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Limnobacter spp. as newly detected phenol-degraders among Baltic Sea surface water bacteria characterised by comparative analysis of catabolic genes

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ABSTRACT

A set of phenol-degrading strains of a collection of bacteria isolated from Baltic Sea surface water was screened for the presence of two key catabolic genes coding for phenol hydroxylases and catechol 2,3-dioxygenases. The multicomponent phenol hydroxylase (LmPH) gene was detected in 70 out of 92 strains studied, and 41 strains among these LmPH$^+$ phenol-degraders were found to exhibit catechol 2,3-dioxygenase (C23O) activity. Comparative phylogenetic analyses of LmPH and C23O sequences from 56 representative strains were performed. The studied strains were mostly affiliated to the genera *Pseudomonas* and *Acinetobacter*. However, the study also widened the range of phenol-degraders by including the genus *Limnobacter*. Furthermore, using a next generation sequencing approach, the LmPH genes of *Limnobacter* strains were found to be the most prevalent ones in the microbial community of the Baltic Sea surface water. Four different *Limnobacter* strains having almost identical 16S rRNA gene sequences (99%) and similar physiological properties formed separate phylogenetic clusters of LmPH and C23O genes in the respective phylogenetic trees.

Introduction

The efficiency of biodegradation processes is assessed by the role of indigenous bacterial populations in the natural environment. Culture-dependent and -independent methods are used in microbial ecology for studies of bacteria in environmental samples but by using culture-dependent community analysis only a small proportion of the community is detected. Nevertheless, the study of cultured isolates remains the most straightforward method for characterising an organism's functions and evaluating its biotechnological potential [2]. Discovering new microbes and characterising their functions are major goals in the study of microbial diversity.

Aromatic hydrocarbons are widespread pollutants in soils and freshwaters, as well as marine environments. These compounds are readily biodegradable by various phylogenetically diverse microorganisms, and the respective catabolic pathways and key enzymes are well described. In the aerobic degradation pathways for phenolic compounds, the rate-limiting step is a hydroxylation reaction resulting in corresponding catechols [12,15]. This step is catalysed by phenol hydroxylase (PH, phenol 2-monooxygenase, EC 1.14.13.7). Among the two different variants of this enzyme found in bacteria, the single- and multicomponent types, multicomponent PH (mPH) is considered to be the major enzyme in natural environments [9,27,33]. All mPHs have a similar enzyme structure and they comprise six subunits, with the catabolic site existing within the largest subunit [15,6,24,25,31]. The second key step in the aerobic degradation of phenolic compounds is the ring cleavage of catechol, which is performed either by intradiol (catechol 1,2-dioxygenase (C12O); EC 1.13.11.1) or extradiol (catechol 2,3-dioxygenase (C23O); EC 1.13.11.2) dioxygenases [12,28], and the respective pathways are called the *ortho* and *meta* pathways. Most commonly, the sequences of the largest mPH subunit (LmPH) and C23O have been used as the tools for characterising the diversity of degraders of phenolic compounds [33,1]. Correlation between phenol-degradation activity and the diversity of genes coding for key catabolic enzymes has been shown several times [1,4].

Despite there being a considerable amount of literature on bacterial phenol-degradation, there is limited knowledge about which organisms degrade phenolic compounds in marine environments. To answer this question, it is important to collect evidence from culture-based studies, which define the catabolic capabilities of candidate organisms living in such environments, and to

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compare the results with the data collected from culture-independent methods. Therefore, this study focused on the characterisation of degraders of aromatic compounds, especially phenol, in the Baltic Sea. This sea is a unique ecosystem, since it is a shallow brackish environment, strongly influenced by the abundant freshwater runoff from the surrounding land [20]. The drainage area of the Baltic Sea is about four times larger than the sea itself. This influences its salinity and other physical conditions, as well as the composition and features of its microbial communities. Many human activities in the region, on land and at sea, pose serious threats and result in substantial impacts on the Baltic Sea. Accidental oil spills from ships, for instance, are becoming an increasing concern (http://www.helcom.fi). Thus, there is an obvious need to study the biodegradative potential of Baltic Sea microbial communities.

In previous research, we described in detail the isolation of 209 bacterial strains from Baltic Sea surface water that were able to degrade different aromatic compounds [18]. The primary aim of the present study was to characterise the phenol-degrading bacteria of this collection and analyse the genetic diversity of their key catabolic enzymes LmPH and C23O. The study also provided the first evidence of phenol-degrading bacteria belonging to the genus *Limnobacter*. Analysis of LmPH sequences amplified from the total community DNA and sequenced with the next generation sequencing (NGS) approach was also performed.

Materials and methods

Identification of phenol-degrading bacteria

As shown in our previous study, a collection of 209 isolates with different BOX-PCR fingerprint patterns was able to utilise the aromatic compounds phenol, benzoate, *m*-toluate, salicylate and/or naphthalene as sole energy and carbon sources [18]. This collection was obtained using both enrichment and direct selection with the above substrates from four sampling sites of Baltic Sea surface water (at depths of approximately 1 m) during two years (2008 and 2009). At sampling sites A (near Tallinn) and B (the Gulf of Finland), sea and river water were less mixed than at sites C (near Narva) and D (near Pärnu). Sampling site C was located ca 2 km from an oil shale mining and processing area. The salinity values of the sampling points were in the range of 3.7‰ (site C in 2008) to 6.3‰ (site B in 2008). A total of 92 strains from this collection formed a set of phenol-degraders, which were studied more precisely in the present study.

For species-level differentiation of phylogenetically related strains some morphological, physiological and biochemical properties (colony morphology, cell shape, motility, growth temperature, oxidase test, amylolytic activity, ability to utilise different carbon sources, the presence of polyhydroxybutyrate granules, denitrification ability, etc.) were examined [3]. The carbon source utilisation tests of the phenol-degrading bacteria were performed on the same selective minimal media previously used during the isolation procedures [18].

