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Review

Family I.3 lipase: bacterial lipases secreted by the type I secretion system

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Abstract. Based on the classification of bacterial lipolytic enzymes, family I.3 lipase is a member of the large group of Gram-negative bacterial true lipases. This lipase family is distinguished from other families not only by the amino acid sequence, but also by the secretion mechanism. Lipases of family I.3 are secreted via the well-known type I secretion system. Like most of proteins secreted via this system, family I.3 lipases are composed of two domains with distinct yet related functions. Recent years have seen an increasing amount of research on this lipase family, in terms of isolation, secretion mechanism, as well as biochemical and biophysical studies. This review describes our current knowledge on the structure-function relationships of family I.3 lipase, with an emphasis on its secretion mechanism.

Keywords. Family I.3 lipase, type I secretion system, β-roll, Ca^{2+} binding, *Pseudomonas*, secretion signal, protein folding.

Introduction

Lipase (triacylglycerol acylhydrolase, EC. 3.1.1.3) is an important class of enzymes with numerous applications in the rapidly growing biotechnology and biomedical fields [1–8]. Lipase hydrolyzes the carboxyl ester bonds in mono-, di- and tri-glycerides to liberate fatty acids and alcohols in aqueous solutions. In micro-aqueous environments, however, lipase possesses the ability to carry out various reactions, such as esterification, alcoholysis, aminolysis, or trans-esterification. Lipase has high regio- and/or enantio-selectivity and, thus, with this unique property, has been widely employed for the production of pure chiral compounds.

All lipases are members of the α/β hydrolase fold superfamily, one of the largest groups of structurally related yet functionally diverse enzymes [9, 10]. Activities of this superfamily of enzymes rely on the presence of a nucleophile-His-acid catalytic triad, which is usually formed by Ser, His, and Asp residues in lipases. The serine residue is usually located within a typical pentapeptide GxSxG sequence motif, located near the center of the enzyme [11]. However, there have been several reports on lipases with a GSDL motif containing the active-site serine residue with relatively distinct properties [12, 13]. This type of lipase might possess a different type of fold, namely the $\alpha/\beta/\alpha$ fold, which is rather different from the α/β fold [14]. Lipases prefer hydrophobic long-chain triacylglycerols as substrates and are inactive under aqueous conditions. Lipases become active as a result of conformational changes upon contact with a water-insoluble substrate at a concentration close to the substrate's solubility limit, a phenomenon known as interfacial activation [1]. Analyses of (3D) three-dimensional-structures of substrate-free [15–17] and substrate- or inhibitor-bound [18–21] forms of lipases reveal that lipase activation results from the

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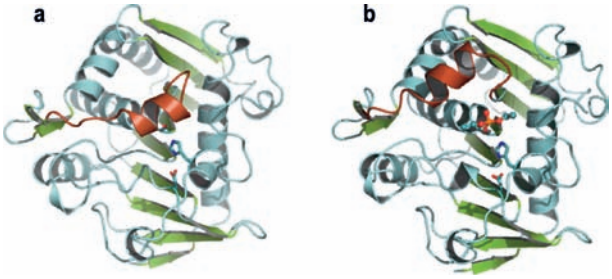

Figure 1. Cartoon representation of the closed (*a*) and open (*b*) conformation of a lipase from *Rhizomucor miehei*, a representative of the α/β hydrolase enzyme (PDB ID 3TGL and 4TGL, respectively), drawn using the program PyMOL (http://pymol.sourceforge.net/). In the closed conformation (*a*), the 17-residue-long lid structure (shown in brown), which consists of amino acid residues 82–96, initially covers the active site that is composed of Ser^{144}, Asp^{233} and His^{257} (shown in stick model). Upon binding of the inhibitor (shown in ball-and-stick model) to the active site Ser^{144}, the lid is moved away from the active site, exposing the active site to the solvent (*b*).

opening of a lid or flap, which initially covers the active site and thus prevents the substrate accessing the active site under aqueous conditions. Figure 1 shows the structure of *Rhizomucor miehei* lipase in a closed and open conformation, depicting the movement of the lid structure.

Almost all of the commonly used lipases are extracellular microbial enzymes, either of fungal or bacterial origin [5]. Although fungal lipases are very useful, bacterial lipases are indispensable research and practical tools. Their ease of production and genetic manipulation, combined with some novel additional features [22], render them versatile in both biotechnology and biomedical applications. A tremendous amount of research has led to some excellent reviews on many of aspects of bacterial lipases [8, 23–26]

Arpigny and Jaeger [23] were the first to classify bacterial lipolytic enzymes into eight families (family I–VIII) based on differences in amino acid sequences and biological properties. Among them, family I, the largest group, was further divided into seven subfamilies (I.1–I.7), of which families I.1, I.2, and I.3 are Gram-negative bacterial true lipases [8]. In addition, a new family of bacterial phospholipases, the patatin-like protein, was recently identified. The phospholipase is produced by pathogenic *Pseudomonas aeruginosa* and has been shown to be involved in the pathogenicity of the bacterium [27]. This phospholipase is unique because it is secreted via the type III secretion system, which transports proteins and toxins directly from the cytoplasm of Gram-negative bacteria to the cytoplasm of their host cells [28].

Family I.1 lipases include lipases from *P. aeruginosa*, *P. fragi*, *P. fluorescens* C9, *P. wisconsinensis*, *Vibrio cholerae*, *Acinetobacter calcoaceticus,* and *Proteus vulgaris*. Family I.2 lipases include lipases from *Pseudomonas luteola*, *Burkholderia glumae*, *B. cepacia,* and *Chromobacterium viscosum*. Family I.1 and I.2 lipases share relatively high amino acid sequence similarities (30–40%) and are secreted via the type II secretion system (T2SS), also termed the general secretory pathway (GSP) [23]. Secretion by this system occurs in two steps. First, the protein is translocated through the inner membrane of Gram-negative bacteria by a Sec-dependent pathway in an unfolded state or a Tat-dependent pathway in a folded state. Both pathways recognize an N-terminal signal sequence of the passenger proteins [29–32]. Proteins, in particular lipases, transported via the Gram-negative bacterial Sec pathway are folded in the periplasmic space and often require the assistance of various molecular chaperones [33–36]. After folding and cleavage of the N-terminal signal peptide in the periplasmic space, the protein is then further secreted to the extracellular medium via the secreton transporter complex.

