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Novel organic solvent-tolerant esterase isolated by metagenomics: insights into the lipase/esterase classification

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ABSTRACT

In order to isolate novel organic solvent-tolerant (OST) lipases, a metagenomic library was built using DNA derived from a temperate forest soil sample. A two-step activity-based screening allowed the isolation of a lipolytic clone active in the presence of organic solvents. Sequencing of the plasmid pRBest recovered from the positive clone revealed the presence of a putative lipase/esterase encoding gene. The deduced amino acid sequence (RBest1) contains the conserved lipolytic enzyme signature and is related to the previously described OST lipase from *Lysinibacillus sphaericus* 205y, which is the sole studied prokaryotic enzyme belonging to the 4.4 α/β hydrolase subgroup (abH04.04). Both *in vivo* and *in vitro* studies of the substrate specificity of RBest1, using triacylglycerols or nitrophenyl-esters, respectively, revealed that the enzyme is highly specific for butyrate (C_4) compounds, behaving as an esterase rather than a lipase. The RBest1 esterase was purified and biochemically characterized. The optimal esterase activity was observed at pH 6.5 and at temperatures ranging from 38 to 45 °C. Enzymatic activity, determined by hydrolysis of *p*-nitrophenyl esters, was found to be affected by the presence of different miscible and non-miscible organic solvents, and salts. Noteworthy, RBest1 remains significantly active at high ionic strength. These findings suggest that RBest1 possesses the ability of OST enzymes to molecular adaptation in the presence of organic compounds and resistance of halophilic proteins.

Key words: hormone-sensitive lipase family, activity-driven metagenomics, metagenomic library, abH4.04, *Lysinibacillus sphaericus*

RESUMEN

Nueva esterasa tolerante a los solventes orgánicos aislada por metagenómica: ideas sobre la clasificación de las esterasas/lipasas. Con el fin de aislar nuevas variantes de lipasas tolerantes a solventes orgánicos (OST), se construyó una librería metagenómica a partir de ADN obtenido de una muestra de suelo de bosque templado. A través de un monitoreo en dos etapas, basado en la detección de actividades, se aisló un clon con actividad lipolítica en presencia de solventes orgánicos. La secuenciación del plásmido pRBest recuperado del clon positivo reveló la presencia de un gen codificante de una hipotética lipasa/esterasa. La secuencia deducida de amino ácidos (RBest1) contiene los motivos conservados de enzimas lipolíticas y está relacionada con la lipasa OST previamente descrita de *Lysinibacillus sphaericus* 205y, que es la única enzima procariota estudiada perteneciente al subgrupo 4.4 de α/β hidrolasas (abH4.04). Estudios *in vivo* e *in vitro* sobre la especificidad de sustratos de RBest1, utilizando triacil-gliceroles o *p*-nitrofenil-ésteres, respectivamente, revelaron que la enzima es altamente específica para compuestos butíricos (C_4), comportándose como una esterasa y no como una lipasa. La esterasa RBest1 fue purificada y caracterizada bioquímicamente. La actividad óptima de esterasa fue observada a pH 6,5 y las temperaturas óptimas fueron entre 38 y 45 °C. Se estableció que la actividad enzimática, determinada por hidrólisis de *p*-nitrofenil ésteres, es afectada en presencia de diferentes solventes orgánicos miscibles y no miscibles, y también sales. Notoriamente, RBest1 permanece significativamente activa a elevadas fuerzas iónicas. Estos hallazgos sugieren que RBest1 posee la capacidad de las enzimas OST de la adaptación molecular en presencia de compuestos orgánicos, así como la resistencia de las proteínas halófilas.

Palabras clave: familia de lipasas sensibles a hormonas, metagenómica funcional, librería metagenómica, abH4.04, *Lysinibacillus sphaericus*

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INTRODUCTION

Metagenomics comprises a series of methodological approaches for accessing the genetic information (the "metagenome") from microbial consortia living in different habitats including uncultured microorganisms that collectively account for more than 95 % of the total, without the need of previous isolation techniques (15, 21). Methodologically, metagenomics is based on the extraction of total DNA from the sample, digestion and cloning of resulting fragments and transformation of ligation mixture into cultivable hosts like *Escherichia coli* (10, 17). The genetic content of the metagenome is finally stored in a metagenomic library from which different target genes or activities can be screened.

This technology paved the way for elucidating the functions of microbial communities, genomic analyses of uncultured soil microorganisms and to search for new genes coding for various proteins from unbiased gene pools.

Since evolution and natural selection have been occurring in the environment for billions of years, the metagenomic approach allows the isolation of enzymes that harbor tailor-made properties, which fit the physicochemical conditions of the habitats studied. Under this perspective, new enzymes with special biophysical features have been isolated from metagenomic libraries that were built from various environments such as temperate soils (18), hot springs (5), sandy ecosystems (29), oceanic waters (33), and cold environments (4). Among these enzymes, several esterases (EC. 3.1.1.1) and lipases (EC. 3.1.1.3) have been isolated (7-9, 19, 20, 23-25, 29, 30, 32).

