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Full Length Research Paper

A deep gold mine metagenome as a source of novel esterases

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New sources of enzymes for biotechnological applications are continually being sought for. While diverse microbial ecosysyems have been demonstrated in the deep subsurfaces, deep mines provide easy access to these specialist communities. Therefore, the aim of this study was to assess a deep mine biofilm as a source of novel esterase enzymes. Biofilm was collected from the Beatrix Mine in South Africa, at a depth of 808 m. Assessment of the diversity revealed a group of previously uncultured bacteria and archaea. A metagenome library was screened for esterolytic activity, producing two esterolytic clones: a phospholipase patatin protein and an isochorismatase family protein. The isochorismatase family protein contained the catalytic Asp and Cys but not the Arg, which is considered as important for catalysis. The patatin showed 55% similarity to its closest relative; the patatin family protein from *Plesiocystis pacifica*. The expressed patatin displayed a preference for the C6 ester and was maximally active at pH 8 and 30°C. This study reported that screening of a relatively small metagenome library from the deep mine biofilm provided two esterolytic clones, which differed from their known counterparts. This indicates that the deep mine ecosystems contain an untapped resource of novel and potentially useful enzymes which may have applications in chemical syntheses.

Key words: Metagenome library, functional screening, lipolytic activity, patatin, isochorismatase.

INTRODUCTION

Diverse microbial ecosystems exist in the subsurface and have been described in the South African gold mines of the Witwatersrand super-group (Moser et al., 2003; Wanger et al., 2008). Biofilms are abundant where water seeps from holes or cracks in the rock face in tunnels which are formed during the course of mining operations. Although the biofilms are exposed to the environment and could be considered to be microbiologically compromised, microbial populations have been found to reflect the geochemistry of the water and high concentrations of contaminating organisms find it difficult to out-compete the indigenous microorganisms once the original geochemical conditions have been restored (Moser et al. 2003; Pederson et al., 1997). Meanwhile, novel microorganisms have been found to inhabit these biofilms (Maclean et al., 2007; Wanger et al, 2008) and they represent a rich source of novel genetic material (Wanger et al., 2008)

Esterolytic enzymes, which include the lipases and patatins, are a large group that catalyses the hydrolysis of ester bonds in a wide variety of substrates and are ubiquitous enzymes found in a wide range of organisms from bacteria to humans (Lee et al., 2006; Litthauer et al., 2010). These enzymes have significant biotechnological importance because of their ability to catalyze regio- and stereo-selective organic reactions. They have no requirements for cofactors, are stable in organic solvents and have broad substrate specificity (Lee et al., 2004; Ranjan et al., 2005; Couto et al., 2010). Although lipolytic

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enzymes belong to many different protein families without sequence similarity, they have the same architecture, the α/β-hydrolase fold, a group of conserved amino acids, including a serine in a highly conserved G-X-S-X-G (X denotes any amino acid) pentapeptide, an aspartate or glutamate residue that is hydrogen bonded to a histidine to form a catalytic triad and the oxyanion hole formed by the backbone amides of two conserved residues (Fischer and Pleiss, 2003; Rhee et al., 2005).

The traditional cultivation-based methods to identify novel biocatalysts are insufficient and cumbersome (Lämmle et al., 2007). In addition, more than 99% of bacteria in such environment cannot be cultured using conventional methods (Handelsman et al., 1998; Rondon et al., 2000; Yun and Ryu, 2005; Couto et al., 2010). The rapidly expanding approach of culture-independent methods (metagenomics) is therefore, useful in understanding and accessing microbial genomes and their functions (Hårdeman and Sjöling, 2007). Metagenomic screening represents an efficient method for isolating novel and useful genes from environmental DNA libraries with high potential for use in pharmaceutical products or production processes from previously uncultured microorganisms (Litthauer et al., 2010). The advantage of this technique is that significantly diverse genes can be isolated directly from the environmental sources (Park et al., 2007), and several novel lipases/esterases have been discovered with the use of metagenomic approach, (Lee et al., 2004, 2006; Rhee et al., 2005; Tirawongsaroj et al., 2008; Kim et al., 2009; Couto et al., 2010). This study similarly reports the construction of a metagenomic library from biofilm collected from a South African deep gold mine, and the identification of different esterolytic enzymes.

MATERIALS AND METHODS

Sample collection

Biofilm was aseptically collected at a depth of 808 m in the Witwatersrand basin from the Beatrix Gold mine (Goldfields Limited) in the Northern Free State at latitude 28°15'S and longitude 26°47'E, near the towns of Welkom and Virginia, South Africa. The temperature, pH and conductivity readings, taken on the site were 31.3°C, 7.31 and 5.03 mS/cm, respectively.

Metagenomic DNA isolation

A modified method of Towner et al. (1991) was used. 2 g of the biofilm sample was mixed with 300 µl phosphate buffer (0.1 M $NaH_2PO_4/NaHPO_4$; pH 6.6), followed by the addition of 100 µl of 100 mM aluminum sulfate (Dong et al., 2006). The pH was adjusted to 8 by the addition of 1 M sodium hydroxide, 500 µl DNA isolation buffer (100 mM Tris-HCl (pH 8); 50 mM EDTA; 1% SDS) was added, was vortexed and 200 µl of glass beads (Sigma, 425-600 µm diameter) was added and the sample was vortexed for 4 min with immediate cooling on ice. Following cooling, 275 µl of 7 M ammonium acetate (pH 7) was added, incubated for 5 min at 65°C and was cooled on ice for 5 min. Thereafter, 500 µl chloroform was added, vortexed and centrifuged at 14 000 rpm for 5 min at 4°C. The supernatant was removed and the DNA was precipitated with one volume of isopropanol overnight at -20°C (Labushagne and Albertyn, 2007). The pellet was collected, washed with 70% (v/v) ethanol, dried and dissolved in 50 µl sterile water containing RNase A and stored at 4°C.

