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Isolation and biochemical characterization of two lipases from a metagenomic library of China Holstein cow rumen

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ABSTRACT

Two novel lipase genes *RlipE1* and *RlipE2* which encoded 361- and 265-amino acid peptides, respectively, were recovered from a metagenomic library of the rumen microbiota of Chinese Holstein cows. A BLAST search revealed a high similarity (90%) between *RlipE2* and a carboxylesterase from *Thermosinus carboxydivorans* Nor1, while there was a low similarity (below 50%) between *RlipE1* and other lipases. Phylogenetic analysis indicated that RlipE2 clustered with the lipolytic enzymes from family V while RlipE1 clustered with six other putative bacterial lipases which might constitute a new subfamily. The recombinant lipases were thermally unstable and retained 60% activity over a pH range of 6.5–8.5. Substrate specificity assay indicated that both enzymes had higher hydrolytic activity toward laurate (C_{12}), palmitate (C_{16}) and stearate (C_{18}). The novel phylogenetic affiliation and high specificity of both enzymes for long-chain fatty acid make them interesting targets for manipulation of rumen lipid metabolism.

Introduction

Ruminant products make an important contribution to the human diet but have caused concern due to their enriched saturated fatty acid content. Ruminant animals are characterized by a pregastric fermentation system which results in extensive transformations of ingested feedstuffs before they reach the duodenum. Previous studies have identified that dietary lipids undergo extensive hydrolysis and biohydrogenation in the rumen [1]. Lipids are readily hydrolyzed to the extent of 85% with the liberation of free long-chain fatty acids, a high proportion of which are unsaturated [2,3]. The extensive biohydrogenation of dietary unsaturated fatty acids in the rumen results in stearic acid as the major fatty acid entering the duodenum. Both the hydrolysis and biohydrogenation processes are accomplished almost entirely by rumen microbes [4,5]. The composition of the rumen microbial community is phylogenetically complex yet highly host-specific, consisting mainly of archaea, ciliate protozoa, bacteria, and fungi [6–8]. The application of 16S rRNA gene-based methods during the last decades has led us to realize that rumen microbial community is much more diverse and complex than ever suspected [6,9]. However, due to the fact that only a small proportion of ruminal bacteria have been cultured [10], little is still known about the physiological properties and metabolic pathways of rumen microorganisms involved in lipid metabolism. In contrast, direct cloning and sequencing of metagenomic DNA from environmental samples provides access to the genetic and metabolic potential of uncultured microorganisms that contribute to rumen metabolism. This approach, referred as environmental genomics or metagenomics, has been applied to explore new catalytic enzymes from a range of environments [11–15].

To recover genes associated with lipase activity in rumen, we have constructed a bacterial artificial chromosome (BAC) metagenomic libraries from rumen samples collected from Chinese Holstein cows. The BAC metagenomic library consisted of 15,360 clones. Restriction analysis revealed a high level of diversity of the cloned DNA fragments, and the average size of inserts was approximately 54.5 kb [16]. In the present study, two novel lipase genes were identified using a selective screening assay for lipolytic activity from this BAC library, followed by the biochemical characterization of the purified protein.

Materials and methods

Screening of the genomic library. Transformants from the library were screened onto Luria–Bertani agar plate containing 1% (w/v) trioleoylglycerol plus 0.001% Rhodamine B dye. Rhodamine B was dissolved in distilled water and sterilized by filtration. LB agar medium containing 1% arabic gum was autoclaved and cooled to approximately 60 °C. Trioleoylglycerol and Rhodamine B were added and emulsified by mixing with a homogenizer. After the medium was allowed to stand for 10 min at 60 °C and thus reduce foaming, 20 ml of the medium was poured into each Petri dish. Transformed cells were spread on the plate and incubated at

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37 °C for 2 d. Lipase production was monitored by irradiating plates with UV light at 350 nm [17].

Whole BAC sequencing. Two whole BAC clones were entirely sequenced by Beijing Genomic Institute as follows: DNA manipulations were carried out according to standard procedures [18]. Mechanically sheared DNA of the BAC was ligated to pBluescript SK- for shotgun sequencing. DNA sequencing reactions were analyzed using an ABI-3100 automated sequencer (Applied Biosystems, USA). After sequence assemblage, the gap was closed by the method of primer-walking.