Lipolytic enzymes represent an important part of the industrial enzymes used for the production of various food products and fine chemicals (27). The importance of their use relies on the possibility to obtain enantiomerically pure molecules starting from mixtures containing several isomers.

Since many substrates for lipolytic enzymes are poorly soluble in water, replacement of aqueous buffers by organic solvent-containing solutions reinforced the interest in organic solvent-tolerant enzymes (26, 37). Moreover, the possibility to perform enzyme-catalyzed reactions in organic solvents avoids microbial contaminations, modifies the substrate specificity and changes the thermodynamic equilibrium favoring synthesis over hydrolysis.

Triacylglycerols are natural substrates of esterases and lipases and are poorly soluble in aqueous buffer solutions. Some of these enzymes are active even in the presence of organic solvents, and are therefore referred to as organic solvent-tolerant (OST) enzymes, being valuable tools for food and fine chemical industry because of their ability to remain active under harsh conditions.

Lipolytic enzymes catalyze both the hydrolysis and the synthesis of ester bonds found in many molecules such as acylglycerols. Esterases prefer short-chain substrates (below 10 carbon chain), whereas lipases are able to hydrolyze either long-chain acylglycerols (beyond 10 carbon chain). Both esterases and lipases from the hormone-sensitive lipase- like family (HSL) share a highly conserved motif (G-X-S-X-G) containing the essential active serine. Additional aspartate or glutamate, and histidine residues are also involved in the formation of a conserved catalytic triad. Also, lipolytic enzymes in the HSL family contain the conserved sequence H-G-G-(G/A) in their oxyanion hole (1).

In this report, we describe the construction of a small-insert metagenomic library derived from a temperate forest soil sample. The library was screened for genes coding for lipolytic enzymes by an activity-based screening system. A novel lipase/esterase encoding gene was discovered, sequenced and analyzed. The enzyme (RBest1) was purified and its substrate specificity and tolerance to various organic solvents was evaluated.

MATERIALS AND METHODS

DNA extraction and metagenomic library construction

In November 2005, 50 g of soil sample were collected in the area of the Göttingen beech forest (51°33' N - 9°57' E), near the Georg-August University in Göttingen (Germany). DNA was extracted using the direct approach described by Zhou *et al.* (41). Purified environmental DNA was partially digested using *Bsp143*I (Fermentas; St.Leon-Rot, Germany) and resulting fragments were resolved by overnight ultracentrifugation in a sucrose density gradient (10-40 %) at 27,000 × g and 4 °C. Fractions containing DNA fragments with molecular sizes between 3 and 5 kb were used for cloning at the *BamH*I site of the pCR2.1-TOPO vector (Invitrogen™) and metagenomic library RB1 was constructed after transformation in competent *Escherichia coli* RR1 cells (an *E. coli* HB101 derived strain, http://openwetware.org/wiki/E._coli_genotypes#RR1). Resulting recombinant cells were selected on Luria Bertani (LB) agar plates containing 100 µg/ml ampicillin, 30 µM IPTG, and 50 µg/ml X-gal (Fermentas).

Screening of lipolytic activity on metagenomic library RB1

In order to isolate clones producing lipolytic enzymes in the metagenomic library RB1, recombinant *E. coli* cells were spread on emulsified spirit blue agar medium (BD-Difco; Franklin-Lake NJ, USA), supplemented with 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 1 % emulsified tributyrin (Sigma; Bornem, Belgium). Colonies producing a blue and clear hydrolytic halo were selected as potential esterase/lipase producers. Plasmids isolated from positive clones were retransformed into competent *E. coli* DH10B cells (Invitrogen) for confirmation of the lipolytic phenotype.

Positive clones were cultivated overnight at 37 °C in LB

medium. Subsequently, cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl (pH 8.0), and disrupted by sonication (3 cycles of 30 s; amplitude: 10-12 µm). After eliminating cell debris by centrifugation, organic solvents were added (DMSO, benzene, toluene and xylene, 25 % v/v final concentration) and the mixture was incubated at 37 °C for 30 min under agitation. Finally, 50 µl of the protein suspension were loaded on apirit blue agar medium containing 1 % (v/v) emulsified tributyrin at pH 7.5. Hydrolysis was observed by formation of a blue hydrolytic halo after 48 h incubation at room temperature.

DNA sequencing and sequence analysis

DNA sequencing was performed at the GIGA sequencing platform of the University of Liège (Belgium) using universal M13 reverse and forward primers. Additional primers were used to fully sequence the insert from the OST-esterase/lipase producing clones (RBest1int1: 5'-CTC TCG TTG CCC GCT GGT CT-3'; RBest1int1rv: 5'-AGA CCA GCG GGC AAC GAG AG-3'). The amino acid sequence of the putative lipolytic enzyme (RBest1) was analyzed with BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and phylogenetic analysis conducted by MUSCLE (11) and Geneious Pro 4.8.2 software (http://www.geneious.com/) for performing the multiple sequence alignment, and MEGA 4.0 for building the phylogenetic tree, using 5,000 replicates in a bootstrap test (40). Alignment was used as template to construct a similarity table using Protdist software from the PHYLIP package (13).

