Bacterial lipolytic enzymes: classification and properties

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Knowledge of bacterial lipolytic enzymes is increasing at a rapid and exciting rate. To obtain an overview of this industrially very important class of enzymes and their characteristics, we have collected and classified the information available from protein and nucleotide databases. Here we propose an updated and extensive classification of bacterial esterases and lipases based mainly on a comparison of their amino acid sequences and some fundamental biological properties. These new insights result in the identification of eight different families with the largest being further divided into six subfamilies. Moreover, the classification enables us to predict (1) important structural features such as residues forming the catalytic site or the presence of disulphide bonds, (2) types of secretion mechanism and requirement for lipase-specific foldases, and (3) the potential relationship to other enzyme families. This work will therefore contribute to a faster identification and to an easier characterization of novel bacterial lipolytic enzymes.

Key words: alignment, carboxylesterase, classification, lipase, structure.

INTRODUCTION

Bacteria produce different classes of lipolytic enzyme, including carboxylesterases (EC 3.1.1.1), which hydrolyse small ester-containing molecules at least partly soluble in water, true lipases (EC 3.1.1.3), which display maximal activity towards water-insoluble long-chain triglycerides, and various types of phospholipase. This paper deals with the former two classes of enzyme. For a description of phospholipases we refer the reader to recent review articles [1,2].

Our knowledge of the structure of lipases and esterases has increased considerably in recent years through the elucidation of many gene sequences and the resolution of numerous crystal structures [3,4]. Efforts accomplished the classification of this large set of data and the identification families and subfamilies of lipolytic enzymes [5,6] (see also the ESTHER database at http://meleze.eusam.inra.fr/cholinesterase). Many attempts have been made to identify sequence motifs conserved in lipolytic enzymes originating from a broad variety of organisms, including higher and lower vertebrates, invertebrates, fungi and bacteria, and to relate them to three-dimensional (3D) structural elements involved in substrate recognition and catalysis, and therefore being essential for the enzyme's function.

The structural superfamily of α/β-hydrolases defined by Ollis et al. [7] comprises a wide variety of enzymes whose activities rely mainly on a catalytic triad usually formed by Ser, His and Asp residues. This triad is functionally (but not structurally) identical with that of trypsin and subtilisin. In the amino acid sequences of α/β-hydrolases the three residues follow the order Ser-Asp-His. The serine residue usually appears in the conserved pentapeptide Gly-Xaa-Ser-Xaa-Gly. α/β-Hydrolases notably include lipolytic enzymes, among which true lipases demand special attention because their peculiar catalytic properties make them very attractive for industrial applications. Their marked preference for water-insoluble substrates and their adsorption on the oil/water interface before hydrolysis involve substantial conformational changes of the enzyme's architecture during catalysis; these have been particularly well documented for lipases of eukaryotic origin [8].

Lipolytic enzymes are currently attracting enormous attention because of their biotechnological potential [9–11]. Most of the lipases used in industry are microbial enzymes, of both fungal and bacterial origin. The great versatility of fungal lipases (from genera such as *Candida*, *Geotrichum*, *Rhizopus* and *Thermomyces*) in biotechnology is illustrated extensively by Gandhi [12], Benjamin and Pandey [13] and Pandey et al. [14]. Among bacterial lipases, attention has usually been focused on particular classes of enzymes such as the lipases from the genus *Pseudomonas*, which are especially interesting for biotechnology [15,16], or esterases possibly involved in bacterial pathogenicity [17]. Unfortunately, information on the relatedness of the numerous bacterial lipases and esterases studied so far is incomplete and scattered in the literature.

Many new bacterial lipolytic enzymes have been studied since the publication of a comprehensive review article in 1994 [18]. However, no attempt has been undertaken to organize this information. Some biochemical properties (such as the dependence of activity on Ca^{2+} ions, pH and temperature) of the best studied families of lipases (from the genera *Bacillus*, *Pseudomonas* and *Staphylococcus*) have been summarized previously [15,16, 18,19]. Usually, lipolytic enzymes are characterized by their ability to catalyse a broad range of reactions. Unfortunately, the wide diversity of methods used for lipase assays (such as the hydrolysis of *p*-nitrophenyl esters, the pH-stat method and the monolayer technique) prevents a direct comparison of results on substrate specificities. In an effort to standardize the measurements, comparative studies have been performed [20,21]. However, only a limited number of bacterial lipases [16] were investigated in these studies.