Gene annotation and analysis of sequence features. GC composition was studied with the GC Content Grapher (http://plantst.sdsc.edu/plantst/html/geneGC.shtml). Open reading frameworks (ORFs) were characterized using the ORF-finder software at the NCBI (www.ncbi.nlm.nih.gov/gorf/gorf.html). Several criteria were used to define ORFs among all the potential ORFs detected. First, only sequences encoding peptides longer than 50 amino acids, preferentially non-overlapping, were retained. Second, when putative ORFs were detected in different reading frames, we selected those that had known homologs and third, in the case of various putative overlapping ORFs in different reading frames with no known homologs, we selected the ORFs that had the longest sequence. We used the programs PSI-BLAST, TBLASTN and BLASTP to look for sequence similarity in each ORF product with published protein sequences [19]. Predicted proteins were classified according to the Clusters of Orthologous Groups of proteins (COGs) database (www.ncbi.nlm.nih.gov/COG/) [20]. Amino acid alignments were performed with the CLUSTAL W program [21]. Phylogenetic analysis was conducted using the program MEGA [22].

Recombinant expression and protein purification. The ORF of the putative lipase gene was amplified by PCR and inserted into pET-22b(+) expression vector (Novagen, USA). The recombinant plasmids were used for the expression of the lipase genes in *Escherichia coli* strain BL21 (DE3). The transformed cells were grown at 37 °C in 500 ml LB medium containing 25 μg ml^{-1} kanamycin. When the cultures reached an optical density of 0.5 at 600 nm (OD_{600}), they were induced for 4 h with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG).

Cells were harvested by centrifugation at 6000*g* for 10 min and washed with 50 mM Tris–HCl buffer (pH 8.0). The cell pellet was re-suspended in the same buffer, and the cells were then disrupted by sonication. Soluble and insoluble fractions were separated by centrifugation at 15,000*g* for 30 min. The recombinant lipase in the insoluble form was denatured with 6 M urea and then refolded by fractional dialysis in 3, 1.5, and 0 M urea in 50 mM Tris–HCl (pH 8.0). The target protein was purified by loading the crude enzyme fraction on a Ni^{2+}-immobilized Chelating Sepharose Fast Flow (Pharmacia Biotech, Sweden). The eluted solution samples showing lipase activity were pooled and concentrated by ultra-filtration using a YM10 membrane (Millipore, USA).

Lipase activity. Lipase activity was measured spectrophotometrically. The reaction mixture consisted of 0.01 ml of 10 mM *p*-nitrophenyl palmitate (*p*NPP) as a substrate in acetonitrile, 0.04 ml of ethanol, and 0.95 ml of 50 mM Tris–HCl buffer (pH 8.0) containing an appropriate amount (10 μl) of the enzyme. The enzyme reaction was performed for 5 min at 25 °C. The amount of *p*-nitrophenol liberated during the reaction was measured by absorbance at 405 nm. One lipase unit was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute.

Substrate specificity of lipase enzyme was assayed using various *p*-nitrophenyl esters with acyl chains of different lengths (butyrate, C_4; valerate, C_5; caproate, C_6; octanoate (C_8); caprate, C_{10}; palmitate, C_{16}, and stearate, C_{18}) under standard assay conditions. Substrate specificity was also examined by titrating free fatty acids liberated from tributyrin (C_4), tricaprylin (C_8), and triolein ($C_{18:1}$ [*cis*-9]). The assay mixture, containing 0.5 ml of triacylglycerols, 5 ml of acetate buffer (pH 5.6), 10 mM $CaCl_2$, and enzyme (2 μg/ml), was incubated at 30 °C for 30 min with magnetic stirring at 500 rpm. The enzyme reaction was stopped by the addition of 2 ml of ethanol. The amount of fatty acids released during the incubation was determined by titrating the mixture with 50 mM KOH to pH 10.0 using an Orion 960 titrator (Thermo Scientific, USA). One unit of lipase activity was defined as the activity required to release 1 μmol of fatty acids per min under the above conditions.

The optimum temperature and pH for enzyme activity were determined spectrophotometrically with *p*-NPP as a substrate. The assay was performed by incubation of the reaction mixture over a temperature and pH range of 0–70 °C and 4–11, respectively.