RBest1 production and purification

RBest1 expression was achieved from the transformed clone containing the environmental DNA fragment that includes the RBest1-encoding gene (pRBest1). Bacterial cells were cultivated overnight in 1 L LB medium at 18 °C under agitation. Soluble proteins from the cytoplasmic fraction were recovered after cell lysis by sonication and centrifugation at 20,000 × g for 40 min and 4 °C.

The RBest1 esterase was purified from the cytoplasmic fraction in a two-step strategy using an AKTA Prime Plus device (GE Healthcare, Uppsala, Sweden). The first purification step was carried out by cation exchange chromatography on a HiLoad S-Sepharose HP column (GE Healthcare), previously equilibrated with 20 mM acetate buffer pH 6.5 (buffer A). Proteins were eluted using a linear gradient of NaCl (0 - 250 mM) in buffer A. Collected fractions were analyzed by testing the activity on *p*-nitrophenyl butyrate (pNPB), and active fractions were further analyzed by 15 % SDS-PAGE gels. The RBest1-containing fractions were pooled and dialyzed overnight against 10 l of 20 mM Tris-HCl, pH 8.0 (buffer B). The dialyzed protein fraction was loaded on a 5-ml HiTrap Q-Sepharose HP column (GE Healthcare) equilibrated in buffer B and eluted using a linear gradient of NaCl (0 - 250 mM). Fractions containing the active esterase were analyzed as described above. Protein concentration and purity were determined by BCA protein quantification assay (Pierce, Rockford, IL, USA) using bovine serum albumin as standard, and by densitometry analysis using the ImageJ software (http://rsb.info.nih.gov/ij/) on 15 % SDS-PAGE gels, respectively.

RBest1 substrate specificity assay

To test the *in vivo* substrate specificity, recombinant *E. coli* RR1clone expressing RBest1 was spread on spirit blue agar containing 1 % of different emulsified triacylglycerols: tributyrin (C_4), tricaprylin (C_8), tricaprin (C_{10}) and triolein (C_{18}) (all purchased from Sigma). Hydrolysis was observed by the formation of a clear blue halo after 48 h incubation at room temperature. To test the *in vitro* substrate specificity, cytoplasmic fractions were prepared as described above and used to measure the enzymatic hydrolytic activity against various nitrophenylesters (pNPE): pNPA, acetate (C_2); pNPB, butyrate (C_4); pNPC, caprylate (C_8); pNPD, decanoate (C_{10}); and pNPS, stearate (C_{18}) (all purchased from Sigma). The standard reaction mixture consisted of 1.25 mM pNPE in 1 ml of 20 mM Tris-HCl, pH 8.0, and the appropriate amount of cell extract capable of releasing at least 0.2 µM of *p*-nitrophenol per minute. When measured at pH higher than 7.5, the release of *p*-nitrophenol was measured at 405 nm (ε_{405nm} pNP = 16,500 /M.cm) for 10 min on a Specord 50 spectrophotometer (Analytik Jena, Jena, Germany). One unit (U) of enzymatic activity corresponds to the release of 1 µmol *p*-nitrophenol per minute.

pH and thermal dependence of RBest1 activity

The pH dependence of RBest1 activity was determined by following the esterase activity at 346 nm (ε_{346nm} pNP = 4,800 /M.cm) (34). Reactions were followed for 3 min in a 20 mM pH-adjusted polybuffer solution (citrate-phosphate-CHES-CAPS) ranging from pH 3.5 to pH 9.5.

The thermal dependence of the activity was determined as described before and at its optimal pH (6.5) using a thermostatized Specord 50 spectrophotometer. The enzyme was preincubated for 5 min at the appropriate temperature before measuring the activity. In order to test the enzymatic thermal stability, stocks of proteins were incubated at temperatures ranging from 30 to 60 °C for up to 6 h. The remaining activity at 40 °C was compared to the initial activity.

Kinetic parameters of RBest1

Kinetic parameters for pNPB hydrolysis were determined under initial rate conditions using a nonlinear regression analysis of the Michaelis-Menten equation. Hydrolysis was measured at 40 °C using pNPB as substrate at final concentrations ranging from 0 to 200 µM in a 20 mM Tris-HCl pH 8.0.

Tolerance to organic solvents and salts

The influence of organic solvents on the RBest1 esterase activity was carried out as previously described with minor modifications (35). Briefly, organic solvents were added to a final concentration of 25 % (v/v) to the protein solution (in 20 mM Tris-HCl pH 8.0) and incubated at 37 °C under rotational agitation for 2 h. Remaining activity was tested under standard conditions using water as control. Tested organic solvents were N,N'-dimethyl-formamide (DMF), dimethyl-sulfoxide (DMSO), acetonitrile, p-xylene, toluene, pentane, benzene and n-hexane. Among the parameters that characterize organic solvents, the log of the partition coefficient (log $K_{o/w}$) is the most commonly used parameter giving the best correlation with enzyme activity and/or stability (28); log $K_{o/w}$ is the distribution of a specific molecule in a two-phase equimolar mixture of water and octanol: the higher the log $K_{o/w}$, the more hydrophobic the molecule is.