In the present paper, 53 sequences of bacterial lipases and esterases are compared and classified according to conserved sequence motifs and the biological properties of these enzymes. Relevant information obtained from the 3D structures is also highlighted when available. This work presents an overview of

Abbreviations used: 3D, three-dimensional; HSL, hormone-sensitive lipase; PAF-AH, platelet-activating-factor acetylhydrolase.

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bacterial lipases and esterases currently known and permits the classification of newly isolated lipolytic enzymes, thereby giving a hint about their general characteristics as a starting point to their investigation.

DATA SEARCH AND ANALYSIS

Sequences were retrieved from protein and nucleotide databases by means of the Entrez server at NCBI (http://www.ncbi.nlm.nih.gov/Entrez/), by using the keywords 'bacteria, archaea, lipase, esterase, carboxylesterase'. Sequence similarity searches were performed with the BLAST 2.0 program [22]. Sequence comparison, sorting and alignment were obtained with the help of the Match-Box server [23] and the CLUSTAL W program [24]. The final presentation of results was prepared with the MEGALIGN program from the Lasergene software package (DNASTAR, Madison, WI, U.S.A.).

RESULTS AND DISCUSSION

True lipases

Pseudomonas lipases

Bacterial true lipases were formerly ordered in the so-called *Pseudomonas* groups 1, 2 and 3 because *Pseudomonas* lipases were probably the first to be studied and have a preponderant role in industry. Because some *Pseudomonas* species that produce important lipases have recently been renamed *Burkholderia* [25] and because many lipases originate from various other genera, we propose a revised classification of true lipases on the basis of six subfamilies (Table 1).

The *Burkholderia glumae* lipase was for a long time the only bacterial lipase with a known 3D structure [26], until the publication of the crystal structures of the lipases from *Chromobacterium viscosum* [27] and from *Burkholderia cepacia* [28,29]. All these enzymes belong to family I.2 of true lipases. Very recently the crystal structure of the *Ps. aeruginosa* lipase was solved (D. Lang, K. E. Jaeger and B. W. Dijkstra, unpublished work) providing the first structure in the lipase family I.1.

Since the publication of comparative studies on *Pseudomonas* lipases [15,16], the sequences of lipases from several bacterial genera were reported that are obviously related to families I.1 and I.2 on the basis of amino acid sequence comparison (Table 1 and Figure 1). Lipases from *Vibrio cholerae*, *Acinetobacter calcoaceticus*, *Ps. wisconsinensis* and *Proteus vulgaris* have molecular masses in the range 30–32 kDa and display a higher sequence similarity to the *Ps. aeruginosa* lipase. Enzymes from subfamily I.2 are characterized by a slightly larger size (33 kDa) owing to an insertion in the amino acid sequence forming an anti-parallel double β-strand at the surface of the molecule [26,28]. The *Ps. luteola* lipase possesses this insertion (residues 254–272 in the preprotein) and shows a high similarity to the *Burkholderia* enzymes, notably in this region (Figure 1).

The expression in an active form of lipases belonging to subfamilies I.1 and I.2 depends on a chaperone protein named lipase-specific foldase ('Lif'). However, such specific helper proteins have yet not been described for *Ps. fluorescens* C9, *Ps. fragi*, *Ps. vulgaris* and *Ps. luteola*. Both subfamilies also share important structural features, which are shown in Figure 1. Apart from the residues forming the catalytic triad, two aspartic residues involved in the Ca^{2+}-binding site described in the crystal structures are found at homologous positions in all sequences. Two cysteine residues forming a disulphide bridge are conserved in a majority of sequences. Because the residues involved in the formation of both the Ca^{2+}-binding site and the disulphide bridge are located in the vicinity of the catalytic His and Asp residues, they are believed to be important in the stabilization of the active centre of these enzymes [28]. The two Cys residues of the *Ps. fluorescens* C9 lipase do not lie at equivalent positions and no information is available on the possible existence of a disulphide bridge in this molecule. *Ps. fragi* and *Ps. vulgaris* lipases contain only one Cys residue.