16S rRNA gene library construction and preliminary diversity assessment

PCR for amplification of the 16S rRNA gene was performed using primers 27F and 1492R for bacteria (Lane, 1991) or A2F (Reysenbach and Pace, 1995) and 20b (Rincón et al., 2006) for archaea with metagenomic DNA as template. The purified PCR products were ligated into the pGEMT-Easy vector and 50 bacterial and 20 archaeal clones were randomly selected and sequenced. Phylogenetic analysis was done using ARB software (Ludwig et al., 2004).

Metagenomic library construction

The metagenomic DNA was partially digested with 1 U *Bam*HI (New England Biolabs) for 1 h at 37°C, 2 to 6 kb DNA was excised from the SYBR Gold stained gel and purified using the GFX Gel Purification Kit (Amersham Biosciences). The fragments were ligated into the linearized pZerO-2 suicide vector (Invitrogen) and ~100 ng of ligation mixture of DNA was transformed into *Escherichia coli* TOP10 host cells by heat shock according to Sambrook et al. (1989). The transformation mix was plated onto LB kanamycin (50 µg/ml) agar plates and incubated overnight at 37°C, colonies were arrayed into 96-well micro titer plates containing 300 µl LB kanamycin (50 µg/ml) medium and grown overnight at 37°C. Glycerol stocks were stored in 96 well plates in LB kanamycin (50 µg/ml) medium supplemented with 40% glycerol at -80°C.

Screening for esterolytic activity

Esterolytic activity was assessed after three days incubation at 37°C using an adapted protocol (Ro et al., 2004) of the tributyrin plate assay (Kouker and Jaeger, 1987). Plasmids were isolated from esterolytic clones and *E. coli* TOP10 cells were transformed with each plasmid using heat shock and re-assessed for estereolytic activity. Clones displaying large zones of clearance after the second round of screening were selected for sequence analysis.

DNA sequencing and analysis

Insert DNA of esterolytic clones were sequenced by primer walking using an ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, CA), incorporating the ABI BigDye® Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Translated ORFs were compared to known sequences using BLASTP (Altschul et al., 1997).

PCR amplification of the patatin gene

The complete patatin gene was amplified by the expand high fidelity

PCR system (Roche) using plasmid DNA from clone pNS6 as the template and the following primer set: PATA-F 5'-GGCATATGGGCCTGACGCTGATC3' (*Nde*I) and PATA-R 5'-TCAAGCTTTCAGGCGCTCTCG-3' (*Hind*III) as derived from the sequence of the patatin ORF. The purified PCR product was ligated into pGEM®-T Easy vector digested with *Nde*I and *Hind*III and 50 ng of the ligated product was used to transform *E. coli* TOP10 competent cells by heat shock (Sambrook et al., 1989). After confirming the sequence of the insert, it was excised with *Nde*I and *Hind*III, purified and ligated into the pET-28b(+) vector to yield pET-28b(+)-P.

Expression and purification of the patatin

E. coli BL21(DE3) (pLysS) cells transformed with pET-28b(+)-P were grown to OD_{600} 0.2 to 0.4 at 30°C and protein production was induced with 0.5 mM isopropyl-dithiogalactopyranoside (IPTG). After 3 h at 37°C, centrifuged cells were treated with the B-PER solution (Pierce, USA), centrifuged and resuspended in 20 mM MOPS (pH 7.4) containing 50 mM imidazole/0.5 M NaCl followed by sonication for 5 min (5 cycles at 80 W). The soluble fraction was loaded onto a HisTrap FF column (5 ml, Amersham Biosciences). Unbound proteins were eluted (5 $ml.min^{-1}$) using 20 mM MOPS (pH 7.4) containing 50 mM imidazole and 0.5 M NaCl. Bound proteins were then eluted in the same buffer using a 0 to 0.5 M linear gradient (100 ml) of imidazole. Fractions were subjected to SDS-PAGE analysis and visualized with Coomassie Blue R-250 (Fairbanks et al., 1971). Western blot analysis for the detection of His-tagged protein was performed using the SuperSignal®West HisProbe™ kit (Pierce, USA) according to the manufacturer's instructions.

Biochemical analysis

Esterolytic activity was assayed using para-nitrophenyl ester substrates with C_4-C_{16} acyl chain lengths according to van Heerden and Litthauer (1999). One unit of enzyme activity was defined as the amount of activity required to release 1 µmol of *p*-nitrophenol/min. The optimal temperature for enzyme activity was determined for a range of 20 to 40°C and the optimum pH of the enzyme was measured for a range of 7 to 10. The buffers used were 100 mM HEPES (pH 7 to 7.5) and 100 mM Tris-HCl (pH 8 to 10).