Nucleotide sequence accession number. The sequences of Rlip1 and Rlip2 were deposited in GenBank under Accession Nos. FJ529693 and FJ529694.

Results and discussion

Screening and sequence analysis

Screening for lipase activity of the whole BAC library yielded eighteen positive clones. Two clones which showed the highest lipolytic activity (largest halos) were designated as Rlip1 and Rlip2, and were used for further analysis.

The inserts of the two BAC clones were sequenced completely in order to identify the genes encoding lipase activity. The insert sequences of clones Rlip1 and Rlip2 were 25,396 and 10,979 bp, had 50.7% and 57.1% G+C content and contained 23 and 11 predicted ORFs, respectively. Most of the predicted protein-coding genes of Rlip1 and Rlip2 (15 of 23 and 7 of 11, respectively) showed significantly similarity to genes of known function, some (3 of 23 ORFs and 2 of 11 ORFs for Rlip1 and Rlip2, respectively) were conserved hypothetical genes, and other ORFs (5 of 23 ORFs and 2 of 11 ORFs for Rlip1 and Rlip2, respectively) did not show any similarity to sequences in the database. The protein coding regions of Rlip1 were not closely related to those of a known bacterial group. The most closely related homologs found in BLAST search for 23 ORFs from Rlip1 resulted in affiliations with 14 different organisms (Table 1). In contrast, the majority of the coding region (64%) of Rlip2 showed typically $<e^{-11}$ *E*-value to *Thermosinus carboxydivorans* ORF homologs (Table 2).

One of the ORFs from Rlip1, which is designated *RlipE1*, encoded a 361-amino-acid putative lipase. *RlipE1* contains the lipase-conserved catalytic nucleophile Ser244 in the consensus pentapeptide GLSFG, which matches the characteristic Gly-X-Ser-X-Gly motif found in lipolytic enzymes [23]. Similarly, an ORF from Rlip2 which encoded a 225-amino-acid putative lipase was named as *RlipE2*. The consensus pentapeptide GLSMG was found in Ser107.

A BLAST search of GenBank revealed a relatively low similarity (below 50%) between *RlipE1* and other bacterial esterases/lipases, including an esterase (EDY81893 from *Verrucomicrobiae bacterium* DG1235 (48%), a lipase (ZP_02066438) from *Bacteroides ovatus* (41%), a glycoside hydrolase (YP_001297384) from *Bacteroides vulgatus* ATCC 8482 (43%), an esterase (ZP_02737217) from *Gemmata obscuriglobus* UQM 2246 (42%), an esterase (YP_592320) from *Acidobacteria bacterium* Ellin345 (41%) and a acetyl xylan esterase (CAJ19122) from an unidentified microorganism (38%). None of these bacterial lipases have been characterized. Arpigny and Jaeger [23] previously reported the extensive classification of bacterial lipolytic enzymes based on a comparison of their amino acid sequences. To classify the phylogenetic position of RlipE1, we selected 50 bacterial lipolytic enzymes representing eight different families and constructed a phylogenetic tree. As shown in Fig. 1, RlipE1 and six other putative bacterial lipases clustered together

Table 1
Predicted protein-coding genes, and remarkable features in the rumen BAC clone Rlip1. ORFs are numbered 1–23 following the sense 5′–3′.