Family I.3 lipases show poor amino acid sequence similarities (< 20%) to either family I.1 or family I.2 lipases. In contrast to family I.1 and I.2 lipases, family I.3 lipases are secreted via the type I secretion system (T1SS) [23, 37]. Secretion by this system occurs in a single step, directly from the cytoplasm to the extracellular medium without any periplasmic intermediate. The proteins secreted by this system usually have a C-terminal secretion signal that remains intact after secretion, and are usually partially unfolded in the cell and are folded in the extracellular medium [38]. Unlike the well-studied family I.1 and I.2 lipases, there is relatively less information on structures and functions of family I.3 lipases. Recently, however, the literature describing the isolation and molecular studies of this lipase family has been growing. This review will summarize recent findings on family I.3 lipases learned from various biochemical and biophysical studies, with emphasis on their secretion mechanism and functionality.

Type I secretion system

The type I secretion system (T1SS) is one of the mechanisms used by Gram-negative bacteria to secrete proteins to the external medium. Secretion occurs in a single, energy-coupled step via an exporter complex that spans the inner and outer membranes (Fig. 2). The relatively simple exporter complex consists of only three protein subunits: an inner membrane-bound ATP-binding cassette (ABC) protein that forms a complex with a membrane fusion protein (MFP) in the periplasmic space, and an outer membrane protein (OMP) that resides in the periplasmic space and is embedded in the outer membrane [38–40]. T1SS is best represented by the *Escherichia coli* hemolysin (Hly) secretion system that consists of HlyB as ABC protein, HlyD as MFP, and TolC as OMP [38].

The ABC protein subunit of the T1SS consists of two transmembrane domains (TMDs) that are embedded in

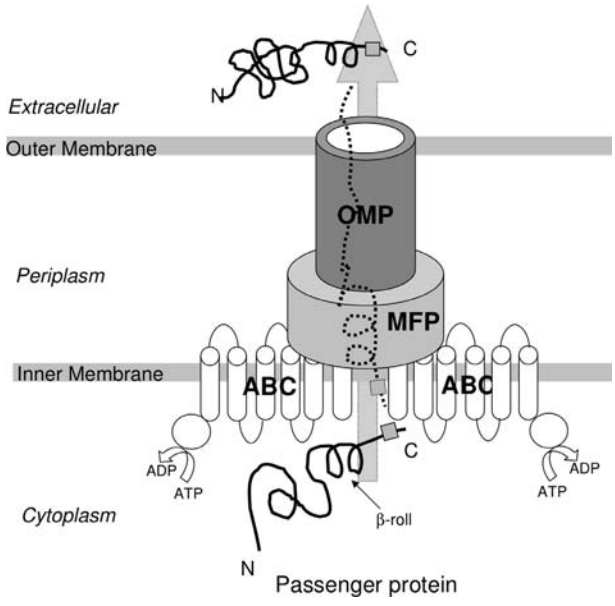

Figure 2. A model of bacterial T1SS. ABC protein (white), membrane fusion protein (MFP; light gray), and outer membrane protein (OMP; dark gray) form a transporter complex protruding through the inner and outer membranes of Gram-negative bacteria [40]. The secretion signal is shown by a gray box near the C-terminal end of the passenger protein [112]. Protein secretion occurs in a single step, bypassing the periplasmic space directly to the extracellular medium (the direction of protein secretion is shown by a large shaded arrow). ATP hydrolysis by the ATPase domain of ABC protein provides energy for protein transport. The C-terminal secretion signal is recognized by the ABC protein, stimulating conformational change of the transporter complex and ATP hydrolysis, which leads to secretion of the passenger protein [40]. A β-roll structure, formed by the repetitive sequences in the presence of Ca^{2+}, is also shown.

the inner membrane, and two nucleotide-binding domains (NBDs) that are exposed to the cytoplasm [41]. The NBD has ATPase activity and provides the energy needed for protein secretion [41–43]. The ABC protein of the T1SS belongs to the well-characterized ABC protein superfamily, the largest protein superfamily found in all kingdoms of life, which is related to the import or export of various molecules, from small ions to polysaccharides and proteins [44].

Relatively little information is available on the MFP of the T1SS, but based on the studies on MexA, an MFP of the multidrug transporter in *P. aeruginosa*, the MFP is known to be located in the periplasm and anchors the inner membrane via fatty acids that are covalently bound to its N-terminal cysteine residue. It is also assumed that the MFP links the ABC protein with the OMP, and that the MFP-OMP interaction provides a tight seal, completely separating the interior of the exporter complex from the periplasm [45, 46]. Furthermore, secondary-structure prediction of HlyD, the MFP of the *E. coli* hemolysin T1SS, showed that this protein has a possible two-domain organization of the periplasmic domain, one that is largely helical and followed by a β strand domain at the C terminus [47]. A recent report suggested that the MFP also plays a role in the folding of the passenger proteins, as several mutations in *E. coli* HlyD have been identified to affect the folding of the secreted *E. coli* HlyA [48].

The OMP of the T1SS is best represented by *E. coli* TolC. TolC is responsible for HlyA secretion and also for multidrug transport in *E. coli* [40]. Determination of its crystal structure reveals that *E. coli* TolC forms a homotrimer with an outer-membrane-embedded β barrel and a long α-helical barrel projecting across the periplasmic space [49]. *E. coli* TolC is a relatively large membrane protein, measuring approximately 140 Å in length and 30 Å in diameter. This protein has been well characterized, and an excellent review on its structure and function is available [50].

ABC protein, in particular its NBD, is responsible for recognition of the passenger protein by the T1SS [51]. Furthermore, a specific interaction between the MFP and OMP of the same or closely related origin is required to form an active exporter, as shown by studies on hybrid T1SSs [52, 53]. In contrast to other types of protein secretion system in Gram-negative bacteria, secretion via the T1SS usually does not require the presence of any molecular chaperone, such as *E. coli* SecB or GroEL/ES [54].