RESULTS

Tables 1 and 2 list the closest relatives of the 16SrRNA clones sequenced. There was no clone whose 16S rRNA gene sequence was 100% identical to those of the known bacterial species. Four clones showed ≥97% sequence identity to the known bacterial species. Sequences with >97% identity were treated as identical (Tamaki et al., 2005). The majority were related to previously uncultured clones from different environments, such as the Sargasso Sea and sea floor basalts, sub-surface ground water, limestone caves, tar pits, alkaliphilic hot springs, mine drainage sites and biofilms (Cho and Giovanni, 2003; Kim and Crowley, 2007; Ikner et al., 2007). Rarefaction analysis using DOTUR (Schloss and Handelsman, 2005) produced an almost linearly increasing graph (not shown), indicating that the biofilm represented a rich mixture of, probably previously uncultured bacteria. Archaeal diversity was smaller as expected.

Analysis of the insert fragments generated by *Xho*I and *Hind*III restriction digestion of 20 of the approximately 10,000 clones produced an average insert size of ~4 kb for the library. Two of the positive clones in pZerO-2 from the second round of screening produced esterase-type sequences. BLAST analysis revealed similarity between pNS1 (GenBank accession no. HM046833) and isochorismatases (YcaC related amidohydrolases) from various bacteria. Isochoristmatase is a cysteine hydrolase that is involved in bacterial siderophore and phenazine biosynthesis. This enzyme is shown to be remarkably similar to the alpha-beta hydrolase family of enzymes (Parsons et al., 2003; Rusnak et al., 1990). Multiple sequence alignment of the 100 best hits with pNS1 showed that the catalytic Asp and Cys, but not the Arg, was conserved in pNS1 (Figure 1).

The patatin clone pNS6 (GenBank accession no. HM046834) is a member of the phospholipase patatin family protein which has been reported to display lipid acyl hydrolase activity but the physiological role of this enzyme remains unclear (Barta and Bartova, 2008). Patatin is the major protein constituent of potato tubers and displays broad esterase activity (Hirschberg et al., 2001). Patatin B2 and phosholipase A2 share conserved domains; both proteins contain the classical lipase/esterase motifs and active sites (G-X-S-X-G) (X-denotes any amino acid), the conserved aspartic acid and histidine. BLASTP (Altschul et al., 1997) analysis of pNS6 revealed moderate similarity between pNS6 and patatin proteins from other bacteria including phospholipase patatin family protein from *Plesiocystis pacifica* (GenBank accession no. ZP_01911272) (55%); patatin from *Roseiflexus* sp. (GenBank accession no. YP_001276494) (34%) and patatin from *Herpetosiphon aurantiacus* (GenBank accession no. YP_001543920) (35%). Further sequence analysis revealed a putative purine rich ribosomal binding site located 5 bp upstream of the ATG start codon. Multiple sequence alignment comparisons of the pNS6 to other phospholipase patatin proteins from various bacteria revealed the presence of five conserved sequence domains (Figure 2). The classical lipase/esterase conserved motif and active site residues [G-X-S-X-G] and the conserved aspartic acid and histidine were identified in pNS6.

Expression and purification of the patatin

The patatin was over-expressed in *E. coli* BL21 (DE3) pLysS at 30°C by induction through the addition of 0.5 mM IPTG to exponentially growing cells, and subsequent growth for another 4 h. SDS-PAGE analysis confirmed

Table 1. Closest BLAST hits of bacterial 16S clones sequenced from Beatrix mine biofilm.

