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Occurrence of diverse alkane hydroxylase *alkB* genes in indigenous oil-degrading bacteria of Baltic Sea surface water

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ABSTRACT

Formation of specific oil degrading bacterial communities in diesel fuel, crude oil, heptane and hexadecane supplemented microcosms of the Baltic Sea surface water samples was revealed. The 475 sequences from constructed alkane hydroxylase *alkB* gene clone libraries were grouped into 30 OPFs. The two largest groups were most similar to *Pedobacter sp.* (245 from 475) and *Limnobacter sp.* (112 from 475) *alkB* gene sequences. From 56 alkane-degrading bacterial strains 41 belonged to the *Pseudomonas* spp. and 8 to the *Rhodococcus* spp. having redundant *alkB* genes. Together 68 *alkB* gene sequences were identified. These genes grouped into 20 OPFs, half of them being specific only to the isolated strains. Altogether 543 diverse *alkB* genes were characterized in the brackish Baltic Sea water; some of them representing novel lineages having very low sequence identities with corresponding genes of the reference strains.

1. Introduction

The intercontinental brackish Baltic Sea, characterized by strong stratification, high nutrient concentration and continuous oxygen deficiency (Koskinen et al., 2011), is a habitat for a mixture of marine, freshwater and brackish water organisms (Höllfors et al., 1981). The coastline of the sea is shared by nine industrial countries that results in intensive marine traffic on the sea. Both the intensive shipping and complications in navigation (narrow traits, shallow depths, crossing shipping lanes) enhance the risk of oil pollution to the marine environment, including bays and beaches (HELCOM (Helsinki Commission. Baltic Marine Environment Protection Commission), 2013). Clean up of oil spills primarily depends on the indigenous microbes present in the environment (Leahy and Colwell, 1990) and therefore it is particularly important to investigate the catabolic capabilities of these organisms. Due to the development of the second generation sequencing techniques a number of studies, dealing with the total microbial community composition of the Baltic Sea, have been performed (Andersson et al., 2010; Koskinen et al., 2011; Tiirik et al., 2014; Dupont et al., 2014) and recently some reports analyzing the communities or/and genes involved in oil-derived hydrocarbons degradation by Baltic Sea bacterioplankton have been published (Jutkina et al., 2011; Reunamo et al., 2013; Vedler et al., 2013; Viggor et al., 2013). Alkanes originating from natural or anthropogenic sources are degraded by several different enzyme systems expressed by phylogenetically diverse bacteria (van Beilen and Funhoff, 2007). The integral-membrane alkane hydroxylases (AlkB; EC 1.14.15.3), which are responsible for hydroxylation of medium-chain-length alkanes (C10 to C16) are the most common and widely distributed genes that have been used for description of the composition of alkane-degrading communities and calculation of the abundance of oil degrading bacteria in various environments (Smith et al., 2013; Nie et al., 2014). The alkane-degradation gene clusters may be plasmid encoded, as for example the well-studied OCT plasmid of *Pseudomonas putida* GPo1 (Kok et al., 1989), but generally they are located in the chromosome (Smits et al., 1999). Usually only one *alkB* gene is found in the genome, but there are several Gram-positive and Gram-negative genera whose representatives have two or more redundant *alkB* genes, for example genus *Rhodococcus* (Whyte et al., 2002) and *Acinetobacter* (Tani et al., 2001), respectively. Although the AlkB sequences may be quite divergent they share a conserved moiety that allows the design of primers applicable for amplification of phylogenetically distant alkane hydroxylases (Smits et al., 1999; Kloos et al., 2006; Viggor et al., 2013).

This study was conducted to evaluate the reactions of indigenous microbial communities in response to a simulated oil spill (heptane, hexadecane, diesel fuel and crude oil were used as model substrates in microcosm experiments), to isolate oil-degrading bacteria, and to analyze the genetic diversity of alkane hydroxylase-encoding *alkB* genes in four different surface water samples of the Baltic Sea.

2. Materials and methods

2.1. Study area and sample collection

A total of eight surface water samples (at depths of approximately 1 m) were collected from four different Baltic Sea regions from August to September in 2008 and 2009 (Fig. 1) using 12-L sterile canisters.

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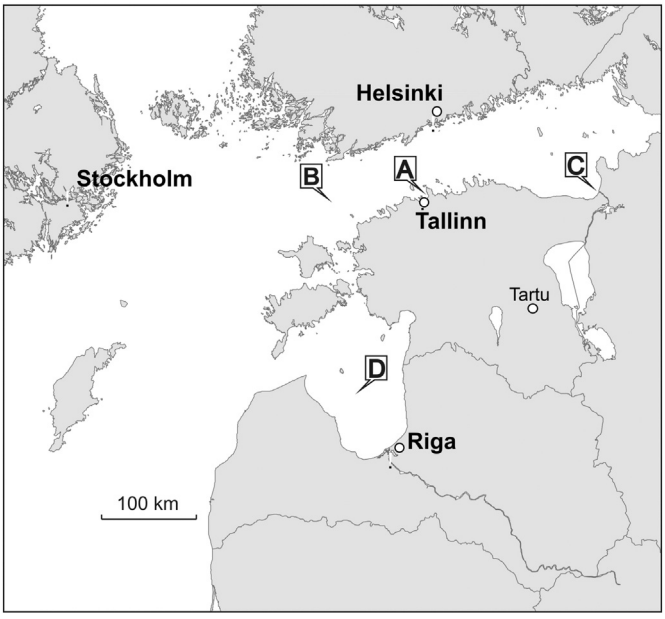

Fig. 1. The location of sampling sites (A — Tallinn Bay, B — Gulf of Finland, C — Narva Bay and D — Gulf of Riga) in Baltic Sea.