The impact of organic solvents on substrate specificity was investigated by incubating the enzyme in the presence of various solvents. Remaining activity on pNPA, pNPB and pNPC was compared to the activity measured for pNPB hydrolysis in 20 mM Tris-HCl (pH 8.0) at 40 °C.

The influence of different salts (NaCl, KCl, $CaCl_2$, $MgCl_2$, $CoCl_2$, $NiCl_2$ and $MnCl_2$) in the esterase activity was studied in 20 mM Tris-HCl pH 8.0 at 40 °C.

RESULTS AND DISCUSSION

Screening for esterase/lipase activity from metagenomic library RB1

A small-insert metagenomic library RB1 was built from a temperate forest soil sample. The library was constructed in pCR2.1-TOPO vector and consisted of ca. 70,000 recombinant *E. coli* bacterial colonies harboring an average insert size of 3.1 kb. The library was screened for lipase/esterase activity, and three clones were found to express a positive phenotype on agar plates containing 1 % emulsified tributyrin. Crude cell extracts were used to test for organic solvent tolerance and allowed the isolation of one recombinant clone, named *E. coli*/pRBest1, exhibiting detectable activity on tributyrin in the presence of DMSO, benzene, toluene and *p*-xylene (data not shown).

Sequence analysis

The DNA insert cloned in recombinant plasmid from esterase/lipase-producing *E. coli*/pRBest1 was sequenced, deposited under GenBank accession number FJ157327 (ACH99848 for deduced aminoacid sequence). A single open reading frame (ORF) of 861 bp was located in the insert, which encodes a 30.7 kDa protein named RBest1.

RBest1 protein shares amino acid sequence similarity (ca. 50 %) to many putative a/b hydrolases, including esterases, peptidases, and other uncharacterized proteins. In addition, RBest1 exhibits all the conserved signatures for the Hormone Sensitive Lipase (HSL)-like family corresponding to the abH4.04 according to the LED classification (1): a catalytic triad including S148 (the active site serine, included in the highly conserved G-E-S148-A-G pentapeptide), D243 and H275, and a conserved H64-G65 sequence that is part of the oxyanion hole (Figure 1).

RBest1 also possesses a unique 11-amino acid insertion at position 99 to 109, not exhibited by other enzymes related to RBest1. Based on the EstE1 structure (PDB: 2C7B), the RBest1's closest metagenome-derived esterase whose structure has been solved (19 % identity, 36 % similarity) (6), this insertion is located at the surface of the protein, in opposition to the active site, between the fourth b-sheet and the third a-helix of the protein (Figure 1). This loop contains an unusually high ratio of charged residues (2 glutamic acids, 1 aspartic acid, 3 lysines), which could strongly interact with polar solvents.

Interestingly, RBest1 is also similar (22 % identity, 36 % similarity) to the lipase from *Lysinibacillus sphaericus* (formerly *Bacillus sphaericus*) 205y (BS-Lip), the model for the abH04.04 subgroup as described on the Lipase Engineering Database (LED: www.led.uni-stuttgart.de/). Both RBest1 and BS-Lip show an unusual conserved active serine embedded in the pentapeptide G-E-S-A-G. Indeed, the lipases from *Bacillus* species are known to possess a particular active serine-containing pentapeptide (A-X-S-X-G) (35). However, there is lack of information on the enzymes from the abH04.04 subgroup, since BS-Lip is the sole previously characterized enzyme and therefore comparison between enzymes is difficult.

Phylogenetic analysis on the abH04 family (including RBest1) using the Neighbor Joining (NJ) method highlighted the existence of two groups or subfamilies (Figure 2). The first group (Group A) is composed by reference members of subfamilies abH04.01, abH04.02, and abH04.03 and sequence similarities with RBest1 range from 15.5 % (*Moraxella* sp.) to 24.2 % (EstE1 of an uncultured *archaeon*).

Group A is divided in two sub clusters. The first one is composed by sequences from the *Acinetobacter* lipase family (abH04.02). In the second cluster, sequences from *Moraxella* lipase 2-like family (abH04.01) and eukaryotic N-deacetylase family (abH04.03) are grouped.