Clone	Closest relative	ID (%)	e-value	Accession
BAC1	Uncultured sludge bacterium 16S rRNA	89	0.0	AF234706.1
BAC2	Uncultured Acidobacteria bacterium 16S rRNA	95	0.0	EU223942
BAC3	Uncultured *Nitrospira sp* clone 16S rRNA	85	0.0	EU084880
BAC4	*Brucella suis*, complete genome	88	0.0.	CP00912.1
BAC5	*Spingomonas yunnanensis* strain YIM 003 partial 16S rRNA	94	0.0	AY894691.1
BAC6	Bacterial species 16S rRNA	88	0.0	Z95709.1
BAC7	Uncultured *Bacteroidetes* bacterium	83	0.0	EU229455.1
BAC8	*Methlocella tundrae* partial 16S rRNA	78	2e-172	AJ563928.1
BAC9	*Marinobacter sp.* 16S rRNA	80	0.0	AJ429499.1
BAC10	Uncultured bacterium gene16S rRNA	90	0.0	AB280279.1
BAC11	Uncultured gamma proteobacterium partial 16S rRNA gene	97	0.0	AJ534675.1
BAC12	Uncultured alpha proteobacterium partial 16S rRNA gene	98	0.0	EF612403.1
BAC13	Uncultured bacterium clone 16S rRNA	88	0.0	EF522844.1
BAC14	Uncultured bacterium gene16S rRNA	94	4e-117	AB240487.1
BAC15	Uncultured soil bacterium partial 16S rRNA	92	0.0	EU589303.1
BAC16	*Aeromonas veronii* partial 16S rRNA	97	0.0	AM184224.1
BAC17	Uncultured bacterium clone partial 16S rRNA	93	0.0	DQ532277.1
BAC18	Uncultured bacterium clone partial 16S rRNA	89	0.0	EF125428.1
BAC19	*Sphingomonas sp.* 16S rRNA	96	0.0	AB047364.1
BAC20	Uncultured soil bacterium partial 16S rRNA	93	0.0	EU589303.1
BAC21	Uncultured *Cytophagales* bacterium clone, 16S rRNA	90	0.0	AF361197.1
BAC22	*Lysobacter taiwanensis* 16S rRNA	94	0.0	DQ314555.1
BAC23	Unidentified bacterium clone 16S rRNA	92	0.0	EF219552
BAC24	Uncultured bacterium clone 16S rRNA	92	0.0	EU083503.1
BAC25	Beta-proteobacterium 16S rRNA	89	0.0	AF236010.1
BAC26	Uncultured bacterium 16S rRNA	93	0.0	AM176872.1
BAC27	*Nitrospira sp.* 16S rRNA	97	0.0	Y14644.1
BAC28	*Methylibium aquaticum* 16S rRNA	97	0.0	DQ664244.1
BAC29	Uncultured deltaproteobacterium 16S rRNA	90	0.0	AJ581552.1
BAC30	Uncultured F*irmicutes* 16S rRNA	97	0.0	EF665782.1
BAC31	Uncultured bacterium clone 16S rRNA	84	3e-119	EF125428.1
BAC32	Uncultured *Cytophagales* bacterium clone, 16S rRNA	83	2e-90	AF361197.1
BAC33	Uncultured bacterium clone 16S rRNA	92	0.0	DQ463246.1
BAC34	Uncultured *Bacillus sp. c*lone 16S rRNA	82	7e-21	AY876909.1
BAC35	Uncultured bacterium clone 16S rRNA	83	1e-106	EU015107.1
BAC36	Uncultured sludge bacterium 16S rRNA	89	1e-131	AF234706.1
BAC37	Uncultured bacterium clone 16S rRNA	94	1e-17	EF173339.1
BAC38	Uncultured bacterium clone 16S rRNA	87	0.0	EF632776.1
BAC39	Uncultured bacterium clone 16S rRNA	86	1e-157	EU386115.1
BAC40	*Plantomycete* 16S rRNA	84	0.0	AY162129.1
BAC41	Uncultured bacterium gene 16S rRNA	96	0.0	AB286376.1
BAC42	*Nitrospira moscoviensis* 16S rRNA	98	0.0	X82558.1
BAC43	Uncultured bacterium clone 16S rRNA	88	0.0	EU617826.1
BAC44	Uncultured bacterium clone 16S rRNA	97	0.0	EU160003.1
BAC45	Uncultured bacterium clone	93	0.0	EU015113.1
BAC46	Uncultured bacterium clone 16S rRNA	95	1e-174	AF392636.1
BAC47	Uncultured bacterium isolate	96	0.0	AY703460.1
BAC48	Uncultured bacterium 16S rRNA	86	0.0	AB280279.1
BAC49	Uncultured bacterium clone	96	0.0	EU148614.1
BAC50	Uncultured *Phaeobacter* 16S rRNA	86	0.0	EU375177.1

Table 2. Closest BLAST hits of archaeal 16S clones sequenced from Beatrix mine biofilm.

Clone	Closest relative	ID (%)	e-value	Accession
ARC2	Uncultured archeon clone ZES-28 partial 16S rRNA	99	0.0	EF367466.1
ARC3	Uncultured archeon clone ZES-28 partial 16S rRNA	99	0.0	EF367466.1
ARC4	Uncultured crenarchaeote partial 16S rRNA	82	1e-142	AJ870317.1
ARC5	Uncultured archeon clone ZES-28 partial 16S rRNA	99	0.0	EF367466.1
ARC6	Uncultured archeon clone ME-59 16S rRNA	94	0.0	DQ641778.1
ARC7	Uncultured crenarchaeote clone partial 16S rRNA	94	0.0	DQ641760.1
ARC8	Uncultured archeon clone ZES-28 partial 16S rRNA	98	0.0	EF367466.1
ARC9	Uncultured archeon clone ME-41 16S rRNA	99	0.0	DQ641760.1
ARC10	Uncultured archeon clone ZES-47 partial 16S rRNA	98	0.0	EF367485.1
ARC11	Uncultured archeon clone ZES-28 partial 16S rRNA	99	0.0	EF367466.1
ARC12	Uncultured archeon clone ZES-28 partial 16S rRNA	99	0.0	EF367466.1
ARC13	Uncultured archeon clone ZES-28 partial 16S rRNA	99	0.0	EF367466.1
ARC14	Uncultured archeon clone ZES-28 partial 16S rRNA	99	0.0	EF367466.1
ARC15	Uncultured archeon clone ZES-28 partial 16S rRNA	96	0.0	EF367466.1
ARC16	Uncultured archeon clone ZES-28 partial 16S rRNA	99	0.0	EF367466.1
ARC17	Uncultured archeon clone ZES-28 partial 16S rRNA	97	0.0	EF367466.1
ARC18	Uncultured archeon clone ZES-28 partial 16S rRNA	97	0.0	EF367466.1
ARC19	Uncultured archeon clone ZES-28 partial 16S rRNA	96	0.0	EF367466.1
ARC20	Uncultured archeon clone ZES-28 partial 16S rRNA	96	0.0	EF367466.1

the appearance of a protein band of the expected size of approximately 60 kDa in the soluble fraction of the *E. coli* (Figure 3) for the recombinant patatin. The protein was partially purified using two steps of Ni-affinity chromatography. Western blotting confirmed the presence of the His-tagged protein (Figure 3).