DNA manipulations and PCR amplification

Genomic DNA was purified from bacterial strains by using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.), according to the manufacturer's instructions. For the extraction of total microbial community DNA, one litre of seawater was run through a sterile filter with a pore size of 0.2 μm (Sartorius AG) and stored at −80 °C. The community DNA was extracted from the filters using a PowerSoil DNA Kit (MoBio Laboratories, Inc, USA), according to the protocol provided by the manufacturer. The extracted DNA was quantified with a spectrophotometer (Nanodrop ND1000) and stored in MilliQ water at −20 °C for subsequent PCR amplification. All PCR reactions were performed in a 25 μL mixture containing approximately 20 ng of template DNA, PCR buffer containing $(NH_4)_2SO_4$, as provided in the Fermentas *Taq*DNA polymerase Kit, 200 μM of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 0.8 μM of each primer, and 0.5 U of *Taq*DNA polymerase (Fermentas).

The amplification and sequence determination of 16S rRNA genes of the isolated strains were performed as described in our previous work [14]. Fragments of the genes encoding LmPH were amplified by PCR using the primer set pheUf (5′-CCAGGSBGARAARGAGARGAARCT-3′) and pheUr (5′-CGGWARCCGCGCCAGAACCA-3′) with the PCR conditions described by Futamata et al. [9]. The *pheA* gene coding for sPH was detected using the primers pheA1 (5′-CAGGATCGAATATCGGTGGCCTCG-3′) and pheA2 (5′-CTTCACGCTGGCGTAACCAATCGC-3′), and the PCR conditions have been described previously [13]. The PCR programme for amplification of the C23O gene fragments with the ORF-F (5′-AGGTGWCGTSATGAAMAAAGG-3′) and ORF-R (5′-TYAGGTSAKMACGTTCAKGAA-3′) primer pair has been described by Junca and Pieper [17]. The *Pseudomonas fluorescens* strains PC18 and PC24 [13] were used as the positive controls for amplification of the genes coding for LmPH and C23O, and the *pheA* gene, respectively. The specific primer pair ORF-FL (5′-AGATCAACATGGCAATGACAGG-3′) and ORF-RL (5′-TTAGGTGAATACGCCCAAAAAG-3′) was designed based on all available C23O sequences of *Limnobacter* strains. The PCR conditions were identical to those of the ORF-F and ORF-R primers, except for the primer annealing temperature that was 57 °C in the case of ORF-FL/ORF-RL. *Limnobacter* sp. strain 2D3 was used as the positive control for amplification with the latter primer pair.

Nucleotide sequencing and in silico analyses

Nucleotide sequencing of PCR products amplified from isolated strains was carried out on a 3730xl DNA Analyzer (Applied Biosystems) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's protocol. Sequences were assembled using BioEdit version 7.0.9.0 [11]. The PCR bands that resulted in ambiguous bases during direct sequencing, suggesting overlapping sequences referring to intraspecies heterogeneity, were cloned into *Escherichia coli* DH5α cells using the InsTAClone™ PCR Cloning Kit (Fermentas), and 20–30 clones of each PCR band were sequenced. One representative of each group of different sequences was chosen for further phylogenetic analyses. The LmPH sequences amplified from the total community DNA were sequenced by default paired-end read protocols using an Illumina HiSeq2000. Reads with low quality at the 3′ end were trimmed with cutoff-value 35 using the program *fastq_quality_trimmer* from the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Trimmed reads shorter than 100 bp and/or containing at least one N were discarded. The sequences of both ends of the LmPH PCR product were analysed separately. The primer sequences were removed and identical sequences were collapsed into one representative sequence. Only the ten most prevalent unique sequences of each water sample were used in further analyses. Multiple alignments were performed and bootstrapped phylogenetic trees were derived using the neighbour-joining method by CLUSTALX version 2.1 [22]. Sequence comparisons were carried out using the BLAST programs from NCBI.

The total DNA from four *Limnobacter* sp. strains was fragmented, and the libraries were prepared and sequenced by default paired-end read protocols using an Illumina HiSeq2000. Whole genome assembly was performed using Velvet version 1.2 [35]. In order

Table 1
Characteristics of the representative phenol-degrading bacteria isolated from Baltic Sea surface water.