ORF	Protein size (aa)	Putative function (COG Accession Number if available)	Most similar homolog (*E*-value)	Apparent phylogenetic affiliation	Identity (overlapped aa)
1	126	Aerotolerance-related membrane protein	*F. bacterium HTCC2170* ZP_01105872.1 (3e−24)	Bacterium	54/124 (43%)
2	201	Aerotolerance-related membrane protein	*F. bacterium HTCC2170* ZP_01105872.1 (6e−36)	Bacterium	74/159 (46%)
3	332	Aerotolerance protein BatA	*P. distasonis ATCC 8503* YP_001302338.1 (4e−100)	Bacterium	188/326 (57%)
4	341	Putative membrane exported protein	*B. fragilis NCTC 9343* YP_212126.1 (9e−31)	Bacterium	94/332 (28%)
5	292	Hypothetical protein BACUNI_01985	*B. uniformis ATCC 8492* ZP_02070564.1(4e−104)	Bacterium	185/288 (64%)
6	330	Magnesium chelatase subunit I COG1239	*P. distasonis ATCC 8503* YP_001302335.1 (7e−135)	Bacterium	241/326 (73%)
7	206	Aspartate kinase COG0527	*F. psychrophilum JIP02/86* YP_001295233.1 (5e−31)	Bacterium	83/203 (40%)
8	60	Unknown	None		
9	97	Aspartate kinase	*F. johnsoniae UW101* YP_001192889.1 (8e−11)	Bacterium	36/93 (38%)
10	1089	Proline dehydrogenase, alpha subunit	*P. distasonis ATCC 8503* YP_001302410.1 (0.0)	Bacterium	684/1092 (62%)
11	51	Unknown	None		
12	472	ATPase COG1672	*P. phaeoclathratiforme BU-1* YP_002019336.1(8e−103)	Bacterium	203/478 (42%)
13	71	Unknown	None		
14	54	Unknown	None		
15	822	Surface antigen BspA-like/ Conserved hypothetical protein	*T. vaginalis G3* XP_001313891.1 (2e−78)	Eukaryota	184/470 (39%)
16	492	TPR-repeat-containing protein	*B. thetaiotaomicron VPI-5482* NP_809153.1 (3e−30)	Bacterium	113/466 (24%)
17	61	Unknown	None		
18	375	ATPase COG1672	*B. intestinalis DSM 17393* ZP_03013366.1 (2e−62)	Bacterium	136/379 (35%)
19	690	Elongation factor G (GTPase) COG0480	*Nitratiruptor sp. SB155-2* YP_001355743.1 (0.0)	Bacterium	384/686 (55%)
20	155	Hypothetical protein Xoryp_05010	*X. oryzae pv. oryzicola BLS256* ZP_02242031.1 (5e−31)	Bacterium	72/160 (45%)
21	401	AAA ATPase COG1373	*B. thetaiotaomicron VPI-5482* NP_811318.1 (2e−109)	Bacterium	203/401 (50%)
22	773	Sialidase COG4409	*P. gingivalis W83* NP_904664.1 (2e−78)	Bacterium	173/415 (41%)
23	361	Lipase	*V. bacterium DG1235* EDY81893.1 (2e−91)	Bacterium	169/348 (48%)

but did not belong to any of the known lipase families. Sequence alignment showed that the motifs at active sites were highly conserved in RlipE1 cluster (Fig. 2A), suggesting that RlipE1 cluster might comprise a new subfamily of bacterial lipolytic enzymes.

In contrast, a relatively high similarity (90%) between RlipE2 and a carboxylesterase (ZP_01667208) from *T. carboxydivorans* Nor1was revealed. However, except for this homolog, the identities between RlipE2 and other esterases/lipases were low (below 50%). Considering that the majority of the most related homologs of Rlip2 coding regions originated from *T. carboxydivorans* Nor1, it was reasonable to assume that Rlip2 represented a genomic segment from a close relative of *T. carboxydivorans*. *Thermosinus carboxydivorans* Nor1 is a novel anaerobic, thermophilic, carbon-monoxide oxidizing, hydrogenogenic bacterium isolated from a hot pool [24]. Phenotypic and phylogenetic studies suggested that strain Nor1 belonged to the *Bacillus–Clostridium* phylum of the Gram-positive bacteria, and could be assigned to a new genus, *Thermosinus* gen. nov. To our knowledge, this is the first evidence to support the existence of a ruminal *Thermosinus* bacterium, while it is also possible that it just attach to the cow's food. On the phylogenetic tree, *RlipE2* clustered with the lipolytic enzymes from family V (Fig. 1). Alignment of *RlipE2* with several representatives of the family V indicate that they share the similar conserved blocks including the GXSXG motif and LIHGF and PTLV sequence conserved around the active-site residues (Fig. 2B).

Biochemical characterization of RlipE1 and RlipE2

To characterize some of the biochemical properties of the RlipE1 and RlipE2 lipases, the genes were heterologously expressed in *E. coli*. The esterase activities monitored using *p*NPP as substrate were detected in both the cell extract and culture supernatant. The His-tagged RlipE1 and RlipE2 protein were purified and represented a 57% and 65% yield from the soluble fraction by a single step of nickel–nitrilotriacetic acid affinity chromatography, respectively (data not shown). The lipase activities of RlipE1 and RlipE2 were found to be 428 and 289 U/mg.