Biochemical analysis of the expressed patatin

The protein could not be purified to homogeneity by Ni-affinity purification and a negative control consisting of cells transformed with an empty pET-28b(+) vector was therefore included in all the assays to correct non-specific baseline activity. The patatin could efficiently hydrolyze short and medium chain (C4 to C10) p-NP esters, with the highest activity towards p-NP caproate (C6) (Figure 4) experimentally suggesting that this enzyme is an esterase. The patatin displayed activity over a temperature range of 20 to 35°C, with the highest activity at 30°C. This was expected as this enzyme was isolated from a mesophilic environment. Optimum activity was observed at pH 8 (Figure 5) which was consistent with esterases, since the majority of lipolytic enzymes are functional at alkaline pH. Similar results were obtained by Tirawongsaroj et al. (2008) who reported on the expression and characterization of a novel bacterial phospholipase patatin isolated from a hot spring in Thailand.

DISCUSSION

Recently, metagenome libraries of fresh water ponds (Ranjan et al., 2005) and hot springs (Tirawongsaroj et al., 2008), as examples, have successfully been screened for esterolytic activities and it has also been known that deep mine biofilms harbor a variety of previously uncultured microbes, which could provide access to novel enzymes. Clone pNS1 encoded a protein designated as an isochoristmatase. Isochoristmatase is a cysteine hydrolase that is involved in bacterial siderophore and phenazine biosynthesis. BLASTP analysis revealed a moderate similarity (60%) between pNS1 and isochorismatases from various bacteria, including isochorismatase family protein (56%) from *Geobacter sulfurreducens* (NP_953629), isochorismatase family protein (55%) from *Nitrosomonas europaea* (NP_842301) and isochorismatase family hydrolase (60%) from *Pseudomonas putida* (YP_001747104). Further sequence analysis revealed a putative purine rich ribosomal binding site located 7 bp upstream of the ATG start codon. The isochorismatases represent a diverse group of enzymes also known as 2,3 dihydro-2,3 dihydroxybenzoate synthase, which catalyse the hydrolysis of isochorismate to 2,3-dihydroxybenzoate and pyruvate. This superfamily has been subdivided by SCOP into six families, N-carbamoylsarcosine amidohydrolase, pyrazinamidase/nicotinamidase, phenazine biosynthesis protein PhzD, YcaC, hypothetical