In most cases, the genes encoding the T1SS subunits form an operon and are clustered adjacently with the gene encoding the passenger protein [55–59], however, this is not always the case. For example, the gene encoding the OMP (TolC) of the *E. coli* hemolysin T1SS is not linked to any export operon, but exists as part of the mar-sox regulon [38].

Passenger proteins of the T1SS

The passenger proteins (also called allocrits) of the T1SS are usually large, monomeric proteins. They include RTX (Repeat in Toxin) toxins, such as *E. coli* HlyA [60, 61], *Pasteurella haemolytica* leukotoxin [62] and *Bordetella pertussis* adenylate cyclase [63], proteases from *Erwinia chrysanthemi* [55], *P. aeruginosa* [64], and *Serratia marcescens* [65], *S. marcescens* heme-binding protein (HasA) [56], *S. marcescens* S-layer protein [66], and family I.3 lipases (see below). These proteins, except for *S. marcescens* HasA, have a C-terminal secretion signal, which remains intact after secretion [40, 60, 67]. This signal is located within the last 60 residues from the C terminus. Furthermore, a nine-residue sequence motif GGxGxDxux (x: any amino acid; u: hydrophobic amino acid) is repeated several times at the upstream region of the C-terminal secretion signal. This sequence motif is also known as the RTX-signature motif, glycine/aspartate-rich repeat region, or hemolysin-type calcium-binding region. These repeti-

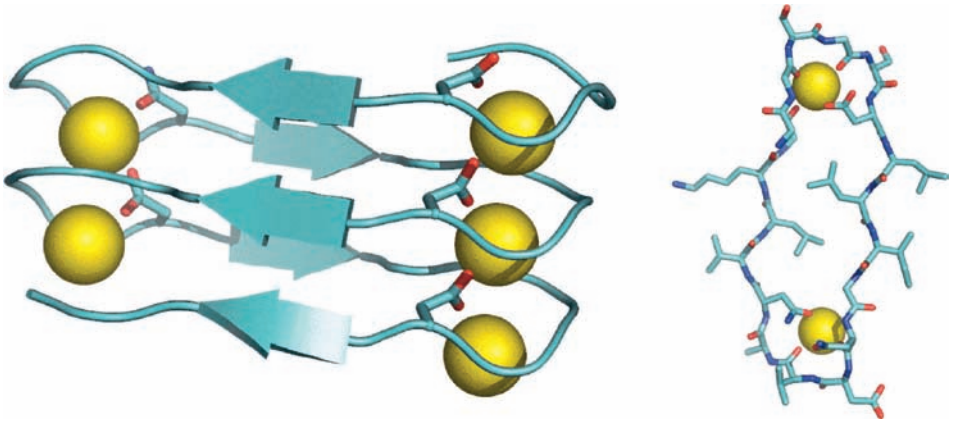

Figure 3. (*a*). Side view of a β-roll structure. The β-roll structure formed by residues 333–379 of *P. aeruginosa* alkaline protease (PDB ID 1AKL) is drawn by the program PYMOL. The β strands and internally bound Ca^{2+} ions are represented by arrows and yellow spheres, respectively. The side chains of the aspartate and asparagine residues, each of which provides two side-chain oxygen atom or one oxygen and one nitrogen atom for Ca^{2+} binding, are shown in a stick model. (*b*) Top view of a β-roll structure. The first turn of the β-roll structure shown in *a* is shown in a stick model.

tive sequences are responsible for Ca^{2+} binding of some passenger proteins [68, 69], and are known to form a β-roll motif (see below for more discussion). The repeats are required to a certain degree for efficient protein secretion, by providing a spacer so that the secretion signal is exposed and can be recognized by the T1SS [70–72], and by conferring intracellular stability against proteolytic degradation to the passenger protein [73, 74]. In contrast to these passenger proteins, *S. marcescens* HasA does not contain any repetitive sequences [75, 76]. Furthermore, unlike other passenger proteins with the repetitive sequences, *S. marcescens* HasA requires a cytoplasmic chaperone, SecB, which plays an important role for Sec-dependent protein secretion, for its efficient secretion [77, 78].

'Natural' passenger proteins of the T1SS do not usually contain any cysteine residue, or in some cases, contain only one cysteine residue, indicating that these proteins do not have any intramolecular disulfide bond. A study on secretion of hybrid eukaryotic proteins fused with C domain of *E. chrysanthemi* protease showed that *E. chrysanthemi* T1SS can only secrete proteins that do not have any intramolecular disulfide bond [79]. Another report, however, showed that *E. coli* alkaline phosphatase, a dimeric periplasmic protein with two intramolecular disulfide bonds (four cysteine residues in one monomer), can be secreted extracellularly when it is fused with the C-terminal secretion signal of α-hemolysin [80]. Thus, the lack of intramolecular disulfide bonds of passenger proteins of the T1SS is not correlated to the secretion competency of these proteins. Proteins with multiple disulfide bonds, including *E. coli* alkaline phosphatase, that are secreted via the Sec-dependent pathway of Gram-negative bacteria are unfolded in the cytoplasm, and their periplasmic folding often requires the assistance of molecular chaperones, which not only function to catalyze the formation of disulfide bonds, but also to shuffle the incorrectly formed disulfide bonds [33, 81]. Therefore, *E. coli* alkaline phosphatase may not fold correctly when it is secreted to the chaperone-free extracellular medium via the T1SS. In fact, the work in our laboratory has shown that when *E. coli* alkaline phosphatase with a C-terminal secretion tag is secreted directly to the extracellular medium via the T1SS, treatment with glutathione redox buffer is required to increase its enzymatic activity to the level similar to that of the wild-type enzyme [82].