Strain[a]	Isolation year/method/ substrate[b]	Closest species in the GenBank database based on 16S rRNA gene sequence identity (%)	GenBank acc. no. of the 16S rRNA gene	Growth on aromatic compounds[c]					LmPH gene group, GenBank acc. no.	C23O gene[d] group, GenBank acc. no.
				Phe	Benz	*m*-Tol	Sal	Nah		
2Aphe2	09/D/Phe	*Pseudomonas stutzeri* NBRC 13596 (100%)	JX177686	+	+	+	–	–	I, JX177800	I, JX177759
2Atol3	09/D/Phe	*P. stutzeri* NBRC 13596 (100%)	JX177688	+	+	+	–	–	I, JX177801	I, JX177760
2A31	09/1R/Benz	*P. stutzeri* NBRC 13596 (100%)	JX177693	+	+	+	–	–	I, JX177797	I, JX177762
2C28	09/1R/Phe	*P. stutzeri* NBRC 13596 (100%)	JX177689	+	+	+	–	–	I, JX177793	I, JX177755
2C30	09/1R/Phe	*P. stutzeri* NBRC 13596 (100%)	JX177691	+	+	+	–	–	I, JX177795	I, JX177764
2A49	09/2R/Phe	*P. stutzeri* NBRC 13596 (100%)	JX177692	+	+	+	–	–	I, JX177796	I, JX177754
2A50	09/2R/Phe	*P. stutzeri* NBRC 13596 (100%)	JX177690	+	+	+	–	–	I, JX177794	I, JX177763
2C47	09/2R/Phe	*P. stutzeri* NBRC 13596 (100%)	JX177682	+	+	+	–	–	I, JX177791	I, JX177756
2C3	09/R/Phe	*P. guineae* LMG 24017 (99%)	JX177687	+	–	–	–	–	I, JX177798	I, JX177757
2C51	09/2R/Phe	*P. guineae* LMG 24017 (99%)	JX177684	+	–	–	–	–	I, JX177792	I, JX177758
D57	08/R/Benz	*P. peli* R-20805 (100%)	JX177685	+	–	–	–	–	I, JX177799	I, JX177761
C10	08/R/Phe	*P. peli* R-20805 (99%)	JX177683	+	–	–	–	–	I, JX177790	VI, JX177784
2Aben1	09/D/Benz	*P. stutzeri* NBRC 13596 (99%)	JX177709	+	+	+	+	–	II, JX177803	II, JX177747
2A1	09/R/Phe	*P. stutzeri* H3 (99%)	JX177707	+	–	–	–	–	II, JX177802	II, JX177746
2A38	09/1R/Nah	*P. stutzeri* NBRC 13596 (99%)	JX177716	+	+	+	+	+	III, JX177805	II, JX177740 III, JX177741
2Anah1	09/D/Nah	*P. stutzeri* PTG4-15 (99%)	JX177710	+	+	+	–	+	III, JX177808	II, JX177745
2C23	09/R/Nah	*P. stutzeri* PTG4-15 (99%)	JX177717	+	+	+	–	+	III, JX177809	II, JX177742
2D37	09/1R/Phe	*P. stutzeri* PTG4-15 (100%)	JX177718	+	+	+	–	+	III, JX177810	II, JX177743
2D54	09/2R/Phe	*P. stutzeri* PTG4-15 (99%)	JX177719	+	+	+	–	+	III, JX177807	II, JX177744
2A33	09/1R/Benz	*P. stutzeri* NBRC 13596 (99%)	JX177724	+	+	+	–	–	III, JX177813	II, JX177753
C52	08/2R/Tol	*P. stutzeri* NBRC 13596 (99%)	JX177708	+	+	+	–	–	III, JX177806	II, JX177739 III, JX177737 IV, JX177738
C70[e]	08/1R/Phe	*P. pseudoalcaligenes* 14.1 (99%) *P. mendocina* FB8 (100%) *P. mendocina* Lma2 (99%)	JX177732 JX177733 JX177734	+	–	–	+	+	III, JX177804	II, JX177736 III, JX177735
2Cben1	09/D/Benz	*P. stutzeri* LC2-8 (100%)	JX177728	+	+	+	–	–	III, JX177815	II, JX177750
2A7[e]	09/R/Phe	*P. stutzeri* LC2-8 (100%)	JN228286	+	+	+	–	–	III, JX177811	II, JX177749
2Aphe4[e]	09/D/Phe	*P. stutzeri* LC2-8 (100%)	JN228285	+	–	–	–	–	III, JX177812	II, JX177752
2Ctol3[e]	08/D/Tol	*P. stutzeri* LC2-8 (99%)	JN228312	+	–	–	–	–	III, JX177816	II, JX177751
2C29	09/1R/Phe	*P. stutzeri* LC2-8 (99%)	JX177730	+	–	–	–	–	III, JX177814	II, JX177748
2Anah4	09/D/Nah	*P. stutzeri* NBRC 13596 (99%)	JX177714	+	+	+	–	–	IV, JX177826	III, JX177772
2A20[e]	09/R/Tol	*P. stutzeri* NBRC 13596 (99%)	JN228288	+	+	+	–	–	IV, JX177825	III, JX177769
2C41	09/1R/Tol	*P. stutzeri* NBRC 13596 (99%)	JX177725	+	+	+	–	–	IV, JX177824	III, JX177771
2B45	09/1R/Tol	*P. stutzeri* NBRC 13596 (99%)	JX177715	+	+	+	–	–	IV, JX177827	III, JX177774
2C63	09/2R/Tol	*P. stutzeri* NBRC 13596 (99%)	JX177731	+	+	+	–	–	IV, JX177823	III, JX177770
B10v[e]	08/R/Tol	*P. stutzeri* H3 (99%)	JN228290	+	+	+	–	–	V, JX177830	III, JX177773
2B57	09/2R/Phe	*P. fluorescens* PC37 (100%)	JX177696	+	+	+	–	+	V, JX177828	ND
2C2	09/R/Phe	*Pseudomonas* sp. LYBRD-7 (99%)	JX177697	+	+	–	–	–	V, JX177829	ND
2C20v[e]	09/R/Tol	*P.* cf.*stutzeri* V4.MO.16 (99%)	JN228303	+	+	–	–	–	VI, JX177817	IV, JX177783
CR2A4	08/D/R2A	*Limnobacter thiooxidans* CS-K2 (99%)	JX177701	+	–	–	-	-	VII, JX177787	V, JX177787
2A6	09/R/Phe	*Limnobacter thiooxidans* CS-K2 (99%)	JX177698	+	-	-	–	–	VII, JX177788	V, JX177788
2C4	09/R/Phe	*Limnobacter thiooxidans* CS-K2 (99%)	JX177699	+	–	–	–	–	VII, JX177786	V, JX177786
2D3	09/R/Phe	*Limnobacter thiooxidans* CS-K2 (99%)	JX177700	+	–	–	–	–	VII, JX177789	V, JX177789
2Dben2	09/D/Benz	*Rhodococcus fascians* NKCM8906 (99%)	JX177726	+	+	–	+	–	VII, JX177834	ND
2Atol2	09/D/Tol	*Hydrocarboniphaga effusa* AP102 (99%)	JX177695	+	+	+	+	–	VIII, JX177833	ND
2A27	09/R/Nah	*Acidovorax radicis* N35 (99%)	JX177703	+	–	–	–	–	VIII, JX177832	ND
C34	08/R/Nah	*Acidovorax soli* BL21 (99%)	JX177702	+	–	–	–	–	IX, JX177831	ND
D52	08/1R/Phe	*Acinetobacter johnsonii* 3B2 (99%)	JX177705	+	+	+	+	+	X, JX177840	ND
C25	08/R/Phe	*A. johnsonii* LB48317/2005 (99%)	JX177713	+	+	+	–	+	X, JX177838	ND
D14[e]	08/R/Tol	*Acinetobacter* sp. zol-02 (99%)	JN228315	+	+	+	–	+	X, JX177836	ND
D19[e]	08/R/Tol	*A. johnsonii* 3B2 (99%)	JN228316	+	+	+	–	+	X, JX177837	ND
D66v	08/R/Tol	*A. johnsonii* 3B2 (99%)	JX177712	+	+	+	–	+	X, JX177841	ND
D36	08/R/Phe	*A. lwoffii* 412 (99%)	JX177706	+	+	+	–	+	X, JX177839	ND
A22	08/1R/Phe	*A. haemolyticus* AY047216 (99%)	JX177704	+	+	+	–	+	X, JX177835	ND
2Bsal[e]	09/D/Sal	*P. collierea* PR212 T (99%)	JN228294	+	+	–	+	+	XI, JX177820	ND
2B1	09/R/Phe	*P. putida* bD1 (100%)	JX177721	+	+	–	+	+	XI, JX177822	ND
2B29	09/R/Nah	*P. putida* bD1 (100%)	JX177694	+	+	–	+	+	XI, JX177821	ND
A8[e]	08/1R/Phe	*P. putida* NBRC 102093 (99%)	JN228275	+	+	–	+	+	XI, JX177818	ND
2B32	09/1R/Phe	*P. collierea* PR212 T (99%)	JX177722	+	+	–	+	+	XI, JX177819	ND