Substrate specificity of both enzymes was initially assayed using various *p*-nitrophenyl esters with acyl chains of different lengths (butyrate, C_4; valerate, C_5; caproate, C_6; octanoate (C_8); caprate, C_{10}; palmitate, C_{16}, and stearate, C_{18}). Under our standard assay conditions, both enzymes showed a narrow chain-length

Table 2
Predicted protein-coding genes, and remarkable features in the rumen BAC clone Rlip2. ORFs are numbered 1–11 following the sense 5′–3′.

ORF	Protein size (aa)	Putative function (COG Accession Number if available)	Most similar homolog (*E*-value)	Apparent phylogenetic affiliation	Identity (overlapped aa)
1	474	SecA, COG0653	*T. carboxydivorans Nor1* ZP_01667322.1(0.0)	Bacterium	332/471 (70%)
2	190	Unknown	None		
3	330	Peptide chain release factor 2 COG1186	*T. carboxydivorans Nor1* ZP_01667321.1 (2e−119)	Bacterium	207/322 (64%)
4	239	Hypothetical protein TcarDRAFT_0551	*T. carboxydivorans Nor1* ZP_01667320.1 (1e−11)	Bacterium	61/226 (26%)
5	681	Hypothetical protein TcarDRAFT_0550	*T. carboxydivorans Nor1* ZP_01667319.1 (3e−154)	Bacterium	304/676 (44%)
6	367	Polysaccharide pyruvyl transferase COG2327	*T. carboxydivorans Nor1* ZP_01667318.1 (2e−114)	Bacterium	219/365 (60%)
7	50	Unknown	None		
8	387	Metallophosphoesterase COG1408	*T. carboxydivorans Nor1* ZP_01666548.1 (6e−54)	Bacterium	132/379 (34%)
9	235	Putative GTPase	*B. fragilis* YCH46(2e−32)	Bacterium	73/133 (54%)
10	225	Lipase COG1647	*T. carboxydivorans Nor1* ZP_01667208.1 (24e−104)	Bacterium	194/215 (90%)
11	60	Unknown	None		

