for presence of these organisms in the water column. The MMC was predominantly found in oxic habitats, suggesting an aerobic metabolism as described for most myxobacteria (Shimkets *et al.*, 2006). On the basis of the presence of MMC bacteria in three different sediment cores from the North Sea down to a sediment depth of about 2 m, however, one can speculate that these organisms can also live under anoxic conditions or produced spores that were buried in the sediment. Oxygen is only present in the upper 3 mm of the sediment at the sampling sites (Köpke *et al.*, 2005). Myxobacteria have been known for a long time to produce spores (Shimkets, 1990; Shimkets *et al.*, 2006), but in contrast to other organisms of the *Deltaproteobacteria*, most myxobacteria are strictly aerobic heterotrophs. *A. dehalogenans* (*Cystobacterineae*), however, was shown to be facultatively anaerobic, using acetate, H₂, succinate, pyruvate, formate and lactate as electron donors, and various chlorophenolic compounds and nitrate as electron acceptors (Sanford *et al.*, 2002). To elucidate how MMC bacteria survive in anoxic sediment, further studies are needed, that is, isolation of these organisms and subsequent physiological characterisation or molecular and gene expression analyses to investigate their metabolic activity.

By applying classical isolation methods, we were able to obtain two isolates affiliated with the *Myxococcaceae* (within *Cystobacterineae*) from the Wadden Sea sediment. *Myxococcus* strains have been obtained from marine environments before and were investigated for their adaptation to marine conditions. Isolates obtained by Wang *et al.* (2007) possessed different levels of salt tolerance, had the

dual motility system and formed fruiting bodies in the presence of suitable seawater concentrations. Some high salt-tolerant strains even lost their fruiting abilities in the absence of seawater Wang *et al.* (2007). Overall, the results suggested an ecological adaptation of myxobacterial social behaviours of these isolates to marine environments (Wang *et al.*, 2007). With the classical methods used in this study (Reichenbach and Dworkin, 1992), no strains of the MMC were obtained what might explain why no organisms of this cluster have been isolated by other researchers so far. Whether the two new *Myxococcus* strains MX1 and MX2 are real marine organisms remains open, because currently we only know that they can grow with and without the addition of 50% of seawater to the medium.

In the mesocosm experiment, we were able to induce at least settlement of MMC bacteria on agar-coated slides. In contrast to the classical methods, the mesocosm experiment provided an environment more similar to natural conditions and thus allowed survival or even growth of MMC bacteria. Settlement occurred not only on slides presettled with cultivated bacteria but also on pure agar. As other bacteria certainly also settled on these slides, we can not determine whether MMC bacteria are attracted by the presence of other microorganisms or if they even hydrolyse other bacteria. The experiment, however, confirmed the preference of MMC bacteria to settle on various surfaces.

Sequence analysis of two MMC fosmids found in this study indicated that both are highly similar but arose from different strains. The GC content of the fosmid sequences is only slightly lower than that of known genomes of other myxobacteria (64 to 72 mol %). Both fosmids have a single 16S rRNA gene and no complete rRNA operon. Split rRNA operons have been identified in bacteria and archaea previously, primarily when an entire genome has been sequenced. In the majority of cases, the 23S and 5S genes form an operon and the 16S rRNA gene is separate, and there can be one or more than one copy of each of the genes (Boyer *et al.*, 2001). Even though unlinked rRNA operons are rare,

they are known for several organisms affiliated with *Planctomycetes*, *Deinococcus-Thermus*, *Alpha-*, *Gamma-* and *Epsilonproteobacteria*, *Poribacteria* or *Euryarchaeota* (<http://www.ncbi.nih.gov/genomes/lproks.cgi>; <https://img.jgi.doe.gov/cgi-bin/er/main.cgi>; Liesack and Stackebrandt, 1989; Ruepp *et al.*, 2000; Tamas *et al.*, 2002; Glöckner *et al.*, 2003; Wu *et al.*, 2003; Henne *et al.*, 2004; Fieseler *et al.*, 2006). For *Deltaproteobacteria*, however, this is to our knowledge the first description of an unlinked rRNA operon. Unusual organisation of rRNA operons is known for myxobacteria. The genome of *S. cellulolum* (account no. AM746676; Schneiker *et al.*, 2007) contains two complete rRNA operons (16S-23S-5S), one incomplete (16S-23S) and one with two 5S rRNA genes (16S-23S-5S-5S).

Comparison of the genomic loci flanking the 16S rRNA genes on the two fosmids MMCf1 and MMCf2 revealed neither a specific genomic locus nor any evidence for a syntenic gene organisation. Although both fosmids have 50% and 61% orthologues in other sequenced myxobacteria, respectively (Supplementary Figure 3), it is striking that about half of the closest homologues of the genes identified on the fosmids by BLASTp analysis were found in other marine bacteria from various taxa and less than one-third of the closest homologues were found within the myxobacteria (Table 2). This might reflect an ancient separation of the MMC from other myxobacterial lineages and adaptation to the marine environment. For nine of the genes identified on the fosmids, no significant BLAST hits in the public databases were found (Table 2). This indicates that at least on a genomic basis bacteria of the MMC are exceptional. Sequencing of the fosmids allowed the identification of other than 16S rRNA genes. Thus, in future studies, highly specific primer systems can be used that target not only the 16S rRNA gene, allowing a higher specificity, as well as expression analyses of functional genes to monitor the activity and to further elucidate details of the lifestyle of these unique bacteria.

Overall, distribution and abundance of the MMC suggest that it comprises important players in marine sediment-associated bacterial communities. Phylogenetic analysis revealed one subcluster (subcluster I, Figure 2) obtained exclusively from tropical and subtropical regions, which might reflect adaptation to specific conditions. Except for a coherence of abundance with the sediment type, and presence with salinity, no further parameters influencing the distribution were identified. Their physiology remains also largely unknown, and we currently do not know whether they hydrolyse biopolymers or even prey on other microorganisms, similar to terrestrial myxobacteria. MMC bacteria also could be a novel source of secondary metabolites because their terrestrial counterparts are one of the prime sources of these bioactive compounds (Gerth *et al.*, 2003; Wenzel and Müller, 2007). Future studies have to reveal this possible activity, the

ecological role of the MMC, parameters having influence on its distribution and whether these organisms are a mortality factor for bacterial growth not considered so far in marine sediments.

Acknowledgements

We thank the crew of the RV Heincke for their valuable support on shipboard, Thomas Badewien and Axel Braun for technical assistance in sampling and Helge Giebel for help with graphical presentations. Furthermore, we thank numerous colleagues and friends for providing samples from various locations. This work was supported by a grant from the Deutsche Forschungsgemeinschaft within the Research Unit 'BioGeoChemistry of the Wadden Sea' (FG-432 TP-5 and TP-B).

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