Subfamily I.3 contains enzymes from at least two distinct species: *Ps. fluorescens* and *Serratia marcescens*. These lipases have in common a higher molecular mass than lipases from subfamilies I.1 and I.2 (*Ps. fluorescens*, 50 kDa; *S. marcescens*, 65 kDa) and the absence of an N-terminal signal peptide and of Cys residues. The secretion of these enzymes occurs in one step through a three-component ATP-binding-cassette transporter system [30,31].

Lipases from Gram-positive organisms

The various *Bacillus* lipases known have in common that an alanine residue replaces the first glycine in the conserved pentapeptide: Ala-Xaa-Ser-Xaa-Gly. However, the lipases from the two mesophilic strains *B. subtilis* and *B. pumilus* stand apart because they are the smallest true lipases known (approx. 20 kDa) and share very little sequence similarity (approx. 15 %) with the other *Bacillus* and *Staphylococcus* lipases.

B. thermocatenulatus and *B. stearothermophilus* produce lipases with similar properties. Their molecular mass is approx. 45 kDa and they display maximal activity at approx. pH 9.0 and 65 °C [32,33].

Staphylococcal lipases are larger enzymes (approx. 75 kDa) that are secreted as precursors and cleaved in the extracellular medium by a specific protease, yielding a mature protein of approx. 400 residues. The propeptide (207–267 residues) presumably acts as an intramolecular chaperone and facilitates the translocation of the lipase across the cell membrane [19]. Interestingly, the lipase from *Staphylococcus hyicus* also displays a remarkable phospholipase activity [34], which is unique among true lipases.

Other lipases

The lipases from *Propionibacterium acnes* (339 residues) [35] and from *Streptomyces cinnamoneus* (275 residues) [36] show significant similarity to each other (39 % identity, 50 % similarity). The central region of these proteins (residues 50–150) is approx. 50 % similar to lipases from *B. subtilis* and from subfamily I.2. No similarity was found between the *Strep. cinnamoneus* lipase and other *Streptomyces* lipases known so far.

The GDSL family

The enzymes grouped in family II do not exhibit the conventional pentapeptide Gly-Xaa-Ser-Xaa-Gly but rather display a Gly-Asp-Ser-(Leu) [GDS(L)] motif containing the active-site serine residue (Figure 2). In these proteins this important residue lies much closer to the N-terminus than in other lipolytic enzymes [17]. We included in this family the esterase from *Strep. scabies* because of its significant similarity to the *Aeromonas hydrophila* esterase (30 %). Convincingly, this similarity is not restricted to the vicinity of functionally important residues but is distributed over the entire sequence. As shown by its crystal structure [37], the catalytic centre of *Strep. scabies* esterase has a particular architecture in that it forms a catalytic dyad instead of a triad. The acidic side chain, which usually stabilizes the positive charge

Table 1 Families of lipolytic enzymes

Amino acid sequence similarities were determined with the program MEGALIGN (DNASTAR), with the first member of each family (subfamily) arbitrary set at 100%. Abbreviations: OM, outer membrane; PHA, polyhydroxyalkanoate.