[a] The capital letter in the strain's code denotes the isolation site: (A) near Tallinn; (B) the Gulf of Finland; (C) near Narva; (D) near Pärnu.
[b] 08 and 09 are 2008 and 2009, respectively; R: first enrichment; 1R: second enrichment; 2R: third enrichment; D: direct plating; Phe: phenol; Benz: benzoate; *m*-Tol: *m*-toluate; Sal: salicylate; Nah: naphthalene.
[c] ± denote growth/absence of growth on phenol (Phe), benzoate (Benz), *m*-toluate (*m*-Tol), salicylate (Sal), naphthalene (Nah), respectively.
[d] ND, genes not detected by PCR.
[e] Plasmid-containing strains [18].

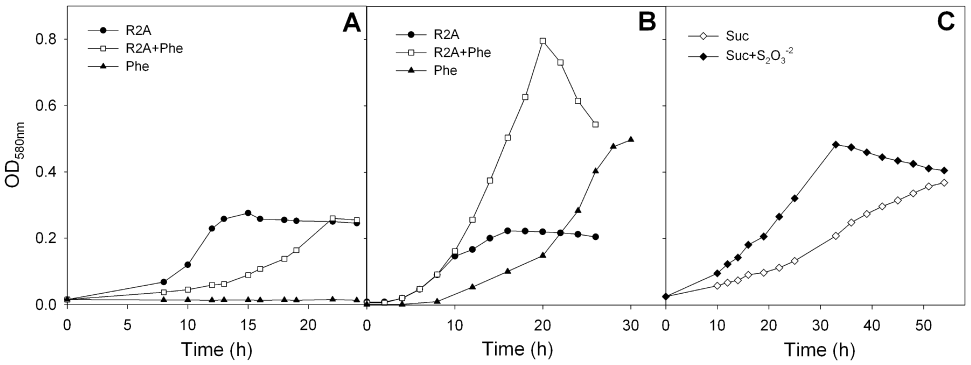

Fig. 1. Growth curves of *Limnobacter thiooxidans* type strain CS-K2 (DSM 13642^T) (A) and the newly isolated *Limnobacter* sp. strain 2D3 (B) in R2A medium, R2A medium supplemented with 2.5 mM phenol (R2A + Phe) and M9 minimal medium supplemented with 2.5 mM phenol (Phe), as well as for strain 2D3 (C) in 10 mM succinate (Suc) and 10 mM succinate mineral medium supplemented with 10 mM thiosulphate (Suc + $S_2O_3^{-2}$). All growth experiments were performed at 30 °C.

to find the assembly with the best N50 value, different quality cutoff-values and different assembly programme parameters were tested for each genome. Before assembly, reads with low quality at the 3′ end were trimmed with cutoff-values 20, 30 and 35 using the programme *fastq_quality_trimmer*. Trimmed reads shorter than 40 bp were discarded. Reads trimmed with quality cutoff-value 30 for strain 2D3 and with quality cutoff-value 35 for strains 2A6, CR2A4 and 2C4 resulted in the best N50 value. Velvet K-mer values 27, 29 and 31 were tested. The best N50 values were achieved using a K-mer value of 31 for all strains. The total length of assembled contigs was 3.34 Mb (ca 2700-fold sequencing coverage), 3.51 Mb (ca 2300-fold sequencing coverage), 3.39 Mb (ca 2200-fold sequencing coverage) and 3.45 Mb (ca 3500-fold sequencing coverage) in 178, 153, 102 and 109 contigs for strains CR2A4, 2D3, 2A6 and 2C4, respectively. The N50 contigs were 944.55 kb, 191.17 kb, 209.97 kb and 213.22 kb with the longest scaffold 1321.02 kb, 966.46 kb, 631.66 kb and 693.16 kb for strains CR2A4, 2D3, 2A6 and 2C4, respectively.

Growth kinetics and analysis of C23O activity

The growth of the type strain *Limnobacter thiooxidans* CS-K2^T and the newly isolated *Limnobacter* sp. strain 2D3 was measured spectrophotometrically at 580 nm. All experiments were run in triplicate. The specific growth rate (μ) of strain 2D3 grown on R2A, R2A supplemented with phenol (2.5 mM), mineral medium supplemented with phenol (2.5 mM), succinate (10 mM), and succinate with 10 mM thiosulphate was calculated from the exponential growth phase of semi-logarithmic absorbance growth curves using the following equation: $\mu = \ln 2/t_d$, where t_d is the doubling time.