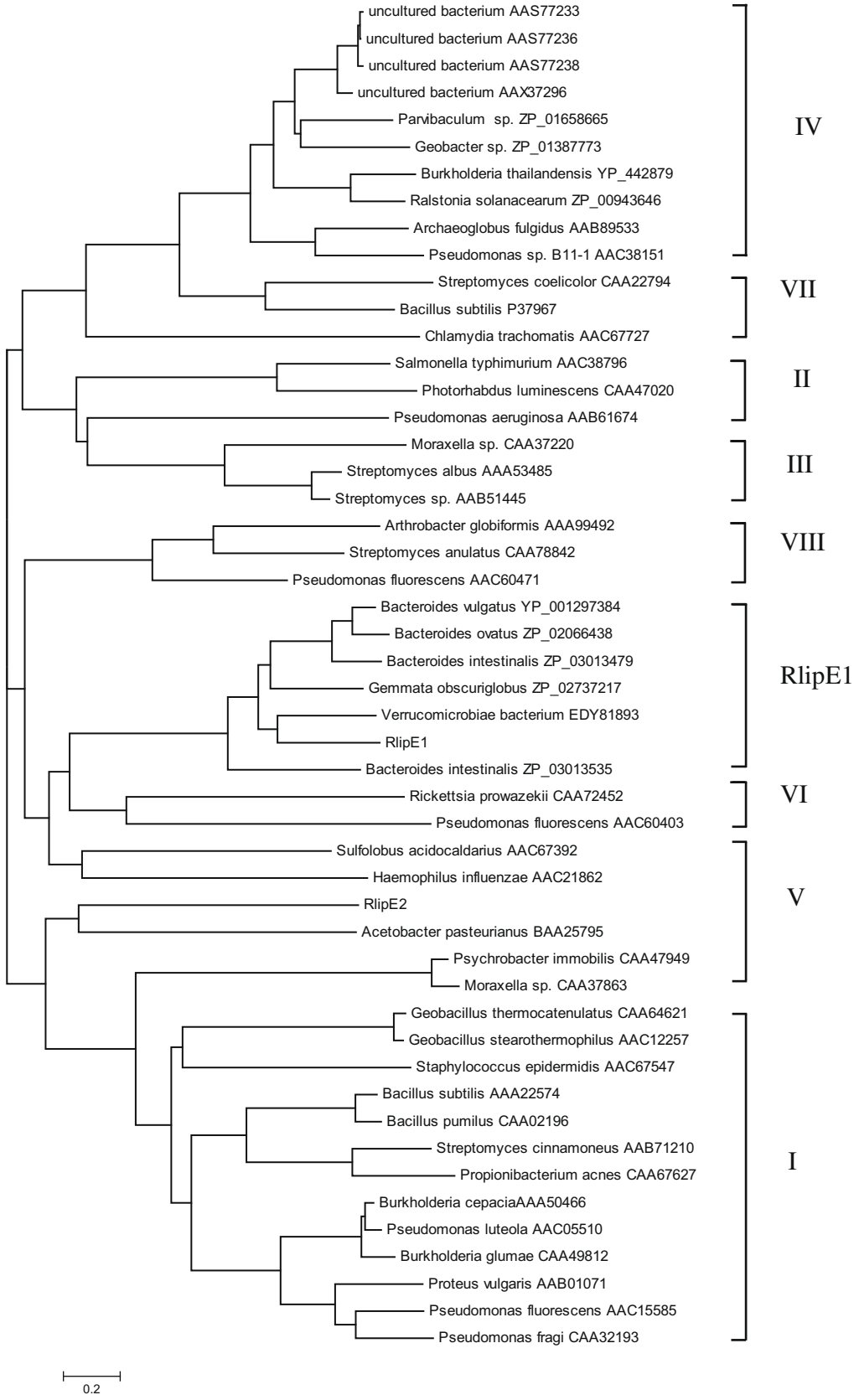

Fig. 1. Phylogenetic analysis of RlipE1 and RlipE2 and closely related proteins. Phylogenetic analysis was conducted using the program MEGA (Version 4.0, [22]). Reference protein sequences were retrieved from GenBank. The units at the bottom of the tree indicate the number of substitution events.

specificity showing a relatively high hydrolytic activity toward laurate (C_{12}), palmitate (C_{16}) and stearate (C_{18}), yet very low activity toward most of the other esters tested (Table 3). The specific activity of RlipE1 for *p*-NP-palmitate (C_{16}) was about seven times higher than that for *p*-NP- octanoate (C_8). Further titration tests using triacylglycerols, namely, tributyrin (C_4), tricaprylin (C_8), and triolein