The second group (Group B, abH04.04) is

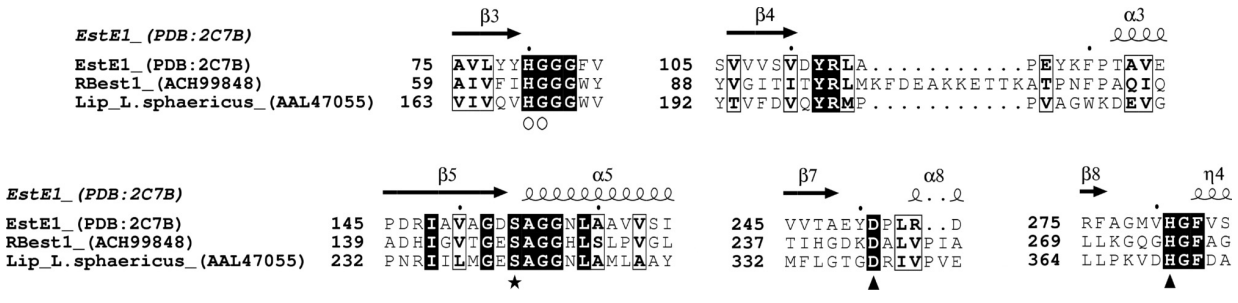

Figure 1. Alignment of amino acid sequences of RBest1 and related characterized lipases/esterases, the crystallized metagenome-derived esterase EstE1 and the OST lipase from *L. sphaericus* 205y. Blocks containing the active serine (★) and the two other residues involved in the catalytic triad formation (▲) are presented. Conserved residues forming the oxyanion hole are presented (○).

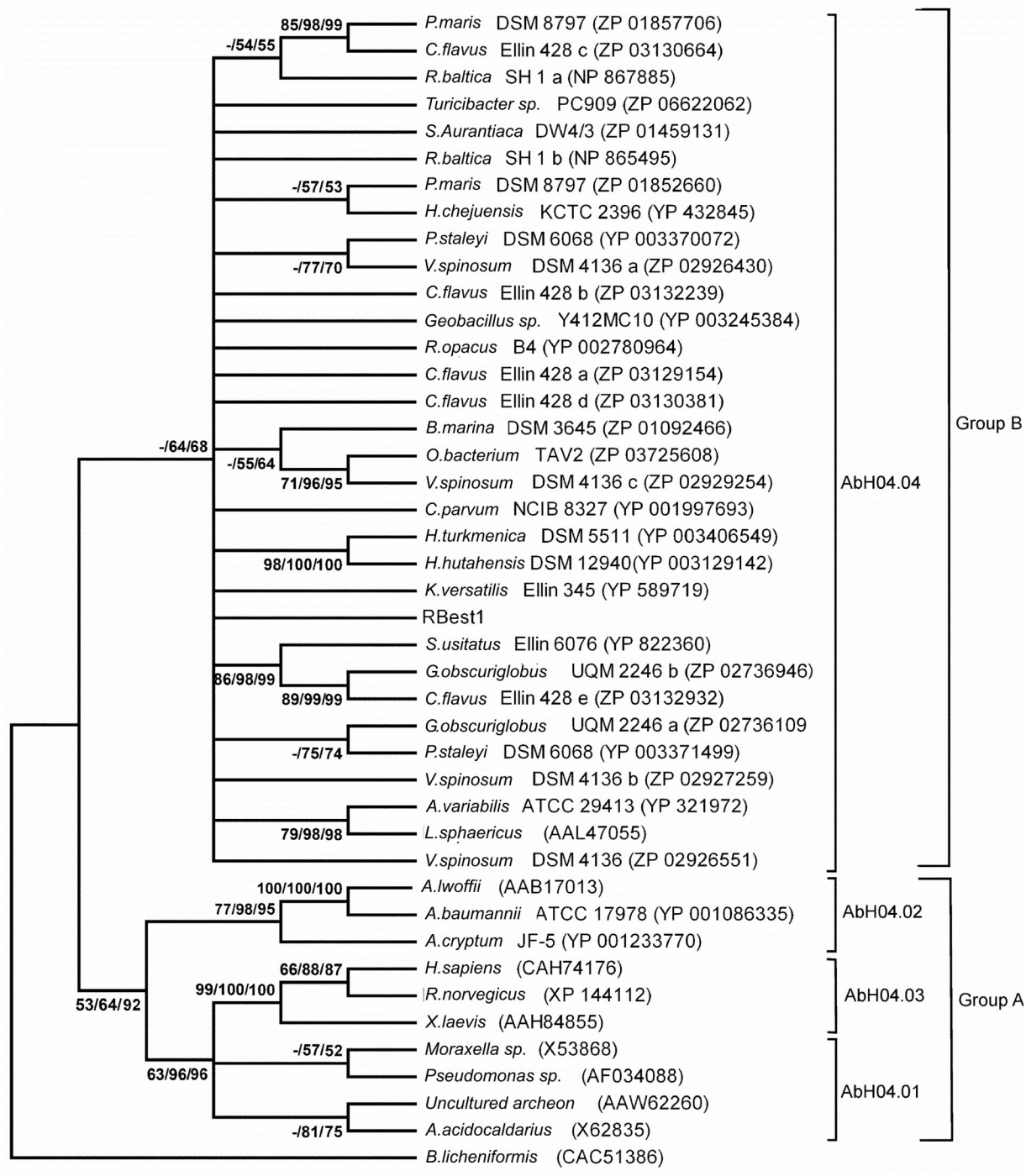

Figure 2. Neighbor Joining tree of the RBest1 sequence and related sequences. Bootstrap values were calculated for MP/ME/NJ method (see text).

composed by RBest1 and other 31 sequences, with similarities ranging from 29.5 % to 41.2 %. The best similarity score (41.2 %) was obtained for the sequence from *Gemmata obscuriglobus* UQM 2246 (ZP_02736946). Group B clusters 15 single branched sequences and seven clusters of two or three sequences.