For qualitative assessment of C23O activity, bacteria were grown on minimal medium agar plates with 2.5 mM phenol for two days, and then the colonies were sprayed with 0.5% catechol (w/v). The appearance of yellow 2-hydroxymuconic semi-aldehyde after 10 min incubation time at room temperature inferred the C23O activity.

Results

Description of the studied set of bacteria

Based on the aim of this study to characterise the genetic diversity of the key catabolic enzymes LmPH and C23O responsible for phenol degradation in previously collected Baltic Sea surface water isolates, 92 strains, able to degrade phenol, were first analysed for the presence of the gene coding for LmPH (described in detail in a separate section below). As a result, 70 PCR-positive strains were found and 56 representative Phe$^+$/LmPH$^+$ strains were selected for further investigation (Table 1).

Comparative sequence analysis of the 16S rRNA genes of the studied strains and their preliminary phenotypic characterisation

The results of comparative sequence analysis of the 16S rRNA genes of the 56 studied strains are shown in Table 1. The majority of the isolates were grouped within the genera *Pseudomonas* (41) and *Acinetobacter* (7) from the class *Gammaproteobacteria*. The class *Betaproteobacteria* was represented by strains of the genera *Acidovorax* (2), *Hydrocarboniphaga* (1) and *Limnobacter* (4). The studied set of biodegraders contained only one Gram-positive phenol-degrading strain (2Dben2) of *Rhodococcus* sp., which was isolated using direct plating on benzoate minimal medium. The four *Limnobacter* strains (the enrichment cultures 2D3, 2A6, 2C4, and the direct plating strain CR2A4) were closely related to *Limnobacter thiooxidans*, a thiosulphate-oxidising bacterium isolated from freshwater lake sediment [29]. Comparative analysis of the newly isolated *Limnobacter* strains and the *Limnobacter thiooxidans* type strain CS-K2^T (DSM 13612^T) indicated that the type strain was not able to degrade phenol (Fig. 1A). The growth of strain 2D3 in R2A medium, R2A medium supplemented with 2.5 mM phenol (R2A + Phe), and M9 minimal medium supplemented with 2.5 mM phenol (Phe) was characterised by the increased length of the lag-phase in Phe, as well as the low and additive growth yield in R2A and R2A + Phe, respectively (Fig. 1B). The specific growth rates of strain 2D3 in these media were similar (approximately 0.41 h^{-1}). In the R2A medium, the type strain grew similarly to strain 2D3 while its growth in R2A + Phe was inhibited (Fig. 1A). All the phenotypic characteristics of the newly isolated *Limnobacter* strains (e.g. cellular morphology, the ability to store polyhydroxybutyrate as a reserve material, and inability to utilise many carbon sources, except for a few carboxylic acids) were very similar to the type strain. In addition, the batch cultures of all the *Limnobacter* strains gave significant increases in growth yields after the addition of thiosulphate (10 mM) to liquid succinate (10 mM) mineral medium (strain 2D3 as an example is shown in Fig. 1C). The specific growth rate values were approximately 0.07 h^{-1} and 0.13 h^{-1} in the succinate medium without and with thiosulphate, respectively. The same phenomenon has been described also in the case of the type strain of *L. thiooxidans* [29]. Therefore, thiosulphate was verified for use as an additional energy source and thus enabled chemolitho-heterotrophic growth of the newly isolated *Limnobacter* sp. strains.

All 56 selected isolates were tested for their ability to use different aromatic compounds as sole sources of carbon and energy (Table 1). The highest number of the studied strains utilised benzoate (41) and the lowest salicylate/naphthalene (11/19). Most