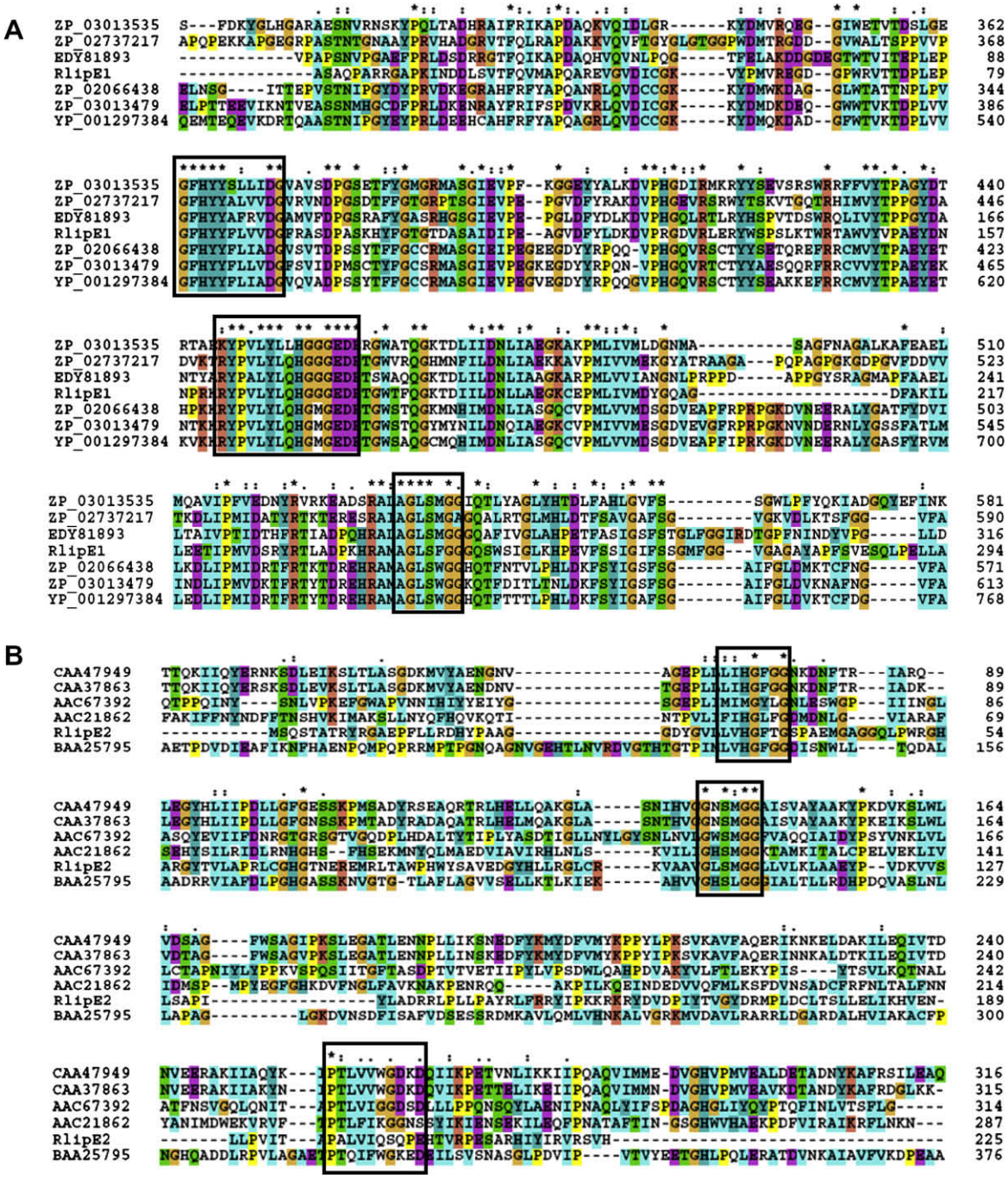

Fig. 2. Conserved blocks in the deduced amino acid sequences of RlipE1 and RlipE2. Sequence alignment was performed by using Clustal W version 1.81. Residues identical to the consensus are shaded. Asterisks indicates amino acid residues belonging to the catalytic triad. (A) RlipE1 and its closely related putative lipolytic enzymes from the following organisms: ZP_03013535, *Bacteroides intestinalis* DSM 17393; ZP_02737217, *G. obscuriglobus* UQM 2246; EDY81893, *Verrucomicrobiae bacterium* DG1235; ZP_02066438, *B. ovatus* ATCC 8483; ZP_03013479, *B. intestinalis* DSM 17393; YP_001297384, *B. vulgatus* ATCC 8482. (B) RlipE2 with representative members of family V from the following organisms: CAA47949, *Psychrobacter immobilis*; CAA37863, *Moraxella sp.*; AAC67392, *Sulfolobus acidocaldarius*; AAC21862, *Haemophilus influenzae* Rd KW20; BAA25795, *Acetobacter pasteurianus*.

($C_{18:1}$ [*cis*-9]) as substrates confirmed the observation that the highest activities for RlipE1 and RlipE2 were for long-chain substrates (Table 3), indicating that these enzymes belong to the lipase class of enzymes rather than to the esterase.

The effect of temperature on lipase activity was determined by using *p*-nitrophenyl caproate as a substrate in the temperature range of 0–70 °C. Both enzymes were active over a wide temperature range, with maximal activity at 30 °C, and then losing almost all their activities above 50 °C (Fig. 3). To examine the thermal stability of these enzymes, enzymes were pre-incubated for 60 min at temperatures ranging from 10 to 70 °C and then residual activity was measured under standard assay conditions. RlipE1 and RlipE2 were stable with the residual activity greater than 80% after incubation at 20 and 30 °C for 60 min. However, both of them were thermally unstable and lost their activities at above 40 °C (data not shown). These results indicate that both RlipE1 and RlipE2 are likely to originate from mesophilic ruminal microorganisms.