Both Groups A and B share conserved HSL lipase signature as described above. Nevertheless, sequences from Group B share the following specific consensus H64-G65-G66, Y95, D120, G146-X147-S148-A149-G150, L153, G240-X-X-D243, and H275 (based on the RBest1 numbering), whereas sequences from Group A show an alternative

consensus, H80-G81-G82, Y112, P116, P121, G152-X153-S154-X155-G256, S231, L253-X254-D255 and H281 (based on the EstE1 numbering). Other methods such as Minimum Evolution (ME) yielded similar results, indicating that the phylogenetic analysis is consistent (data not shown).

Although accurate phylogenetic analysis highlights the vast diversity of previously annotated lipase-related sequences, it is assumed that the major part of the environmental enzyme diversity still remains untapped.

RBest1 production and purification

RBest1 production was performed using the pRBest1 plasmid isolated from the metagenomic library. A purification protocol using two different chromatographic steps was set up and allowed the purification of 1.4 mg of pure esterase (ca. 28 kDa) from the cytoplasmic fraction obtained from 1 L culture.

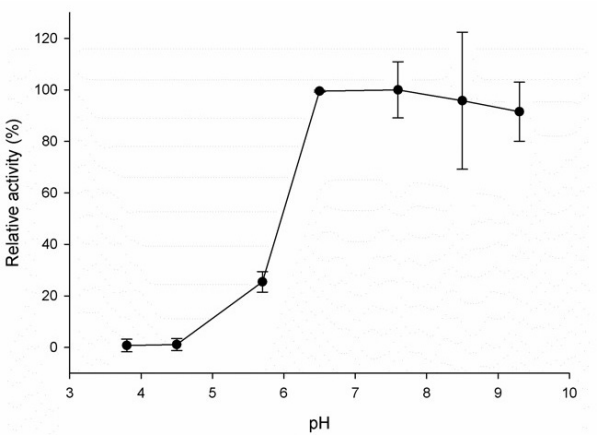

Figure 3. Influence of pH on the RBest1 activity. The maximum measured activity at pH 6.5 was taken as 100 %. Enzyme activity was determined under standard conditions. Presented results are the mean values obtained from triplicate experiments.

Biochemical characterization of RBest1

Although RBest1 was isolated on tributyrin-containing medium, its ability to hydrolyze other substrates was further investigated. Substrate specificity was studied both *in vivo* and *in vitro*, using either triacylglycerol emulsions (1 % of tributyrin, tricaprylin, tricaprin and triolein) or chromogenic nitrophenyl esters (pNPE), respectively. For *in vivo* analysis, formation of a blue halo around the colonies in emulsified spirit blue agar medium after 48 h incubation was only observed for substrate tributyrin. To test *in vitro* substrate specificity, the highest activity was recorded with pNPB (C_4: 300 mU/mg) followed by pNPA (C_2: ca. 25 mU/mg). The relative activity of pNPC (C_8), pNPD (C_{10}) and pNPS (C_{18}) as substrates was also determined (< 1 % of that recorded with pNPB).

Regarding the overall substrate specificity, RBest1 enzyme showed higher specificity for C4 substrates, confirming that RBest1 is an esterase rather than a lipase. On the other hand, BS-Lip displays the highest activity towards C_8, C_{10} and C_{12} substrates (39).

The effect of pH on the activity was investigated using purified RBest1 and pNPB as substrate in different pH-adjusted buffers. Maximum activity was measured in the range of pH 6.5 and remained significant when pH increased (Figure 3). No activity was recorded at pH values lower than 5.5.

Thermal dependence of the RBest1 activity was determined under standard conditions. The activity increased from 20 to 45 °C, being the latter the apparent maximum activity, and then quickly decreased (Figure 4A).

The thermal stability of RBest1 esterase was investigated by incubating the esterase at different temperatures for up to 6 h. Residual activity on pNPB at each temperature was determined. The enzyme remained significantly active when incubated at temperatures below 40 °C for up to 6 h. Nevertheless, when incubated at higher temperatures, rapid inactivation was observed (Figure 4B).

Kinetic parameters K_m and k_{cat} for pNPB hydrolysis were deduced to be 0.020 ± 0.002 mM and 88.1 ± 8.0 /min, respectively. The catalytic efficiency (k_{cat}/K_m) was calculated as 4,400 /mM.min.

Organic Solvent and Salt Tolerance

Protein stability and activity in the presence of organic solvents were tested in order to determine the OST behavior of RBest1.

Remaining activity against several pNPE was investigated after incubation for 30 min in the presence of organic solvents (Figure 5A). All the tested solvents appeared to affect the RBest1 activity. Non-miscible solvents ($\log K_{o/w} > 0$) improved the pNPE hydrolysis whereas miscible solvents ($\log K_{o/w} < 0$) had different effects. Inactivation occurred in the presence of acetonitrile (0 % remaining activity) whereas DMSO showed a slight activation of the hydrolytic activity. DMF appeared to moderately decrease the pNPE hydrolysis catalyzed by RBest1.