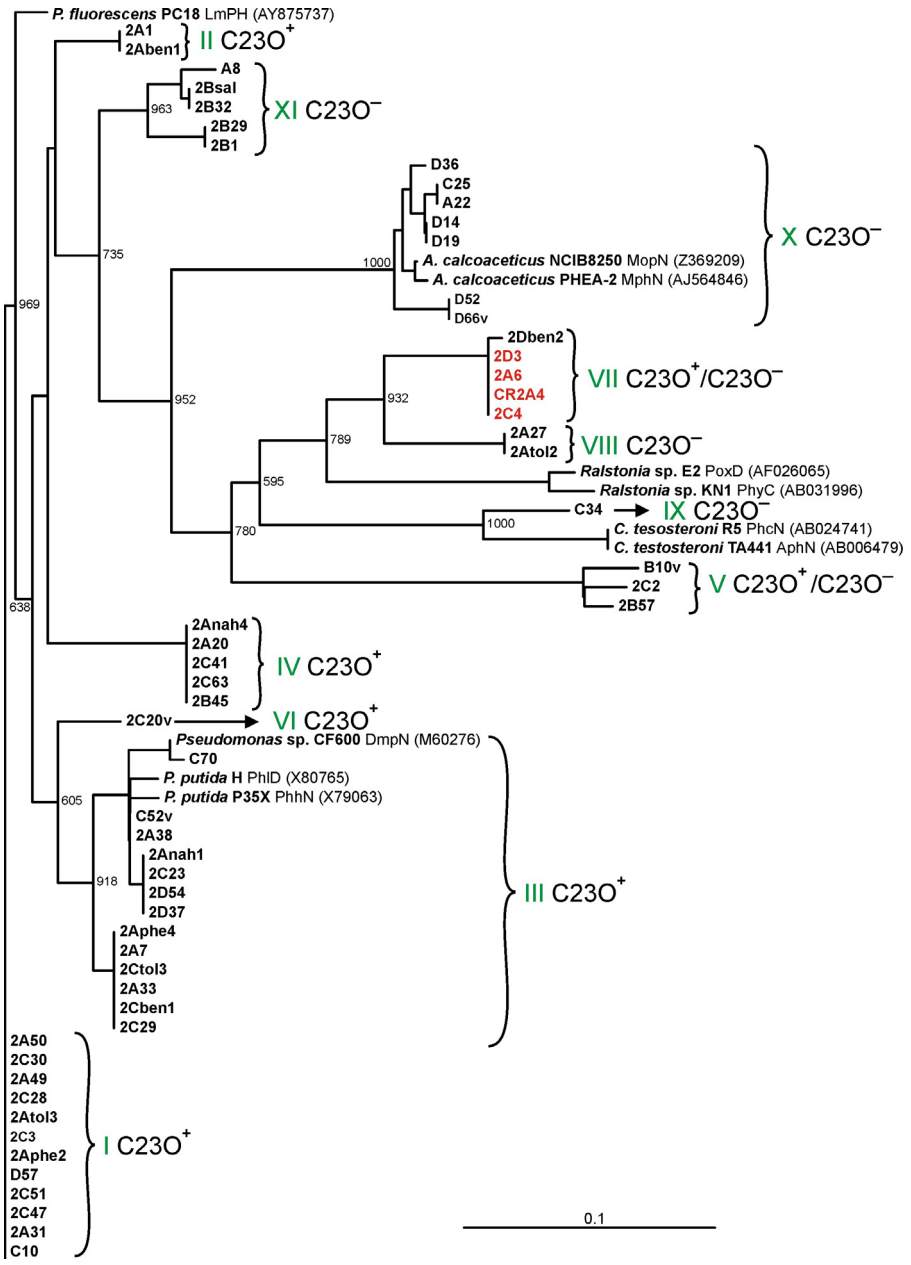

Fig. 2. Neighbour-joining tree based on the deduced amino acid sequences of the large subunit of multicomponent phenol hydroxylases (179 aa) of the isolated phenol degraders (the *Limnobacter* strains are in red letters) and the reference strains obtained from GenBank (accession numbers are indicated in parentheses). Bootstrap values (per 1000 trials) higher than 500 are indicated at the relevant nodes. The scale bar represents 0.1 substitutions per amino acid site. Different clusters are denoted by brackets and green roman numerals, and the presence (+) or absence (−) of the catechol 2,3-dioxygenase gene (C23O) in the strains belonging to the given cluster is indicated.

strains grew on at least three of the studied aromatic compounds, whereas the strains of the genus *Acinetobacter* exhibited a broad range of degradation (Phe, Benz, Tol and Nah; but not Sal). The strains from the genus *Limnobacter* and *Acidovorax*, as well as the four *Pseudomonas anguilliseptica* strains (2C3, 2C51, D57, C10), had a very narrow degradation range (only Phe).

The detection of PH and C23O genes in the isolated strains and their phylogenetic analysis

All 92 phenol-degrading isolates of this study were tested for the presence of single- and multicomponent PHs. While a number of bacterial isolates having the single-component PH (sPH) coded by the *pheA* gene were previously detected by us in phenol-polluted river water [13], all Baltic Sea isolates were PCR negative for the *pheA* gene. For the detection of the mPH genes in our isolates, the primers pheUf and pheUr, targeting different phylogenetic groups of mPHs [9], were used to amplify the fragment of the LmPH gene with a product size of approximately 620 bp. As already mentioned, 70 strains gave a PCR product in this analysis. The phylogenetic analysis of the 56 representative amino acid sequences of these LmPHs (179 aa) identified eleven LmPH groups (Fig. 2). LmPHs of the four *Limnobacter* strains together with the only Gram-positive strain (2Dben2) formed a separate cluster (VII) in this phylogenetic tree.

All strains (38 from 56) with a biomass that turned yellow when sprayed with catechol (i.e. that possessed the *meta*-cleavage activity) were studied by PCR for the presence of the genes coding for C23O. The primers ORF-F and ORF-R were used to amplify the fragment of the C23O gene with a product size of approximately 924 bp.

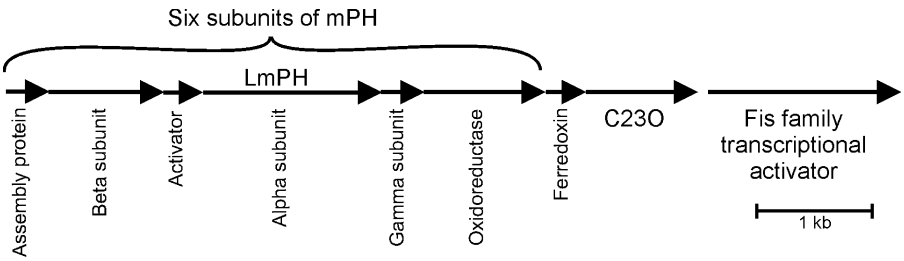

Fig. 3. Genomic organisation of the six genes coding for the subunits of multicomponent phenol hydroxylase (mPH) in *Limnobacter* strains, including the largest subunit of mPH (LmPH), and catechol 2,3-dioxygenase (C23O) together with the genes coding for a ferredoxin and a Fis family transcriptional activator. Names of the mPH subunits are given below the respective genes. The scale bar of 1 kb is given. The sizes of the genes and intergenic regions are to scale. The GenBank accession numbers of these regions for the four *Limnobacter* strains are JX177786-9.

This primer pair was chosen because it targets the subfamily I.2.A of C23Os [17], which includes the largest group of C23Os thought to be widely distributed in contaminated environments. All these strains, except for the four *Limnobacter* strains, yielded a PCR product. As the latter strains had been subjected to complete genome sequencing, the preliminary incomplete sequencing results were used to find C23O genes in their genomes using a BLAST search. In fact, one copy of the C23O gene was found in all four strains and these were included in the phylogenetic analysis as well. Furthermore, in the case of all these strains, the six genes coding for the mPH subunits were found on the same contig that likely formed one operon together with the C23O-encoding gene, which would be regulated by a Fis family transcriptional activator (Fig. 3). In all these gene clusters, the C23O gene is preceded by a gene coding for a ferredoxin that has been reported to promote reductive reactivation of C23O [16]. All the studied strains that did not express C23O activity gave a negative result in the respective PCR analysis. The type strain CS-K2^T of *L. thiooxidans* resulted in no PCR products with all studied primer pairs targeting PH and C23O genes, including the newly designed primers ORF-FL and ORF-RL specifically targeting the C23Os of *Limnobacter* strains. Two strains (C70 and 2A38) were found to have two phylogenetically different copies of the C23O gene, and one strain (C52) even had three, and they were all included in the phylogenetic analysis. The groupings of the 42 representative amino acid sequences deduced from the C23O genes (270 aa) are shown in Fig. 4, and the majority of the C23O sequences were grouped into three clusters. The most distant cluster V contained only the *Limnobacter* strains. When the LmPH and C23O phylogenetic trees were compared, it appeared that these catabolic enzymes mostly clustered similarly. Interestingly, LmPHs from all strains that did not express C23O activity formed separate clusters (VIII-XI) distinct from other sequences, with the exception of the four *Limnobacter* strains and strain B10v that carried a C23O gene.

