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## Bacterial lipases <sup>☆</sup>

Karl-Erich Jaeger <sup>a,\*</sup>, Stéphane Ransac <sup>b</sup>, Bauke W. Dijkstra <sup>b</sup>, Charles Colson <sup>c</sup>,  
Margreet van Heuvel <sup>d</sup> and Onno Misset <sup>d</sup>

<sup>a</sup> *Lehrstuhl Biologie der Mikroorganismen, Ruhr-Universität, D-44780 Bochum, FRG*, <sup>b</sup> *Laboratory of Biophysical Chemistry, University of Groningen, NL-9747 AG Groningen, the Netherlands*, <sup>c</sup> *Laboratoire de Génétique microbienne, Université Catholique de Louvain, B-1348 Louvain-La-Neuve, Belgium*, and <sup>d</sup> *Research and Development, Gist-brocades, NL-2600 MA Delft, the Netherlands*

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**Abstract:** Many different bacterial species produce lipases which hydrolyze esters of glycerol with preferably long-chain fatty acids. They act at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium. A characteristic property of lipases is called interfacial activation, meaning a sharp increase in lipase activity observed when the substrate starts to form an emulsion, thereby presenting to the enzyme an interfacial area. As a consequence, the kinetics of a lipase reaction do not follow the classical Michaelis-Menten model. With only a few exceptions, bacterial lipases are able to completely hydrolyze a triacylglycerol substrate although a certain preference for primary ester bonds has been observed. Numerous lipase assay methods are available using coloured or fluorescent substrates which allow spectroscopic and fluorimetric detection of lipase activity. Another important assay is based on titration of fatty acids released from the substrate. Newly developed methods allow to exactly determine lipase activity via controlled surface pressure or by means of a computer-controlled oil drop tensiometer. The synthesis and secretion of lipases by bacteria is influenced by a variety of environmental factors like ions, carbon sources, or presence of non-metabolizable polysaccharides. The secretion pathway is known for *Pseudomonas* lipases with *P. aeruginosa* lipase using a two-step mechanism and *P. fluorescens* lipase using a one-step mechanism. Additionally, some *Pseudomonas* lipases need specific chaperone-like proteins assisting their correct folding in the periplasm. These lipase-specific foldases (Lif-proteins) which show a high degree of amino acid sequence homology among different *Pseudomonas* species are coded for by genes located immediately downstream the lipase structural genes. A comparison of different bacterial lipases on the basis of primary structure revealed only very limited sequence homology. However, determination of the three-dimensional structure of the *P. glumae* lipase indicated that at least some of the bacterial lipases will presumably reveal a conserved folding pattern called the  $\alpha/\beta$ -hydrolase fold, which has been described for other microbial and human lipases. The catalytic site of lipases is buried inside the protein and contains a serine-protease-like catalytic triad consisting of the amino acids serine, histidine, and aspartate (or glutamate). The Ser-residue is located in a strictly conserved  $\beta$ - $\epsilon$ Ser- $\alpha$  motif. The active site is covered by a lid-like  $\alpha$ -helical structure which moves away upon contact of the lipase with its substrate, thereby exposing hydrophobic residues at the protein's surface mediating the contact between protein and substrate. This movable lid-like  $\alpha$ -helix explains at a molecular level the lipase-specific phenomenon of interfacial activation. At least some of the pathogenic bacterial species produce a lipase which has been studied with respect to its role as a virulence factor. Lipases of *Propionibacterium acnes* and *Staphylococcus epidermidis* may be involved in colonization and persistence of these bacteria on the human skin. Lipases of *S. aureus* and *P. aeruginosa* are produced during the bacterial infection process and, at least in vitro, considerably impair the function of different cell types involved in the human immune response like

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<sup>☆</sup> The authors dedicate this article to Prof. Dr. Uli Winkler, a pioneer and continuous supporter of research on bacterial lipases, on the occasion of his 65th birthday.

\* Corresponding author. Tel.: (0234) 700 3101; Fax: (0234) 709 4114

macrophages or platelets. The present state of knowledge suggests to classify the lipases as important bacterial virulence factors which exert their harmful effects in combination with other bacterial enzymes, in particular the phospholipases C. Most of the steadily increasing interest in bacterial lipases is based on their biotechnological applications which are partly based on their potential to catalyze not only hydrolysis but also synthesis of a variety of industrially valuable products. Optically active compounds, various esters and lactones are among the substances synthesized using bacterial lipases. Recently, an important application emerged with the addition of bacterial lipases to household detergents in order to reduce or even replace synthetic detergent chemicals which pose considerable environmental problems. As a main conclusion, lipases represent an extremely versatile group of bacterial extracellular enzymes that are capable of performing a variety of important reactions, thereby presenting a fascinating field for future research.