Family	Subfamily	Enzyme-producing strain	Accession no.	Similarity (%)		Properties
				Family	Subfamily	
I	1	*Pseudomonas aeruginosa**	D50587	100		True lipases
		Pseudomonas fluorescens C9	AF031226	95		
		Vibrio cholerae	X16945	57		
		Acinetobacter calcoaceticus	X80800	43		
		Pseudomonas fragi	X14033	40		
		Pseudomonas wisconsinensis	U88907	39		
		Proteus vulgaris	U33845	38		
	2	*Burkholderia glumae**	X70354	35	100	
		*Chromobacterium viscosum**	Q05489	35	100	
		*Burkholderia cepacia**	M58494	33	78	
		Pseudomonas luteola	AF050153	33	77	
	3	*Pseudomonas fluorescens SIK W1*	D11455	14	100	
		Serratia marcescens	D13253	15	51	
	4	*Bacillus subtilis*	M74010	16	100	
		Bacillus pumilus	A34992	13	80	
	5	*Bacillus stearothermophilus*	U78785	15	100	
		Bacillus thermocatenulatus	X95309	14	94	
		Staphylococcus hyicus	X02844	15	29	Phospholipase
		Staphylococcus aureus	M12715	14	28	
		Staphylococcus epidermidis	AF090142	13	26	
	6	*Propionibacterium acnes*	X99255	14	100	
		Streptomyces cinnamoneus	U80063	14	50	
II (GDSL)		*Aeromonas hydrophila*	P10480	100		Secreted acyltransferase
		*Streptomyces scabies**	M57297	36		Secreted esterase
		Pseudomonas aeruginosa	AF005091	35		OM-bound esterase
		Salmonella typhimurium	AF047014	28		OM-bound esterase
		Photorhabdus luminescens	X66379	28		Secreted esterase
III		*Streptomyces exfoliatus**	M86351	100		Extracellular lipase
		Streptomyces albus	U03114	82		Extracellular lipase
		Moraxella sp.	X53053	33		Extracellular esterase 1
IV (HSL)		*Alicyclobacillus acidocaldarius*	X62835	100		Esterase
		Pseudomonas sp. B11-1	AF034088	54		Lipase
		Archaeoglobus fulgidus	AE000985	48		Carboxylesterase
		Alcaligenes eutrophus	L36817	40		Putative lipase
		Escherichia coli	AE000153	36		Carboxylesterase
		Moraxella sp.	X53868	25		Extracellular esterase 2
V		*Pseudomonas oleovorans*	M58445	100		PHA-depolymerase
		Haemophilus influenzae	U32704	41		Putative esterase
		Psychrobacter immobilis	X67712	34		Extracellular esterase
		Moraxella sp.	X53869	34		Extracellular esterase 3
		Sulfolobus acidocaldarius	AF071233	32		Esterase
		Acetobacter pasteurianus	AB013096	20		Esterase
VI		*Synechocystis sp.*	D90904	100		Carboxylesterases
		Spirulina platensis	S70419	50		
		*Pseudomonas fluorescens**	S79600	24		
		Rickettsia prowazekii	Y11778	20		
		Chlamydia trachomatis	AE001287	16		
VII		*Arthrobacter oxydans*	Q01470	100		Carbamate hydrolase
		Bacillus subtilis	P37967	48		*p-Nitrobenzyl esterase*
		Streptomyces coelicolor	CAA22794	45		Putative carboxylesterase
VIII		*Arthrobacter globiformis*	AAA99492	100		Stereoselective esterase
		Streptomyces chrysomallus	CAA78842	43		Cell-bound esterase
		Pseudomonas fluorescens SIK W1	AAC60471	40		Esterase III

* Lipolytic enzyme with known 3D structure.

of the active-site histidine residue, is replaced by the backbone carbonyl of the residue located three positions upstream of the histidine itself, namely Trp-315. Interestingly, a second enzyme displaying the GDSL motif, the α1 subunit of the platelet-activating-factor acetylhydrolase (α1PAF-AH) from bovine brain, shows a catalytic triad in which an aspartic residue also lies three positions upstream of the active-site histidine [38]. Both enzymes have an α/β tertiary fold substantially different from that of the α/β-hydrolase family and share conserved sequence blocks with at least four other bacterial esterases, as shown in Figure 2.