β-Roll motif

The β-roll motif, which was first identified in the crystal structure of *P. aeruginosa* alkaline protease [83], is built of a succession of the GGxGxDxux sequence, with the first six residues forming two half-sites for Ca^{2+} binding and the last three forming a short β strand (Fig. 3). Other metal ions such as Mg^{2+} or Tb^{2+} cannot induce the formation of this motif, indicating that Ca^{2+} specifically induces this conformation [84]. It is worth noting though, that Sr^{2+} and Ba^{2+} can bind to *E. coli* hemolysin and restore its hemolytic activity, albeit at lower affinities than Ca^{2+} [69]. The tightly bound, internal Ca^{2+} ions are proposed to 'lock' the structure together. As shown in Figure 3b, the binding sites of Ca^{2+} are provided by the main-chain carbonyl oxygen atoms of the glycine residues and the side-chain carboxyl oxygen atoms of the aspartate residues or the carboxamidyl oxygen and nitrogen atoms of the asparagine residues. Each aspartate or asparagine residue coordinates with two Ca^{2+} ions through two oxygen atoms or one oxygen and one nitrogen atom, respectively. As a result, a β-roll structure is stabilized by an Asp (Asn)-Ca^{2+}-Asp (Asn)-Ca^{2+}-Asp (Asn) coordination (Figure 3a). In fact, mutation of the aspartate residues, which are located in the central region of this motif, to

Ala abolishes the ability of the repeats to bind Ca^{2+} and thereby to form a β-roll structure [74].

Amino acid sequences of family I.3 lipases

Family I.1 and I.2 lipases are produced by a relatively large variety of bacterial species [23], while so far only two genera of bacteria, *Pseudomonas* and *Serratia*, have been reported to produce family I.3 lipase. First studies on this lipase family were conducted on lipases from *P. fluorescens* LS107d2 [85], *P. fluorescens* B52 [86], *S. marcescens* Sr41 [67], *P. fluorescens* SIK W1 [59, 87] and *S. marcescens* SM6 [88]. *S. marcescens* SM6 lipase shares a high amino acid sequence similarity (97%) to *S. marcescens* Sr41 lipase. *P. fluorescens* B52 was later reported to produce another lipase, which is also a family I.3 lipase [89]. In this review, this latter lipase will be designated as *P. fluorescens* B52 (LipB52) lipase to distinguish it from the first *P. fluorescens* B52 lipase identified. Reports on *P. fluorescens* SIK W1 lipase are rather confusing since the deduced amino acid sequence in the first report (GenBank accession no. D11455) [87] differs from that of the later (GenBank accession no. AF083061) [59]. In this review, we use the latter as the correct sequence, since the first sequence contains nine cysteine residues, a feature uncommon in proteins secreted via the T1SS [79].

In addition to the aforementioned strains, *Pseudomonas* sp. KB700A [90] and *P. fluorescens* HU380 [91] have been reported to produce family I.3 lipases. The work in our laboratory has also identified a novel member of this lipase family, produced by *Pseudomonas* sp. MIS38 [92]. This lipase consists of 617 amino acid residues and has a molecular mass of 68 kDa. Amino acid alignment using the CLUSTALW program with BLOSUM similarity matrix [93], shows that *Pseudomonas* sp. MIS38 lipase has amino acid sequence similarities of 95% with *P. fluorescens* HU380 lipase, 92% with *P. fluorescens* B52 (LipB52) lipase, 76% with *P. fluorescens* B52 lipase, 75% with *Pseudomonas* sp. KB700A lipase, 74% with *P. fluorescens* SIK W1 lipase, 72% with *P. fluorescens* LS107d2 lipase, and 61% with *S. marcescens* Sr41 and SM6 lipases.

Family I.1 and I.2 lipases have molecular masses of 30–33 kDa [23]. Compared to these lipases, family I.3 lipases are relatively large, with molecular masses of 50–68 kDa (Table 1). The amino acid sequences of the representative members of family I.3 lipases are shown in Figure 4. Family I.3 lipases have no cysteine residue, indicating that, like other proteins secreted via the T1SS, they do not have a disulfide bond. The active-site residues of family I.3 lipases have been identified by site-directed mutagenesis as Ser^{207}, Asp^{255}, and His^{313} for *Pseudomonas* sp. MIS38 lipase [92, 94]. These residues are fully conserved in the family I.3 lipase sequences, suggesting that these residues form a catalytic triad in this lipase family.

Enzymatic properties of family I.3 lipase

The optimum pH and temperature for activity are available for several family I.3 lipases (Table 1). All of these lipases are produced by mesophillic or psychrotrophic Gram-negative bacteria [67, 85–92]. Interestingly, all of them have optimum temperatures for activity in the mesophilic or thermophilic range (35–55 °C), which is considerably higher than the optimal growth temperatures of their source organisms. In fact, studies on *P. fluorescens* SIK W1 lipase showed that the optimal temperature range for its activity (45–55 °C) [87] is much higher than the optimal growth temperatures of its source organism (20–25 °C) [95] or its optimal secretion temperature by a reconstituted system in *P. fluorescens* SIK W1 or *E. coli* (25 °C) [59, 96]. The optimum pH range for this lipase family is a mildly alkaline pH (pH 7.5–8.5). Three members of this family, *P. fluorescens* SIK W1, *Pseudomonas*

Table 1. Properties of family I.3 lipases.

Producer strain	GenBank accession no.	Molecular mass (kDa)	Optimum pH	Optimum temperature (°C)	Reference
Pseudomonas sp. MIS38	AB025596	68	7.5	55	92
P. fluorescens B52 (LipB52)	AY623009	68	8.0	40	89
S. marcescens Sr41	D13253	65	8.0	45	67, 97
S. marcescens SM6	U11258	65	NA	NA	88
P. fluorescens HU380	AB109033	64	8.5	45	91
P. fluorescens B52	M86350	50	NA	NA	86
Pseudomonas sp. KB700A	AB063391	50	8.0–8.5	35	90
P. fluorescens SIK W1	AF083061	50	8.5	45–55	87, 102
P. fluorescens LS107d2	M74125	50	NA	NA	32

NA, not available.