Analysis of LmPH gene fragments amplified from the total community DNA

The isolated total community DNA from all eight water samples was used to amplify the fragment of the LmPH gene using the same primer pair pheUf/pheUr. Altogether, 1,207,933 (site A, 2008) to 9,010,545 (site D, 2008) quality trimmed and N-filtered sequences of LmPHs were obtained from the two ends of the respective PCR products of the eight water samples using NGS. After collapsing all identical sequences, the ten most prevalent unique sequences per water sample were used in further analysis (analysis of both ends was performed separately). The sequences of the pheUf and pheUr ends were 75 bp and 80 bp long, respectively. As the pheUr end is longer, the BLAST analysis with the blastn program was performed with this end. The results showed that 62 out of 80 sequences were ≥96% identical to the LmPH sequences of the *Limnobacter* strains. When the count of each sequence in the non-collapsed pool of quality trimmed reads was also considered, it appeared that 87% of the sequences from the pheUr end were ≥96% identical to the LmPH sequences of the *Limnobacter* strains.

Discussion

The wide range of microorganisms able to degrade phenolic compounds includes genera such as *Acidovorax*, *Acinetobacter*, *Arthrobacter*, *Azoarcus*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Comamonas*, *Delftia, Hydrogenophaga*, *Microbacterium*, *Mycobacterium*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas* and *Variovorax* [15,33,31,5,7,10,19,34]. Our study widened this range by including the genus *Limnobacter*. The strains belonging to this genus have never been reported to have phenol-degradation activity. All the strains of this genus were isolated either from the direct platings or first enrichments from all four sampling points, indicating their significance in the Baltic Sea. The NGS approach showed that the LmPHs of this genus were indeed abundant in the microbial communities of the water samples studied.

Detection of key catabolic genes and functional assays are essential for estimating the natural biodegradation potential of bacteria in a specific environment. The assessment of C23O activity provided evidence that the phenol-degraders studied possessed either the *meta* or the *ortho* catabolic pathway. To date, no *Acinetobacter* strains have been shown to exhibit C23O activity [5]. In fact, in all our studied *Acinetobacter* strains (D36, C25, A22, D14, D19, D66v and D52), as well as in the strains from the genera *Pseudomonas* (A8, 2Bsal, 2B32, 2B29, 2B1, 2C2, 2B57), *Acidovorax* (2A27, C34), *Hydrocarboniphaga* (2Atol2) and *Rhodococcus* (2Dben2), the LmPHs clustered separately (Fig. 2), and degradation of catechol must occur by the action of C12O. We have shown previously that six *Pseudomonas* strains from river water possessed LmPHs that formed a separate cluster together with the Mop type of LmPHs [23]. Catechols produced by this type of mPHs have also been shown by Ehrt et al. [5] to be channelled to the *ortho* pathway. However, the *Limnobacter* sp. strains CR2A4, 2C4, 2A6 and 2D3, and the *P. stutzeri* strain B10v demonstrated that this type of mPH may also be connected to C23O. Similar genomic organisation of mPH and C23O genes, as in our studied *Limnobacter* strains, has also been described in a set of bacterial strains belonging to the order *Burkholderiales*, including *Limnobacter* sp. MED105 (NCBI's whole genome shotgun project with accession number ABCT01000000), in which the gene coding for C23O is located in the same cluster as the genes coding for mPH, while all the other genes for the *meta* pathway are coded several kilobases away [26]. In the latter strain, this gene cluster is the only aromatic catabolic trait observed, and this is probably true also in the case of our *Limnobacter* strains as they show a very narrow range of degradation of aromatic compounds. The usage of the *meta*- or the *ortho*-fission of catechols depends not only on the bacterial species but also on the growth