For the *Aeromonas hydrophila* esterase, Brumlik and Buckley

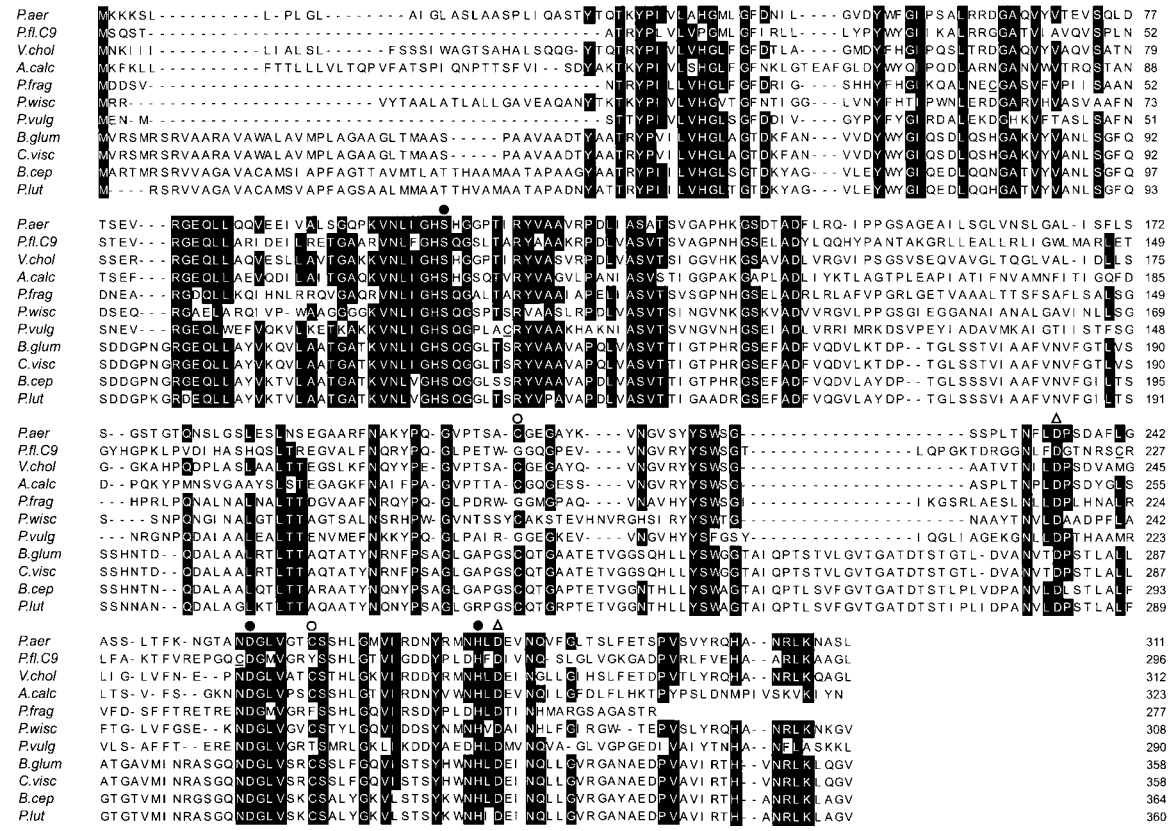

Figure 1 Alignment of amino acid sequences of true lipases from subfamilies I.1 and I.2 (see Table 1)

Symbols: ●, amino acid residues belonging to the catalytic triad; ○, cysteine residues forming the disulphide bridge; △, aspartic residues involved in the Ca^{2+}-binding site. Cysteine residues in *Ps. fluorescens* C9, *Ps. fragi* and *Proteus vulgaris* lipases are underlined.

Figure 2 Blocks of sequence conserved in the GDSL esterase family (family II; see Table 1)

Symbols: ●, amino acid residues belonging to the catalytic triad; ○, location of the active-site Asp-116 (position 134 in the precursor) proposed by Brumlik and Buckley [39] for the *Aeromonas hydrophila* enzyme.

[39] proposed that the active-site aspartic residue is Asp-116 located in block III (Figure 2), arguing that the Asp-116 → Asn mutant is completely inactive. However, both the absence of activity of the enzyme and its severely impaired secretion, which was also reported, might be due to the misfolding of the protein in the periplasm and to its subsequent proteolytic degradation without implying that Asp-116 belongs to the catalytic triad. However, it was shown in the above-mentioned bovine α1PAF-AH [38] that a second conserved aspartic residue located three positions upstream from the active histidine (Figure 2) can take part in the active site and that this third acidic residue is not essential for the enzyme's function, as in the *Strep. scabies* esterase [37]. Obviously, more structural information is needed to establish whether these enzymes share a common fold and a common architecture of their catalytic triad (or dyad) in addition to the similarity of their sequences.

Another interesting feature of the GDSL esterases from *Ps. aeruginosa*, *Salmonella typhimurium* and *Photorhabdus luminescens* is an additional C-terminal domain that encompasses approximately one-third of their entire sequence and is similar to that of a newly identified family of autotransporting bacterial virulence factors ([40,41], and S. Wilhelm, J. Tommassen and K.-E. Jaeger, unpublished work). In these proteins the C-terminal domain is presumably folded into approx. 12 amphipathic β-sheets forming an aqueous pore in the outer membrane. The catalytic N-terminal domain transits through this pore and is in some instances released in the extracellular medium by a specific proteolytic process.