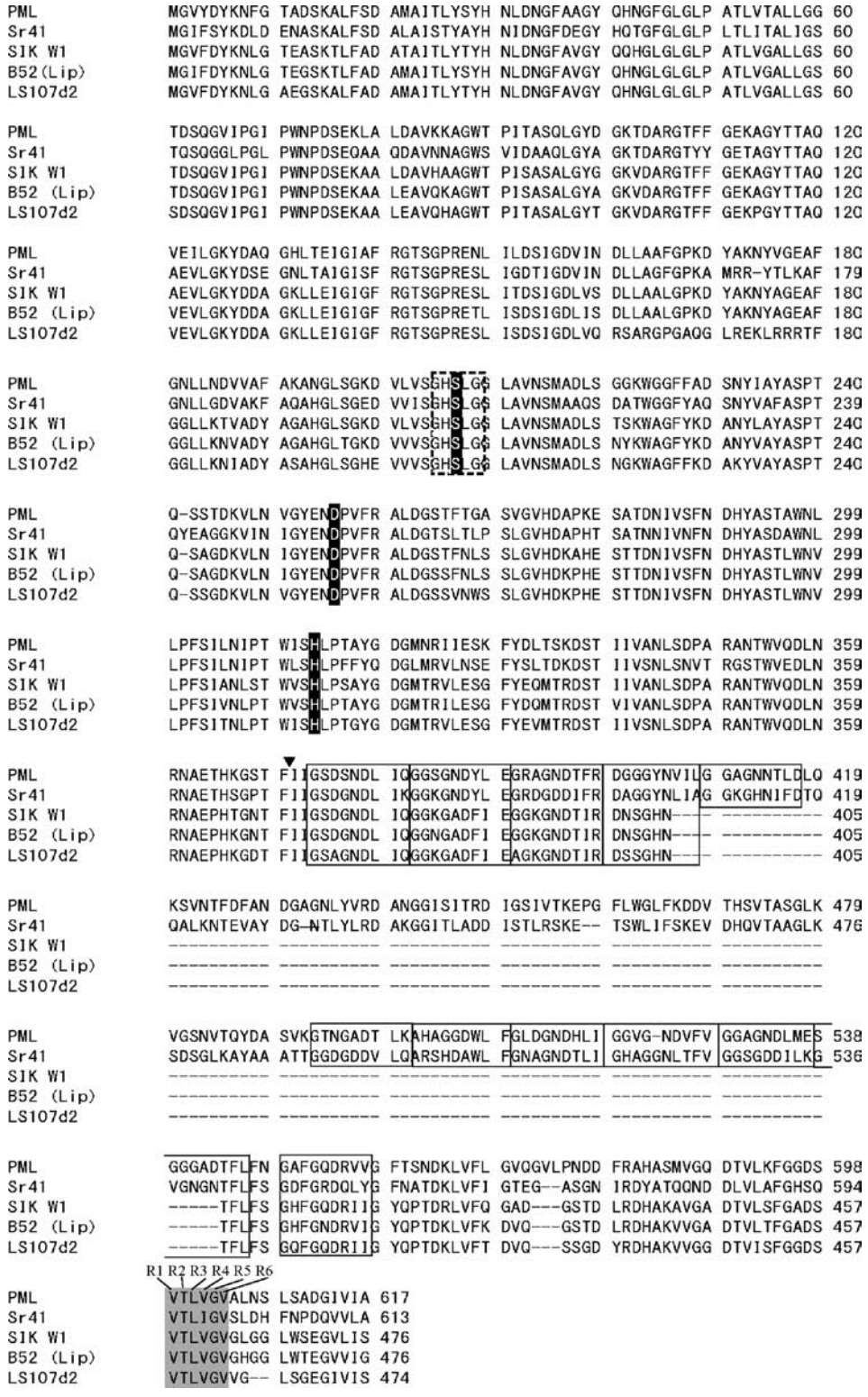

Figure 4. Amino acid sequences of representative members of family I.3 lipase. The amino acid sequences of lipases from *Pseudomonas* sp. MIS38 (PML), *S. marcescens* Sr41 (Sr41), *P. fluorescens* SIK W1 (SIK W1), *P. fluorescens* B52 [B52 (lip)], and *P. fluorescens* LS107d2 (LS107d2) are aligned using the program CLUSTAL W [93]. The consensus GxSxG sequence, containing the active-site serine residue, is boxed by broken lines. The amino acid residues forming a catalytic triad, Ser, Asp, and His, are highlighted in black. The repetitive nine-residue sequence motif, GGxGxDxux, is boxed by solid line. A putative C-terminal secretion signal (R1–R6) is shaded [112]. The cleavage site with limited chymotryptic digestion [92] is shown by a solid arrowhead. Numbers represent the positions of the amino acid residues that start from the initiator methionine residue for each protein. The GenBank accession numbers for these sequences are summarized in Table 1.

sp. MIS38, and *S. marcescens* Sr41 lipases, have been shown to have similar substrate specificities, the highest being toward C_4-C_8 triglycerides [87, 92, 97]. *Pseudomonas* sp. KB700A lipase, however, shows a substrate preference toward C_{10} or longer triglycerides [90]. All lipases of this family are strongly inhibited by EDTA and their activities are highly dependent on Ca^{2+} [87, 90, 92]. Other divalent cations such as Zn^{2+}, Mg^{2+}, Mn^{2+} or Co^{2+} do not significantly restore the activities of EDTA-treated enzymes. Furthermore, the stability of *P. fluorescens* SIK W1 lipase against chemical denaturation has been shown to be increased in the presence of Ca^{2+} [98].

Several members of family I.3 lipase exhibit esterase activities and can hydrolyze fatty acid monoesters [90, 92]. These esterase activities are, however, much less efficient than their lipase activities toward triacylglycerols, suggesting that family I.3 lipases have the properties of true lipases. In fact, *Pseudomonas* sp. MIS38 lipase has been reported to show interfacial activation, as its activity increases sharply at a saturated substrate concentration [92].

Folding of family I.3 lipases without assistance of molecular chaperones

In contrast to family I.1 and I.2 lipases, which are secreted to the periplasmic space and are folded with the assistance of molecular chaperones [33, 34, 99], family I.3 lipases are secreted directly to the extracellular medium in an unfolded or partially folded state, and are folded outside the cell [37–39,70,100]. All the reported molecular chaperones are localized in the cytoplasmic or periplasmic space, and so far no extracellular molecular chaperones have been identified in Gram-negative bacteria [81, 101], suggesting that the folding of family I.3 lipases does not require the assistance of any molecular chaperone. When the genes encoding family I.3 lipases are expressed in a plasmid designed for protein overproduction in *E. coli*, the lipases usually accumulate abundantly in the cells as inclusion bodies [92, 102]. Following chemical denaturation, the solubilized lipases can be efficiently refolded *in vitro* by dialysis against Ca^{2+}-containing buffer [92] or size-exclusion protein refolding chromatography [98]. Following further purifications, the resulting refolded lipases exhibit enzymatic activities [73, 92, 98] and assume secondary or tertiary structures similar to those of the secreted ones [73], indicating that family I.3 lipases can be refolded efficiently *in vitro* without the assistance of any molecular chaperone.