The activities of RlipE1 and RlipE2 under buffered conditions over the pH range of 4–11 were measured under standard assay conditions to determine the optimal pH for the lipases. Using *p*-

Table 3
Specific enzyme activities of RlipE1 and RlipE2 on different substrates.

Substrate	Specific activity (U/mg)	
	RlipE1	RlipE2
pNP-acylesters		
Acetate (C_2)	ND	ND
Butyrate (C_4)	ND	ND
Valerate (C_5)	4.3	1.4
Caproate (C_6)	23.5	19.6
Octanoate (C_8)	49.3	56.4
Decanoate (C_{10})	78.1	64.3
Laurate (C_{12})	342.6	328.7
Palmitate (C_{16})	428.4	289.2
Stearate (C_{18})	267.9	186.5
Triglycerides		
Tributyrin (C_4)	0	1.3
Tricaprylin (C_8)	37.3	28.9
Triolein ($C_{18:1}$)	345.7	231.6

ND, not determined.

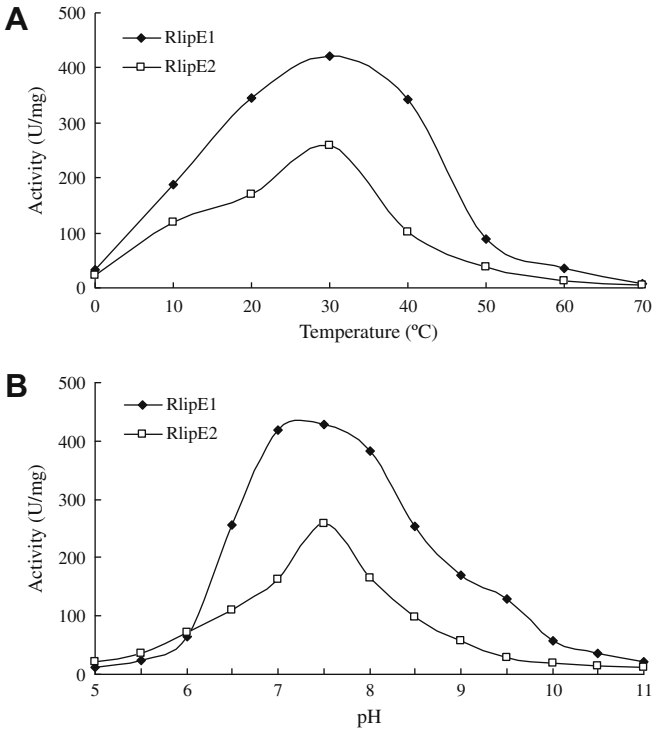

Fig. 3. Influence of temperature and pH on the lipolytic activities of RlipE1 and RlipE2. Enzyme activities were measured at different temperatures (A) and a constant pH of 7.5, or different pH (B) and a constant temperature of 25 °C.

nitrophenyl palmitate as substrate, we found that Rlip1 exhibited at least 60% of its maximal activity in the pH range of 6.5–8.5, with the highest activity at a pH of 7.5. A similar pattern of pH-dependent enzyme activities was also observed for RlipE2 (Fig. 3B).

Conclusion

The metagenome of a rumen microbial community contains an immense pool of genes, most of which are not represented in pure and enrichment cultures due to the selective growth conditions. Metagenomic technology has provided greater access to a wide range of 'uncultured' microorganisms which enabled us to investigate the genes associated with rumen lipid metabolism that are not represented in culture collections. In the present study, two novel lipases, Rlip1 and Rlip2 were identified from the rumen of a Holstein cow by using a metagenomic approach. The novel phylogenetic affiliation of RlipE1 and high specificity of both enzymes for long-chain fatty acid are interesting traits that highlight RlipE1 and RlipE2 as potential targets for manipulating rumen lipid metabolism and supply of lipids to the animal. Future work will establish the contribution of both enzymes to rumen lipid metabolism and thus provide more information on their importance in this gut ecosystem.

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