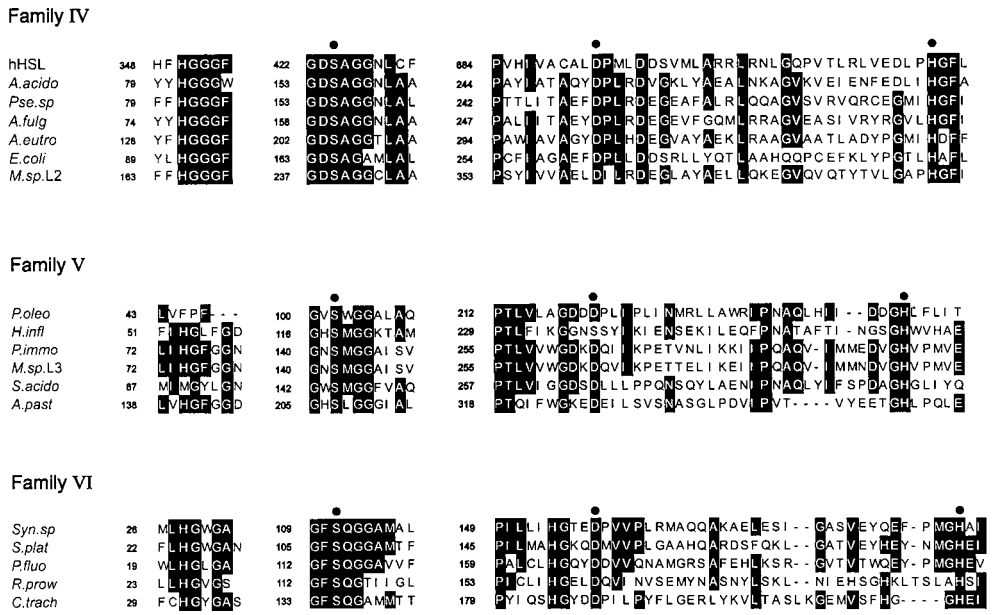

Figure 3 Sequence blocks conserved in families IV, V and VI (see Table 1)

Symbol: ●, amino acid residues belonging to the catalytic triad. Compare the motifs surrounding the active-site serine residue.

Family III

This family of lipases was identified primarily by Cruz et al. [43] and mentioned by Wei et al. [44], who solved the 3D structure of the *Strep. exfoliatus* (M11) lipase. This enzyme displays the canonical fold of α/β-hydrolases and contains a typical catalytic triad. It also shows approx. 20% amino acid sequence identity with the intracellular and plasma isoforms of the human PAF-AH. These PAF-AHs are monomer proteins, in contrast with the heterotrimeric PAF-AH from bovine brain. Their tertiary fold was modelled on the basis of the *Strep. exfoliatus* lipase structure [44]. Their active-site aspartic residue, identified primarily by site-directed mutagenesis, was shown to be located in the sequence at a position non-equivalent to that found in the *Strep. exfoliatus* enzyme, again underlining the great functional versatility of the α/β-hydrolase scaffold.

The hormone-sensitive lipase (HSL) family

A number of bacterial enzymes (family IV) display a striking amino acid sequence similarity to the mammalian HSL [45]. Figure 3 shows sequence blocks that are highly conserved in HSL and six lipolytic enzymes from distantly related prokaryotes. The proposed active-site residues, which were inferred from the three-dimensional model of the human HSL [46], are also highlighted. The mammalian HSL seems to derive from a catalytic domain, homologous with the bacterial enzymes, merged with an additional N-terminal domain and a regulatory module inserted in the central part of the sequence. The relatively high activity at low temperature (less than 15 °C) retained by HSL and the lipase from *Moraxella* sp. [47] was once thought to derive from the conserved sequence motifs of these enzymes [48]. However, the marked sequence similarity (Table 1 and Figure 3) between esterases from psychrophilic (*Moraxella* sp., *Psychrobacter immobilis*), mesophilic (*Escherichia coli*, *Alcaligenes eutrophus*) and thermophilic (*Alicyclobacillus acidocaldarius*, *Archeoglobus fulgidus*) origins indicates that temperature adaptation is not responsible for such an extensive sequence conservation. A comparative study of these enzymes would therefore be very valuable for the determination of the distinctive properties of this family of hydrolases.