Two distinct domains of family I.3 lipases

Proteins, in particular proteases, which are secreted by the T1SS are proposed to have two domains, as shown by crystal structures of *P. aeruginosa* alkaline protease (PDB ID 1AKL) [83], *S. marcescens* metalloprotease (PDB ID 1SAT) [103], *Pseudomonas* TAC II 18 sp. alkaline protease (PDB ID 1H71) [104], and *E. chrysanthemi* protease C (PDB ID 1KAP) [105]. The active-site residues of these proteases are all located in the N-terminal domains (N domains). The C-terminal domains (C domains) of these proteins contain the secretion signal, and several repeats of the GGxGxDxux sequence motif that form a β-roll motif in the presence of Ca^{2+} [64–67].

Family I.3 lipases have also been proposed to consist of two domains, based on the limited proteolysis of *Pseudomonas* sp. MIS38 lipase [92]. Upon limited proteolysis of this protein with chymotrypsin, two peptides, with molecular masses of 39 and 28 kDa, are generated as the primary products. Determination of the N-terminal amino acid sequences revealed that the 39- and 28-kDa fragments represent the N and C domains generated upon cleavage of the peptide bond between Phe^{370} and Ile^{371}, respectively (Fig. 4). All of the amino acid residues that form a catalytic triad of *Pseudomonas* sp. MIS38 lipase, as well as those of family I.3 lipases, are located in the N domain. The C domain contains 12 repeats of the GGxGxDxux sequence motif and the putative secretion signal. These repeats are not located consecutively, but are interrupted by the insertion of a peptide with 75 amino acid residues between the fifth and sixth repeats. *S. marcescens* Sr41, *S. marcescens* SM6, and *P. fluorescens* B52 (LipB52) lipases also contain 12 repeats which are not located consecutively, but are interrupted by a similar insertion sequence. In contrast, lipases from *P. fluorescens* B52 (B52 lipase), *Pseudomonas* sp. KB700A, *P. fluorescens* SIK W1, and *P. fluorescens* LS107d2 contain only five repeats that are located consecutively.

The T1SS of family I.3 lipase

The genes encoding the T1SS for family I.3 lipases have been cloned from *Pseudomonas* sp. No. 33 [58], *P. fluorescens* SIK W1 [59], and *S. marcescens* Sr41 [106]. In all cases, the three genes that encode the T1SS subunits form an operon and are clustered with the lipase gene [58, 59]. The nomenclature of the genes encoding the T1SS for family I.3 lipase follows that of the genes encoding other T1SSs, using letter order starting from the ABC protein, followed by MFP and OMP. Reconstitution of these T1SSs in *E. coli* has been shown to permit the secretion of family I.3 lipases co-expressed with T1SSs [59, 73, 106]. Likewise, overproduction of these T1SSs in the original bacterial hosts enhances the secretion level of family I.3 lipases present in these hosts [96, 107].

Beside being secreted by their cognate T1SS, family I.3 lipases have been shown to be secreted by heterologous T1SSs, reconstituted in *E. coli* or in their original bac-

terial hosts. *Pseudomonas* sp. MIS38 lipase can be efficiently secreted via the T1SS for *S. marcescens* Sr41 lipase (LipBCD or Lip system) [73]. *P. fluorescens* B52 lipase can be secreted via the T1SS for alkaline protease of the same strain [108]. *S. marcescens* SM6 and Sr41 lipases can be secreted via the T1SS for *E. chrysanthemi* protease (PrtDEF), albeit at a lower efficiency compared to secretion by their cognate T1SSs [52, 86].

It has recently been shown that the secretion ability of the T1SS can be improved by introducing amino acid substitutions to the T1SS [109]. *In vitro* random mutagenesis of the ABC protein (TliD) of the T1SS (Tli system) for *P. fluorescens* SIK W1 lipase, followed by screening for mutants with increased secretion efficiency, has led to the identification of several single mutations that enhance the secretion level of lipase. All mutations are located at the cytoplasmic region of the transmembrane domain. These results suggest that the mutations change the overall conformation of *P. fluorescens* TliD, resulting in a more efficient secretion capacity of the T1SS in general. Alternatively, the mutations increase the ability of *P. fluorescens* TliD to interact with the signal sequence of *P. fluorescens* SIK W1 lipase, and thereby increase the secretion efficiency of lipase. However, *P. fluorescens* TliD mutants that can increase the secretion level of *P. fluorescens* SIK W1 lipase did not significantly increase the secretion level of the protease from the same strain, PrtA. PrtA shares the T1SS (Tli system) with *P. fluorescens* SIK W1 lipase for its secretion, but has a different signal sequence. Therefore, it is more likely that the mutations improve the recognition of the signal sequence by *P. fluorescens* TliD. A similar result has been reported for the T1SS for *E. coli* hemolysin [110]. The mutation L448F in the cytoplasmic region of *E. coli* HlyB (the ABC protein), at the linker position between the TMD and NBD, increases the secretion level of heterologous proteins, such as subtilisin E and scFv antibody, fused with the *E. coli* HlyA signal sequence.