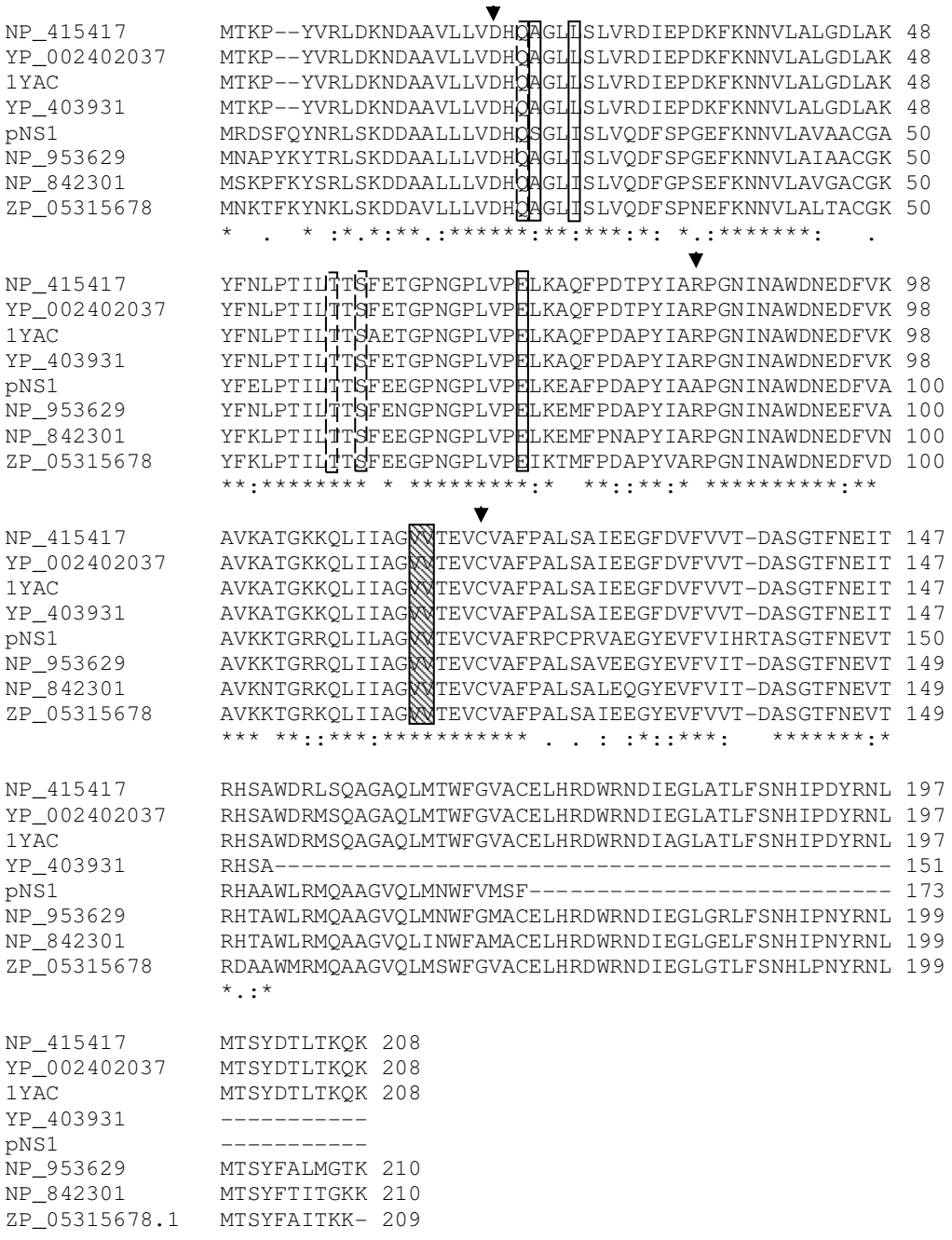

Figure 1. Alignment of pNS1 with six isochorismatase proteins. Sequences were identified by their GenBank accession numbers (NP_415417= *E. coli* str. K-12 substr. MG1655; YP_002402037= *E. coli* 55989; 1YAC = *E. coli* 1YAC (YcaC gene product); YP_403931 = *Shigella dysenteriae* Sd197 ; NP_953629 = *Geobacter sulfurreducens*; NP_842301 = *Nitrosomonas europeae*). The boxes indicate the dimer interface residues, the hatched box indicates the *cis* peptide bond and the broken boxes indicates the conserved Gln23, Thr59 and Ser61 (pNS1 numbering). The triangles denote catalytic residues.

protein YecD and ribonuclease MAR1. There is, however, no universal conservation of catalytic residues across the multiple families (Caruthers et al., 2005). The isochorismatase pNS1 contained two (D21 and C120) of the three expected catalytic residues, the basic Arg being absent but the conserved Gln23, Thr59 and Ser6 were all found in the pNS1 (Figure 1). Although bacteria commonly possess the isochorismatase enzyme, its