Family V

Like proteins in the HSL family, enzymes grouped in family V originate from mesophilic bacteria (*Pseudomonas oleovorans*, *Haemophilus influenzae*, *Acetobacter pasteurianus*) as well as from cold-adapted (*Moraxella* sp., *Psy. immobilis*) or heat-adapted (*Sulfolobus acidocaldarius*) organisms. They share significant amino acid sequence similarity (20–25%) to various bacterial non-lipolytic enzymes, namely epoxide hydrolases, dehalogenases and haloperoxidase, which also possess the typical α/β-hydrolase fold and a catalytic triad [49,50]. On the basis of the crystal structure of the *Xanthobacter autotrophicus* dehalogenase [49], the tertiary fold and the active site residues of the *Psy. immobilis* lipase were predicted by molecular modelling [51]. The sequence patterns conserved around the active-site residues of family V enzymes are presented in Figure 3.

Family VI

With a molecular mass in the range 23–26 kDa, the enzymes presented here are among the smallest esterases known. The 3D structure of the *Ps. fluorescens* carboxylesterase was solved [52]. The active form of this enzyme is a dimer. The subunit has the α/β-hydrolase fold and a classical Ser-Asp-His catalytic triad. This carboxylesterase hydrolyses small substrates with a broad specificity and displays no activity towards long-chain triglycerides [53]. Very little is known about the other enzymes in this family. Their amino acid sequences were derived from whole-genome sequences except that for the *Spirulina platensis* esterase, which was cloned specifically [54]. The enzymes in family VI display approx. 40% sequence similarity to eukaryotic lysophospholipases (Ca^{2+}-independent phospholipases A_2). Their major conserved sequence motifs are shown in Figure 3.

Family VII

A number of rather large bacterial esterases (55 kDa) share significant amino acid sequence homology (30 % identity, 40 % similarity) with eukaryotic acetylcholine esterases and intestine/liver carboxylesterases. The esterase from *Arthrobacter oxydans* is particularly active against phenylcarbamate herbicides by hydrolysing their central carbamate bond [55]. It is plasmid-encoded and is therefore potentially transmissible to other strains or species. The *B. subtilis* esterase was found to efficiently hydrolyse *p*-nitrobenzyl esters. It can therefore be used to advantage in the final removal of the *p*-nitrobenzyl ester used as a protecting group in the synthesis of β-lactam antibiotics [56]. The genome sequencing project of *Strep. coelicolor* revealed a putative open reading frame corresponding to a carboxylesterase; however, this protein has not yet been characterized.

Family VIII

The three enzymes forming this family are approximately 380 residues long and show a striking similarity to several class C β-lactamases. A stretch of 150 residues (from positions 50 to 200) is, notably, 45 % similar to an *Enterobacter cloacae ampC* gene product [57]. This feature suggests that the esterases in family VIII possess an active site more reminiscent of that found in class C β-lactamases, which involves a Ser-Xaa-Xaa-Lys motif conserved in the N-terminal part of both enzyme categories [58,59]. In contrast, Kim et al. [60] proposed that the esterase/lipase consensus sequence Gly-Xaa-Ser-Xaa-Gly that appears in the *Ps. fluorescens* esterase would be involved in the active site of the enzyme. However, this motif, which is also present in the *Strep. chrysomallus* esterase, is not conserved in the *Arthrobacter globiformis* enzyme. Moreover, the motif lies near the C-terminus of the *Ps. fluorescens* and *Strep. chrysomallus* enzymes and no histidine residue follows it in the sequence. This implies that the order of the catalytic residues in the sequence (Ser-Asp-His) that is conserved throughout the entire superfamily of lipases and esterases would not be respected in this case. Obviously, more structural information is needed to describe unambiguously the catalytic mechanism of the family VIII esterases.

Conclusions

Despite a highly conserved tertiary fold and obvious sequence similarities, lipolytic enzymes display a wide diversity of properties and of relatedness to other protein families. In an attempt to help the microbiologist confronted with a new bacterial lipolytic enzyme, we have tried to distinguish between subgroups in this large family and to summarize the current knowledge available for each group.

By consulting the protein and gene databases and using the keywords 'lipase, esterase, carboxylesterase' combined with 'bacteria, archaea' we found 217 entries, of which many turned out to be redundant, corrected or closely related sequences. We therefore restricted our analysis to the 53 sequences listed in Table 1. We are aware that some relevant sequences might have been overlooked in this procedure and that many others will appear, especially from the continuing genome-sequencing projects. Nevertheless we hope that this work will serve as a basis for a more complete and evolving classification of bacterial lipolytic enzymes as more structural and kinetic information becomes available.

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