Secretion signal of family I.3 lipase

Like most proteins that are secreted via the T1SS, the N and C termini of family I.3 lipase are not cleaved after secretion [67, 92]. Furthermore, as mentioned above, the secretion signal of proteins secreted via the T1SS is located within the last 60 amino acid residues of the protein. A specific interaction between the NBD of the ABC protein, *E. coli* HlyB, and the last 60 amino acid residues of *E. coli* HlyA has been reported [51]. Work in our laboratory also showed that the last 19 amino acid residues of *Pseudomonas* sp. MIS38 lipase are important for its secretion via the Lip system [73]. The first study to identify the exact location of the secretion signal of proteins secreted via the T1SS was done on the *E. chrysanthemi* metalloprotease PrtG [111]. It showed that *E. chrysanthemi* PrtG (the intact protein or its C-terminal peptide fragment with 56 amino acid residues) lacking the last four amino acid residues almost completely loses its secretion competency through its cognate transporter. Likewise, addition of one amino acid residue to the C-terminal end of *E. chrysanthemi* PrtG greatly reduced or abolished its ability to be secreted via the T1SS. These results suggest that the last four amino acid residues of the protein, consisting of an aspartate residue followed by three hydrophobic residues (Duuu; u: hydrophobic amino acid), are important for the secretion of proteins via the T1SS. A similar motif, an acidic residue (D or E) followed by four or more hydrophobic residues (D/Euuuu), is also conserved in the C-terminal end of family I.3 lipases (Fig. 4), suggesting that this motif is also important for secretion of family I.3 lipases via the T1SS.

More recently, however, a motif consisting of six amino acid residues (R1–R6), located upstream of the aforementioned C-terminal D/Euuuu motif, was identified as the secretion signal, by analyzing the secretion of *S. marcescens* Sr41 lipase via its cognate T1SS (Lip system) [112]. In *S. marcescens* Sr41 lipase, the amino acid sequence of this motif is VTLIGV (Fig. 4). HasA from the same strain, which is not secreted via the Lip system, becomes secretion competent when the VTLIGV motif is inserted into a specific position near the C terminus of *S. marcescens* HasA. Based on the mutational studies of the R1–R6 residues, as well as the comparison of the C-terminal amino acid sequences of proteins that are secreted by the Lip system, the sequence of a six-residue secretion signal, recognized by the Lip system, has been proposed to be (V/I)-x-(L/I/V)-(V/I/M/T)-(G/A)-(u/q). In this sequence, u represents any hydrophobic residue and q represents any uncharged polar residue. The mutational studies of *S. marcescens* Sr41 lipase also showed that the extreme C-terminal motif (D/Euuuu) is not essential for secretion of *S. marcescens* Sr41 lipase, but the distance between the six-residue secretion signal and the C terminus is important for its secretion. This may be the reason why the *E. chrysanthemi* PrtG mutants with the addition or deletion of the C-terminal amino acid residues have lost their secretion competency [111].

Pseudomonas sp. MIS38 lipase is secreted efficiently through the T1SS for *S. marcescens* Sr41 lipase (Lip system) when the genes encoding *Pseudomonas* sp. MIS38 lipase and the Lip system are co-expressed constitutively in *E. coli* [73]. Although the lipases from *Pseudomonas* sp. MIS38 and *S. marcescens* Sr41 share only 61% amino acid sequence similarity to each other (relatively low compared with those between the other two family I.3 lipases), both proteins have a very similar six-residue secretion signal located at the same position (Fig. 4). Furthermore, *Pseudomonas* sp. MIS38 lipase lacking the last 19 residues, which contains the entire six-residue

secretion signal, cannot be secreted via the Lip system (PMLΔ19; Fig. 5) [73]. These results suggest that T1SS for a given family I.3 lipase can secrete efficiently any family I.3 lipase, because a six-residue secretion signal is highly conserved among various family I.3 lipases (Fig. 4).

It is worth mentioning that structural studies on the peptides that encompass the C-terminal ends of *E. coli* HlyA [113, 114], *E. chrysanthemi* PrtG [115], and *S. marcescens* HasA [76, 116] show that these peptides are relatively unstructured in an aqueous environment but undergo a conformational change, to assume two α helical structures separated by an unstructured linker, in a membrane mimetic environment. Such a conformational change has been proposed to be an important feature for recognition of the secretion signal of passenger proteins by their cognate T1SSs [113]. A combinatorial study on the secretion signal of *E. coli* HlyA also showed that the first α helix and a part of the unstructured linker (functional domain I) are important for efficient *E. coli* HlyA secretion [117]. Furthermore, the C-terminal nine amino acid residues (functional domain II) have also been shown to be somehow important, although not as significant as the functional domain I, for efficient *E. coli* HlyA secretion [118]. The functional domain I contains an ISAAG sequence motif. This sequence is different from but similar to the aforementioned six-residue secretion signal of *S. marcescens* Sr41 lipase [112], suggesting that the T1SS for *E. coli* HlyA also recognizes its passenger protein through this signal motif. Meanwhile, the functional domain II may play a role similar to that of the Duuu sequence motif found in *E. chrysanthemi* PrtG [111]. This motif may not be directly involved in secretion of proteins through T1SS, but may be required to provide a space between the secretion signal and the C terminus of the protein, because most of the mutations in the functional domain II do not significantly reduce or abolish the secretion competency of HlyA.

Requirement of functional repetitive sequences for efficient folding of family I.3 lipase into an active conformation

Family I.3 lipases specifically require Ca^{2+} for activity [87, 90, 92]. This is because the C domain of family I.3 lipases, which contains the repetitive nine-residue sequences, needs to be folded in order for the whole protein to fold into its active conformation. The N domain of *Pseudomonas* sp. MIS38 lipase is inactive when it is overproduced as a polypeptide chain separate from the C domain [119]. Measurement of the far-UV circular dichroism (CD) spectra indicates that the secondary structure of the N domain is not changed in the presence of Ca^{2+}. In contrast, full-length PML exhibits Ca^{2+}-dependent activity and its secondary structure is changed in the presence of Ca^{2+}. At least five repeats are required for *Pseudomonas* sp. MIS38 lipase to be active (Fig. 5), to