After longer incubations (2 h) in the presence of 25 % benzene at 37 °C under agitation, activity against pNPB appeared to be stabilized in the previously observed enhanced state (Figure 5B). On the other hand, when the enzyme was incubated under the same conditions in the presence of DMSO, the esterase activity apparently became stabilized to the control state beyond 2 h. Incubation in the

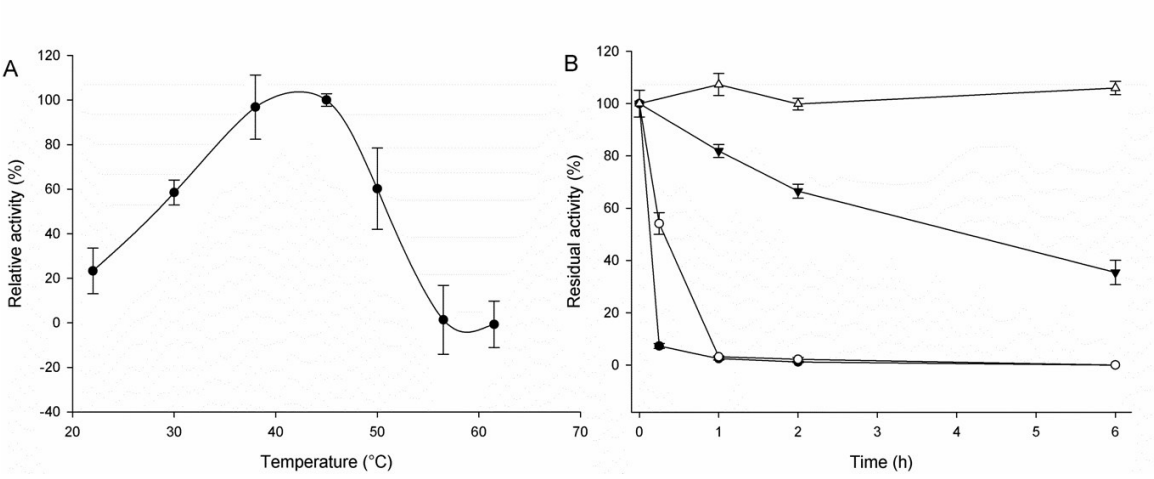

Figure 4. (A) Influence of temperature on the RBest1 activity. The enzyme was pre-incubated for 5 min at temperatures ranging from 20 - 60 °C. Enzyme activity was determined under standard conditions. The maximum measured activity at 45 °C was taken as 100 %. **(B)** Influence of temperature on the RBest1 enzyme stability. Enzyme was pre-incubated for up to 6 h at 30 °C (△), 40 °C (▼), 50 °C (○) and 60 °C (●). Remaining activity was measured at 40 °C. Presented results are the mean values obtained from triplicate experiments.

presence of *p*-xylene or DMF for a longer period seemed to increase the solvent effect; whereas the activity towards the first further increased, the latter reinforced the inactivation process (Figure 5B).

For both RBest1 and BS-Lip enzymes, it is noteworthy that organic solvents like benzene, toluene and xylene increase the stability and the activity of both crude extracts and purified enzymes (22, 35, 39). On the other hand, while acetonitrile has a deleterious effect on both enzymes, DMSO and DMF affect more slightly their hydrolytic activity.

The RBest1 hydrolytic activity on pNPB was stable over a wide range of NaCl concentrations; while the enzyme was not significantly affected by 1 M NaCl, the activity rate decreased to 50 % when NaCl concentration increased to 5 M (Figure 6). Several other salts were tested, showing that they were able to more severely affect the RBest1 activity