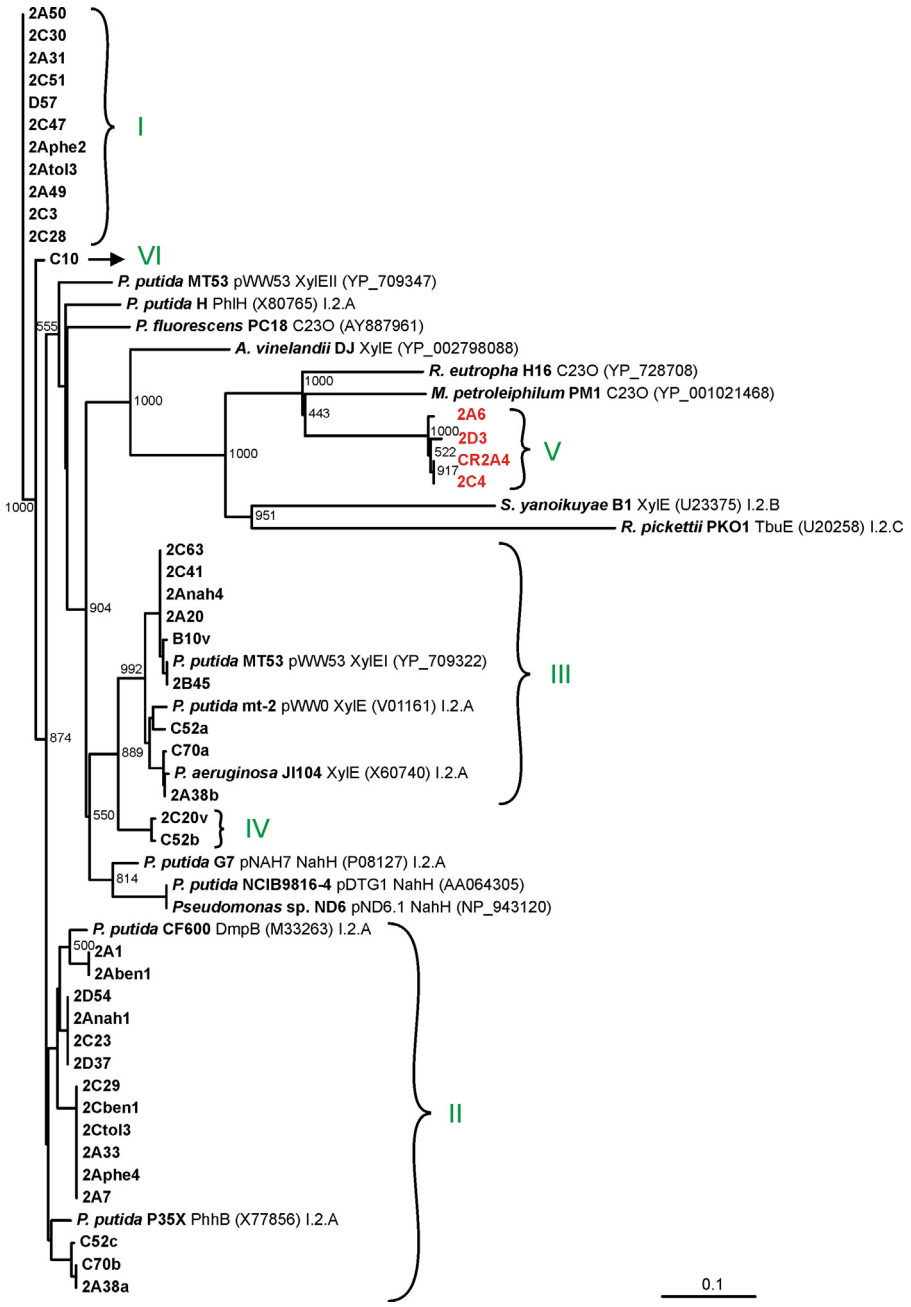

Fig. 4. Neighbour-joining tree based on the deduced amino acid sequences of the catechol 2,3-dioxygenases (270 aa) of the isolated phenol degraders (the *Limnobacter* strains are in red letters) and the reference strains obtained from GenBank (accession numbers are indicated in parentheses). The codes I.2.A, I.2.B and I.2.C designate the classification of Eltis and Bolin [8]. Bootstrap values (per 1000 trials) higher than 500 are indicated at the relevant nodes. The scale bar represents 0.1 substitutions per amino acid site. Different clusters are denoted by brackets and green roman numerals.

substrates. A total of 45% of all the phenol-degrading bacteria studied here expressed the *meta*-cleavage pathway. As expected, the majority of our isolates (70 out of 92) able to degrade phenol possessed mPH genes, and the rest of the strains must have contained sPHs or mPHs with sequences not closely related to the primers used in this study. The *pheA* gene coding for the sPH, found in several freshwater strains, was not detected in the Baltic Sea isolates. We have described previously that the strains having different PH types show different kinetic parameters [32]. Essentially, sPH-harbouring strains have higher K_S values and lower μ_{max} values than the strains with mPH. These results allow us to speculate that, due to the low concentration of substrates, the phenol-degraders in Baltic Sea surface water must have mPHs with a high affinity for phenol.

Phylogenetic groupings of the studied phenol-degrading bacteria did not coincide well with groupings based on their LmPH and C23O amino acid sequences, especially in the case of the genus *Pseudomonas*. This is likely to be the result of frequent horizontal gene transfer between the strains of *Pseudomonas*, which is conducted mostly by plasmids. Indeed, as shown by us previously [18], 9 out of 11 plasmid-bearing strains in this study belonged to this genus (Table 1). The finding that the Gram-positive strain 2Dben2 contained the LmPH gene that was almost identical to those of *Limnobacteria* also reflects lateral gene transfer in which not only plasmids but also, for instance, bacteriophages and genomic islands could play important roles.

The slow growth rate of the *Limnobacter* strains with a high concentration of phenol (2.5 mM) as the sole carbon and energy

source could be explained by high affinity of these strains for phenol. This whole-cell kinetics is characteristic to phenol-degraders from *Betaproteobacteria* [9]. Low concentration of dissolved oxygen in subsurface environments is a limiting factor for microbial degradation of aromatic compounds [30]. The Baltic Sea is characterised by continuous oxygen deficiency and this may cause the prevalence of bacteria which can grow under hypoxic conditions. Betaproteobacterial C23Os (e.g. TbuE from *Ralstonia pickettii* PKO1) have been shown to have significantly higher affinities for catechol and molecular oxygen [21]. According to the phylogenetic classification of C23Os by Eltis and Bolin [8], these proteins belong to the subfamily I.2.C. However, the *Limnobacter* strains widespread in the Baltic Sea possess C23Os that, together with the respective enzymes of the other betaproteobacterial strains *R. eutropha* H16 and *Methylibium petroleiphilum* PM1, belong to a cluster which is distinct from both subfamilies I.2.A and I.2.C (Fig. 4). Hence, our further investigations will be focused on the *Limnobacter* strains and especially on the analysis of the kinetic parameters of their growth and biodegradation capabilities under hypoxic conditions. Finishing the analysis of their complete genomes and comparative genomics will provide valuable information about the diversity of the genus *Limnobacter*, including the description of the novel species.

Authors' contribution

Eve Vedler and Eeva Heinaru contributed equally to this paper.

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