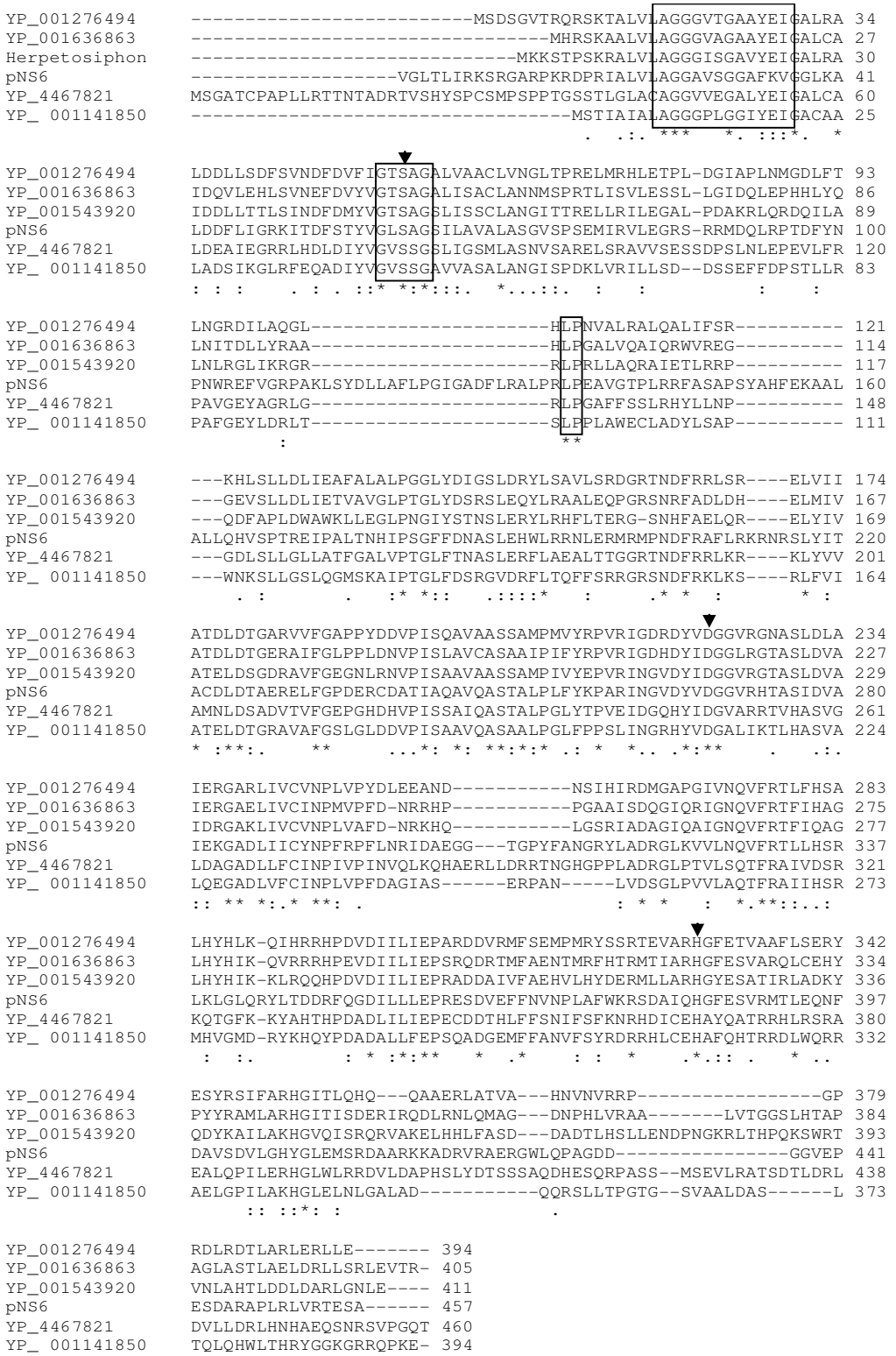

Figure 2. Multiple alignment of selected bacterial phospholipase patatin family proteins. Sequences were identified by their GenBank accession numbers (YP_001276494 = *Roseiflexus*; YP_001636863 = *Chloroflexus*; YP_001543920 = *Herpetosiphon*; YP_4467821 = *Salinibacter*; YP_ 001141850 = *Aeromonas*). The regions demarcated by boxes are the oxyanion hole, serine hydrolase motif and conserved proline. The triangles denote the catalytic triad.

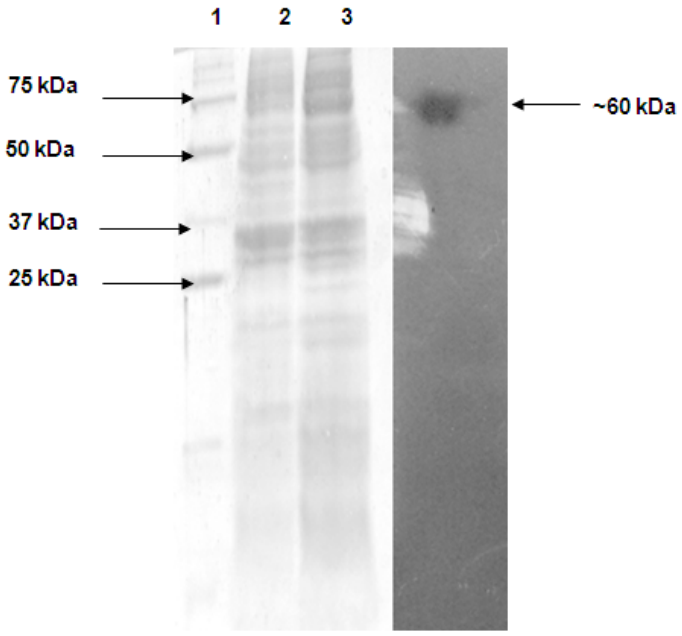

Figure 3. Expression of patatin in *E. coli.* Lane 1, Molecular marker (BioRad); lane 2, uninduced control; lane 3, expressed patatin after 4 h of induction at 30°C with 0.5 mM IPTG. Western blot analysis using anti-His (6) antibodies.

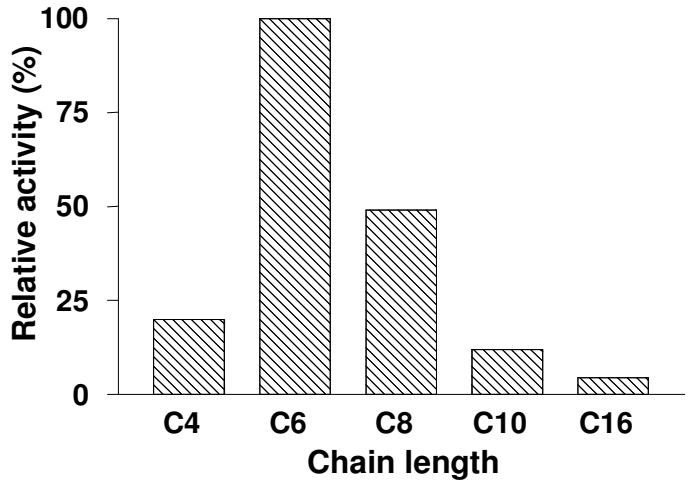

Figure 4. Substrate specificity for *p*-nitrophenyl esters with varying carbon lengths.

natural substrates and physiological functions are unknown and pNS1 could conceivably display a function which is not related to hydrolytic activity. An isochorismatase with almost no sequence identity to pNS1, SttH streptothricin hydrolase from *Streptomyces albulus*, was shown to be a novel mechanism of streptothricin resistance (Hamano et al., 2006).

BLASTP analysis of pNS6 revealed moderate similarity (<55%) between pNS6 and patatin proteins from other bacteria including phospholipase, patatin family protein (55%) from *P. pacifica* (ZP_01911272), patatin (34%) from *Roseiflexus* sp. (YP_001276494) and patatin (35%) from *H. aurantiacus* (YP_001543920). According to the blast analysis (55% similarity to the closest hit), we have obtained a new patatin protein. Further sequence analysis revealed a putative purine rich ribosomal binding site located 5 bp upstream of the ATG start codon.