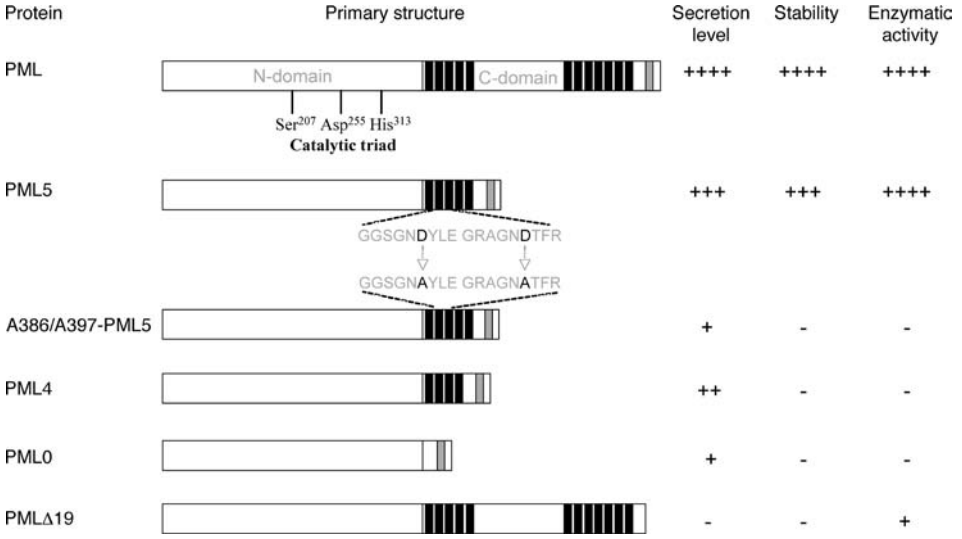

Figure 5. Comparison of the secretion level, stability, and activity of *Pseudomonas* sp. MIS38 lipase (PML) and its variants [73, 74]. The amino acid sequences of these proteins are schematically shown. In these sequences, the GGxGxDxux repetitive sequences and the putative six-residue secretion signal are represented by solid and shaded boxes, respectively. The secretion level, stability (resistance to chymotryptic digestion), and enzymatic activity of each protein relative to those of PML are shown by ++++, +++, ++, +, and –, in which ++++ and – represent 'very high' and 'very low', respectively. Secretion of these proteins was examined using *E. coli* DH5 cells carrying the Lip system. Stability and activity of these proteins were examined using the refolded protein, which was overproduced in the *E. coli* cells in inclusion bodies, solubilized in the presence of urea, refolded, and purified. 'Enzymatic activity' represents the specific activity, which is defined as the enzymatic activity per milligram of protein. The specific activity of the refolded protein of PML was nearly identical to that of the secreted protein.

bind Ca^{2+} ions, and to fold into a putative β-roll motif [73, 74]. These results suggest that folding of family I.3 lipases into their active conformation requires the presence of at least five functional repetitive sequences. Similarly, *E. coli* HlyA loses its cytolytic activity when the repeats are deleted [120]. The β-roll motif probably acts as a kind of intramolecular chaperone which helps the folding of the catalytic lipase domain.

Requirement of functional repetitive sequences for efficient secretion of family I.3 lipase via the T1SS

Proteins secreted via the T1SS have a C-terminal secretion signal, and therefore have to be fully translated before they can be recognized by the ABC protein subunit. In contrast, proteins secreted via the Sec-dependent pathway have an N-terminal signal peptide and it has been proposed that the recognition of the signal peptide by the membrane ATPase SecA can occur during or after protein synthesis by ribosome [30]. Proteins secreted via the T1SS and Sec system have a similar requirement, in which their physical size should not exceed the pore size of either transporter. In the Sec system, the limiting size seems to be the internal diameter of the membrane-bound SecY complex, which was determined to be about 20–25 Å. This size is enough to accommodate the passage of unfolded proteins, however, with a little expansion it may be enough to allow passage of an α helix [121]. Likewise, the internal diameter of *E. coli* TolC, the model of the OMP subunit of the T1SS, has been proposed to be about 30–35 Å in an open conformation [49]. Therefore, globular proteins transported via these systems should be unfolded or at least only partially folded prior to secretion.

Studies on the mutants of *Pseudomonas* sp. MIS38 lipase with a decreased number of the repetitive sequences suggest that these repeats are required to some extent for efficient protein secretion (Fig. 5) [73]. These studies showed that at least five repeats are required to maintain its secretion efficiency and intracellular stability against proteolytic degradation. Other family I.3 lipases, such as lipases from *P. fluorescens* SIK W1, *P. fluorescens* LS107d2, and *P. fluorescens* KB700A, also contain only five repeats (Fig. 4), suggesting that five repeats are enough to promote the folding of family I.3 lipase into an active and stable conformation, presumably because five repeats are enough to make a stable β-roll structure.

It has also been shown that these repeats have to be functional, because the substitution of the aspartate residues located within the repeats to Ala seriously affects the enzymatic activity and stability of *Pseudomonas* sp. MIS38 lipase (Fig. 5) [74]. The secretion level and the intracellular amount of the *Pseudomonas* sp. MIS38 lipase mutant with the defective repeats (A387/A396-PML5) are greatly reduced compared with those of the parent protein (PML5), indicating that the secretion efficiency of this mutant protein is reduced because less protein is available to be secreted via the T1SS. Biochemical characterizations of these proteins indicate that PML5 is more resistant to proteolytic degradation than A387/A396-PML5, only when a β-roll structure is formed. These results may suggest that, beside functioning as a spacer, the repetitive sequences also need to be folded intracellularly to protect the protein from proteolytic degradation. However, the intracellular free Ca^{2+} concentration in *E. coli*, which is estimated to be around 0.6 µM [122], is three orders of magnitude lower than that required for formation of a β-roll structure [68, 69]. Further studies will be required to understand the role of the repetitive sequences.

Summary

Family I.3 lipases have gained enormous attention in recent years because of their unique secretion mechanism. The fact that these lipases are secreted directly into the extracellular medium and function without the need for molecular chaperones simplifies their recombinant production in Gram-negative bacteria. Studies of these lipases at the molecular level have also shed light on many aspects of their secretion mechanism as well as biochemical and biophysical properties. However, to date, no crystal structures of family I.3 lipases have been solved, and so many questions on structural aspects remain unanswered. Studies on the requirements of protein secretion via the T1SS have also given rise to valuable information for the growing interest in exploiting this system for secretory production of recombinant proteins in Gram-negative bacteria. Here, further questions, such as the mechanism by which the ABC protein recognizes and interacts with the signal sequence, and how the β-roll motif alters protein stability against intracellular degradation, still need to be resolved.

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