The sample code in this study consists from capital letter (A — Tallinn Bay, B — Gulf of Finland, C — Narva Bay and D — Gulf of Riga) and samples from the year 2009 have the number 2 before the letter. The sample taken ca 6 km from the port of Tallinn, A (59°32′12″ N, 24°41′18″ E), is in high sea traffic area. Sampling point C (59°28′30″ N, 28°00′30″ E) is affected by fresh water more than other sites because it is taken ca 2 km from the coast, near the estuary of the river Narva. Distances of offshore sampling sites B (59°28′60″ N, 22°57′00″ E) and D (57°37′00″ N, 23°37′00″ E) from coast were ca 37 km and 41 km, respectively. The salinity values of the sampling points were as follows A/2A — 5.8/6.0‰, B/2B — 6.3/6.1‰, C/2C — 3.7/4.1‰ and D/2D — 5.4/5.3‰. Samples were stored at 4 °C until analysis (within 24 h after collection).

2.2. Isolation of bacteria: direct selection and microcosm experiments

Hydrocarbon-degrading bacterial strains were isolated from plated-out cultures of collected seawater on selective M9 mineral agar plates (Adams, 1959; Bauchop and Elsden, 1960) containing crude oil (CO; Lukoil Oil Company OAO), diesel fuel (DF; Neste Oil), heptane (Hp; Fluka; ≥96% purity) or hexadecane (Hd; Fluka; 99% purity) as the only growth substrate (vapor phase). Plates were sealed and incubated at 15 °C for 14 days.

The microcosms (enrichment and control) experiments were performed as described in Jutkina et al. (2011) using crude oil (CO), diesel fuel (DF), heptane (Hp) or hexadecane (Hd) at final concentration 1% (*v*/*v*) as the only growth substrate in enrichments. The experiments were done with each seawater sample in 250-mL sterile flasks containing 50 mL of liquid media at 15 °C on rotary shaker (130 rpm) for three weeks. Isolation of hydrocarbon-degrading bacteria from different microcosms was performed after serial dilution and plating on selective media (containing the same carbon source that was used in microcosm), followed by a 2 week incubation period at 15 °C.

Purified, morphologically different bacterial cultures having unique BOX-PCR fingerprint pattern (Heinaru et al., 2000) and *alkB* gene were stored in 20% glycerol at −80 °C.

The carbon source utilization tests of the bacteria were performed on the minimal media supplemented with octane (C8; AlfaAesar; ≥98% purity), hexadecane (C16/Hd; Fluka; 99% purity), octadecane (C18; AlfaAesar; 99% purity) or docosane (C22; Sigma-Aldrich; 99% purity) as the only growth substrate (vapor phase).

2.3. Extraction of total community DNA

Total community DNA was extracted from initial seawater and from the first enrichment step of microcosm experiments as described by Viggor et al. (2013) using a PowerSoil DNA Kit (MoBio Laboratories, Inc., USA), according to the protocol provided by the manufacturer. The extracted DNA was quantified with spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies, USA) and stored in MilliQ water at −20 °C.

2.4. PCR amplification of target molecules

The 16S rRNA genes and membrane-bound alkane hydroxylase encoding *alkB* gene fragments of the isolated bacterial strains, and the 16S rRNA gene fragments of bacterial communities (eight samples of the initial water and samples obtained from microcosm enrichment experiments with all seawater samples each supplemented with heptane, hexadecane, diesel fuel or crude oil) for denaturing gradient gel electrophoresis (DGGE) analysis were amplified as described in our previous work (Viggor et al., 2013). The specific primer pair HydroAlkBF (5′-CCTACGGCCATTTCTTCATTGA-3′) and HydroAlkBR (5′-GGCTGTAATG CTCGAGATAATT-3′) was designed based on *Hydrocarboniphaga effusa* AP103 *alkB* gene sequence (EIT68388). The PCR conditions were identical to those of the alkBF and alkBR primers (Viggor et al., 2013).

2.5. Clone libraries

Gel purified (Qiaquick Gel Extraction Kit, Qiagen) PCR products of the *alkB* genes amplified from the total community DNAs of the initially collected seawater samples were cloned into a pTZ57R/T vector and transformed into competent *Escherichia coli* DH5α cells, following the manufacturer's protocol of InsT/Aclone product Cloning Kit (Thermo Fisher Scientific Inc). Screening and thereafter grouping of correctly amplified inserts obtained from community DNAs were done using DGGE analysis (Viggor et al., 2013). The PCR products of the *alkB* genes amplified from the isolated strains that resulted in ambiguous bases during direct sequencing, suggesting overlapping sequences referring to intra-species gene redundancy, were cloned similarly. In the case of *alkB* clone libraries of the strains the screening of inserts from white colonies of transformants was performed using the vector specific primer pair (M13-R 5′-AACAGCTATGACCATG-3′ and M13-F 5′-CATTTTGCTTGC CGG-3′; Thermo Fisher Scientific Inc.). At least ten correctly amplified inserts were randomly picked and sequenced. One representative of each group of different sequences was chosen for further phylogenetic analyses.

2.6. DGGE

The INGENYphorU-2 × 2 (Ingeny International, Netherlands) DGGE system was used for analysis of the *alkB* gene clone libraries of initially collected seawater and 16S rRNA genes amplified from the community DNAs of initially collected seawater and enrichment experiments, following the recommendations of the manufacturer. Detailed description of used DGGE conditions and subsequent analysis of DGGE gels are described earlier in Viggor et al. (2013). The cluster analysis of PCR-DGGE fingerprints of the partial 16S rRNA genes of the control sample (initial seawater without additional substrate) communities and enrichment communities revealed that after three week incubation the community profiles of the Baltic Sea water without substrate formed separate cluster from enrichment communities (data not shown) as was reported in Viggor et al., 2013.

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