For most of the lipolytic enzymes, the catalytic triad consists of serine in a conserved pentapeptide G-X-S-X-G aspartate and a highly conserved histidine. Multiple sequence alignment comparisons of the pNS6 to other phospholipase patatin proteins from various bacteria revealed the presence of five conserved domains. The classical lipase/esterase conserved motif and active sites residues (G-X-S-X-G) (X-denotes any amino acid) and the conserved aspartic acid and histidine were identified in pNS6 (Figure 2). In addition, we aligned pNS6 with other bacterial lipolytic enzymes. However, there was no significant alignment. A phylogenetic tree was constructed in order to observe where pNS6 clusters with respect to the 8 different lipolytic families (data not shown). pNS6 did not cluster within any of the 8 families.

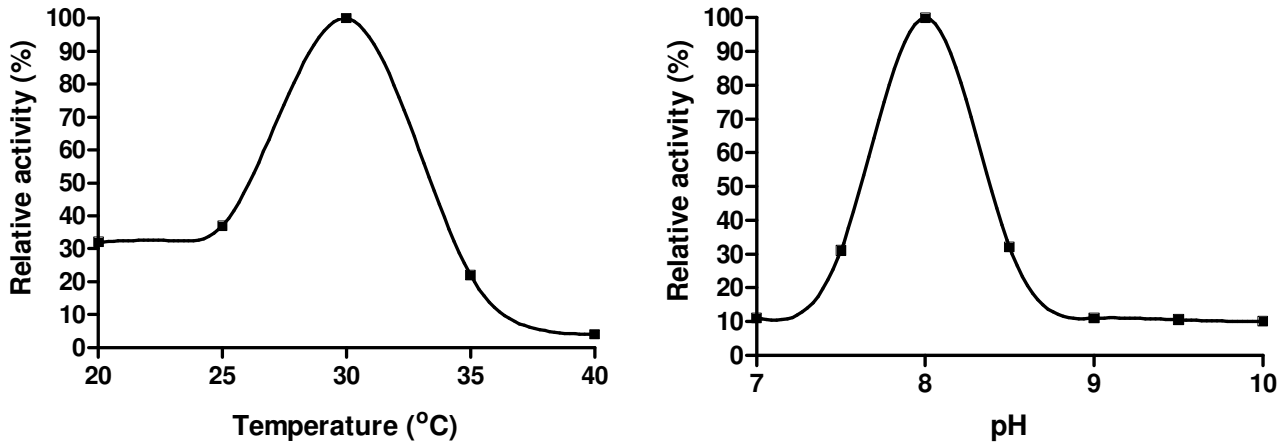

Figure 5. Temperature and pH profiles of the expressed patatin. Error bars indicating standard deviations after performing the experiment in triplicate are covered by the symbols.

Our findings were supported by literature which reported that bacterial PLPs do not show any homology to the known groups of bacterial lipases as evaluated by Blast (Altschul et al., 1997) or PFAM domain search (Banerji and Flieger, 2004), indicating that the amino acid sequences of PLPs and established groups of lipolytic enzymes are not closely related. Secondly, although bacterial PLPs contained the G-X-S-X-G lipase motif which is common even in distinct lipase families, bacterial PLPs do not exhibit the same features as found for other bacterial lipases (Arpigny and Jaeger, 1999). Since the prokaryotic PLPs appear to be more related to their eukaryotic counterparts than to any other group of bacterial lipases, it was proposed that they comprise a new group of bacterial lipolytic enzymes (Banerji and Flieger, 2004).

Although the patatin has only distant identity with its closest relative, the catalytic residues in pNS6 could be identified, but its true substrate is not known. According to the biochemical analysis, pNS6 (patatin) had a low optimum temperature of 30°C and an optimum pH of 8.0 in the same range as most other bacterial esterases; for example the PLP from a Thailand hot spring (Tirawongsaroj et al., 2008) had an optimum pH of 9. pNS6 was not thermally stable (data not shown). It had a preference for short to medium chain length *p*-nitrophenyl esters, displaying the highest activity with pNP-caproate. This was similar to the PLP phospholipase described by Tirawongsaroj et al. (2008) which displayed highest activity with pNP-butyrate and *p*NP-valerate. The patatin proteins do not cluster in any of the known eight classes representative of lipolytic enzymes (Arpigny and Jaeger, 1999) that have been detected in plants, as plant storage glycoproteins (Andrews et al., 1988), and are found in bacteria (Banerji and Flieger, 2004) that catalyse the hydrolysis of a range of phospho and acryl-lipids (Hirschberg et al., 2001; Sharma et al., 2004). According to the studies conducted by Hirschberg et al. (2001), patatin provides an interesting opportunity for application of its catalytic power as a mono-acyl esterase, or esterase synthase at low water activities. In addition, patatin has also been shown to inhibit the growth of corn rootworm in a dose dependent manner when it was fed to insects on an artificial diet, and therefore could be of potential use in the control strategies of corn rootworm (Rydel et al., 2003). Similarly, patatin forms part of the defense mechanisms in potato crops (Barta and Bartova, 2008), as well as having antifungal effect on spore germination of *P. infestans* (Sharma et al., 2004) and can therefore, be applied in the biological control of the pathogen due to its antimicrobial activity.

The unique geochemistry and extreme conditions of certain environments which are often energy or nutrient limiting (Onstott et al., 2006) are selected for specialist populations, which can serve as a source of novel enzymes with unique catalytic capabilities. This study also concludes that an enormous untapped genetic resource can be found in the deep subsurface, especially in South Africa, as illustrated by the detection of two novel esterase activities after a relatively limited screening.

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