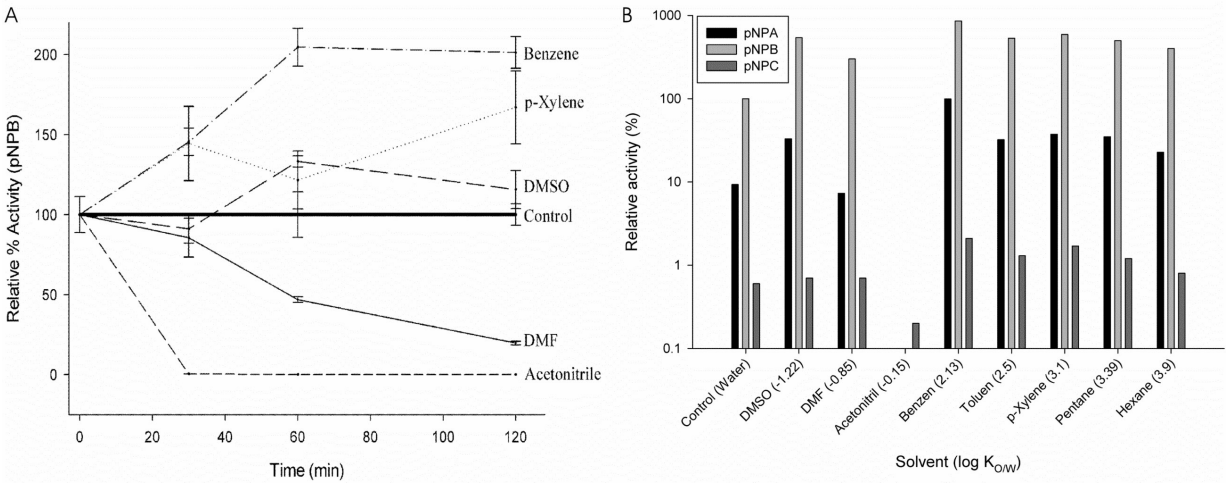

Figure 5. (A) RBest1 Organic Solvent Tolerance, activity against pNPB in the presence of various organic solvents after incubation at 40 °C for 0 to 2 h under rotational agitation. Depicted results are the mean value obtained from triplicate experiments and relative to the activity measured in the buffer-containing control. **(B)** Influence of organic solvents on the RBest1 activity on pNPA, pNPB and pNPC. The enzyme was preincubated for 30 min at 40 °C under agitation in presence of organic solvent (25 %). Enzyme activity was determined under standard conditions. The measured activity for pNPB hydrolysis in aqueous buffer was taken as 100 %. Presented results are the mean values obtained from triplicate experiments.

on pNPB. In addition, divalent cations such as Ca^{2+}, Mg^{2+}, Co^{2+} and Ni^{2+} had a deleterious effect on the RBest1 activity (Figure 6).

This halophilic behavior seems to correlate with the previously reported for many OST enzymes. Indeed, molecular adaptation to organic solvents converges to some extent with halophilic adaptation since increasing salt concentrations causes reduction of water activity (2, 36).

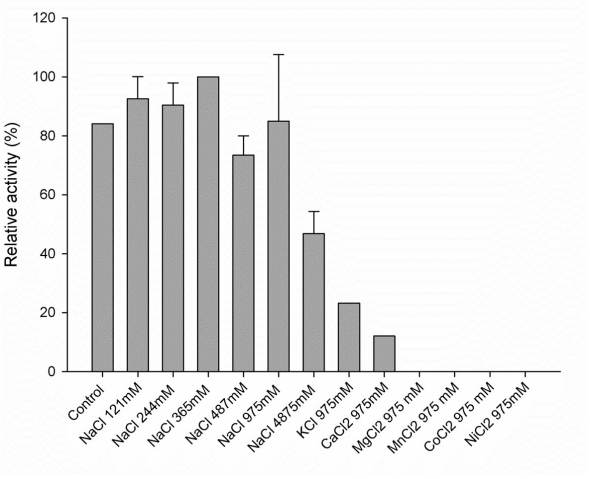

Figure 6. Effect of various salts on the RBest1 activity, measured at 40 °C under standard conditions. The optimal hydrolysis condition was defined as 100 % activity; presented values are means of triplicate experiments.

Bacteria growing in organic solvent-containing media circumvent the solvent's toxic effects by degradation and transformation of solvents or by active efflux of solvents (16). *Lysinibacillus sphaericus* is a common environmental organism which produces an insecticidal toxin similar to that produced by *Bacillus thuringiensis* (12). In fact, *L. sphaericus* 205y, producing the BS-Lip lipase, was isolated for its ability to grow in the presence of benzene, toluene, and *p*-xylene-containing media (22).

Nevertheless, although both RBest1 and BS-Lip are OST enzymes sharing significant similarities and displaying the same range of tolerance against organic solvents, no clear evidence for their phylogenetic association could be established.

Lysinibacillus sphaericus 205y was isolated out of a presumably contaminated soil sample from Malaysia (22), while RBest1 is derived from a pristine German soil sample. In addition, the sample used for the metagenomic library construction had not been exposed to any organic solvent or to high salt concentration. Nonetheless, enzymes remaining active in low water-containing environments were observed in many physiological conditions including frozen ecosystems. Noteworthy, it is reported that *Bacillus* dormant spores are resistant to harsh conditions including desiccation for long periods of time (3, 38). Their reactivation involved many factors and the production of esterase/lipase was correlated with spore germination (3, 14, 31). However, functional analyses of lipase/esterase involved in spore germination still remain incomplete, and additional information is required to better understand their biophysical features.

As described here, the metagenomic approach gives the possibility to isolate enzymes displaying specific properties. The RBest1 ability to remain active in the presence of various organic solvents and the possibility to modulate its substrate specificity in the presence of organic solvents makes RBest1 a versatile new enzyme with potential biotechnological applications or in bioremediation processes.

Nevertheless, a better understanding of the microbial environmental diversity requires not only important efforts on DNA sequencing but also protein analysis in order to elucidate their physiological function. A major effort on the functional analysis of the metagenomederived enzymes remains to be done to better understand the role of individual genes and proteins in the environment. Indeed, attempts to elucidate the physiological function of proteins derived from metagenomes sometimes lead to unexpected results.

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