**Key words:** Lipase (EC 3.1.1.3); Mechanism of secretion; Interfacial activation; Three-dimensional structure; Virulence factor; Biotechnological applications

## Introduction

Lipases (EC 3.1.1.3) are distributed throughout the living organisms which form two primary divisions of the phylogenetic tree, namely the bacteria and a second division branching into both the eukarya, including animals, plants and fungi, and the archaea, with the former archaeobacteria [1]. The scope of this review is the description of lipases which are produced and secreted by bacteria and are therefore called extracellular enzymes. Their presence had been observed as early as in 1901 for *Bacillus prodigiosus*, *B. pyocyaneus*, and *B. fluorescens* [2] which represent some of today's best studied lipase-producing bacteria now named *Serratia marcescens*, *Pseudomonas aeruginosa*, and *P. fluorescens*, respectively. The main reason for the steadily growing interest in lipases, reflected by an average of

1000 publications appearing per year, is the biotechnological versatility of these enzymes including their potential to catalyze the hydrolysis and also the synthesis of esters (Fig. 1), which was also recognized nearly 70 years ago [3]. Important aspects related to bacterial lipases have been covered by excellent review articles describing purification, biochemistry and molecular biology of selected species [4–7], a comparative description of *Pseudomonas* lipases [8] and especially the biotechnological applications of lipases [9–13]. This article will describe lipases from both Gram-positive and Gram-negative bacteria covering mainly the period from 1990 to 1993. Following a general introduction into the mechanism of lipolysis and lipase assay systems we discuss factors regulating synthesis, secretion and release of lipases and some of their biochemical properties. We further present the general concept of three-

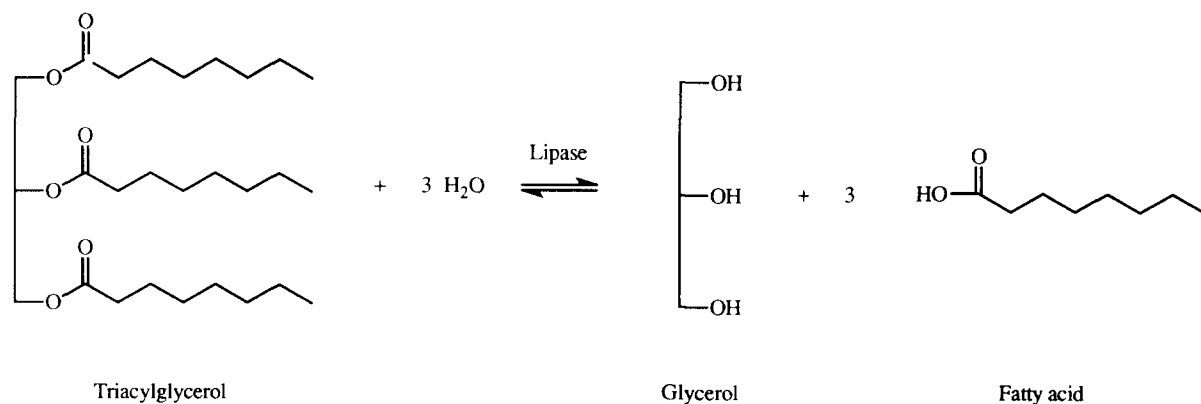


Fig. 1. Enzymatic reaction of a lipase catalyzing hydrolysis or synthesis of a triacylglycerol substrate.

dimensional structure for lipases as it emerges from the present structural knowledge which is mainly based on lipases of non-bacterial origin. Finally, we review the most important aspects of applied lipase research, i.e. their role as virulence factors in bacterial infections and their significance for biotechnological applications. It was primarily this latter fact that led the Commission of the former European Community (which is now called European Union) to launch in the framework of the program BRIDGE (Biotechnology Research for Innovation, Development and Growth in Europe) a research project on lipases of mammalian, fungal and bacterial origin which was funded with a total of 4.3 million ECU [14]. This project was scheduled to run from 1990 to 1993 with participants including University laboratories from nine European countries and the companies Gist-Brocades, Novo-Nordisk, and Unilever. Although it is not yet concluded the EU set up a new project on lipases which is foreseen to start in 1994 in the framework of the BIOTECHNOLOGY program.

### **Mechanism of lipolysis**

Lipases (glycerol ester hydrolases, E.C. 3.1.1.3) are hydrolases acting on the carboxyl ester bonds present in acylglycerols to liberate organic acids and glycerol. Their major substrates are long-chain triacylglycerols, and this property is the basis of an old definition of lipases as 'long-chain fatty acid ester hydrolases' or 'esterases capable of hydrolyzing esters of oleic acid' [15].

#### *Definition of the interface*

Triacylglycerols are uncharged lipids. Although those with short-chain fatty acids are slightly soluble in water, compounds with longer-chain fatty acids esterified to glycerol are insoluble. The maximum concentration of monomers in aqueous solution has been called the saturation value, which is the point where triacylglycerols start to form emulsions. In contrast, phospholipids, which are also insoluble in water, form micelles when exceeding the maximum concen-

tration of dissolved monomer at a point called the cmc (critical micelle concentration). While the maximum saturation value for triacylglycerols can be as high as 0.330 M in the case of triacetin [16], it can be less than 1  $\mu$ M for long-chain triacylglycerols. Lipolysis occurs exclusively at the lipid-water interface, implying that the concentration of substrate molecules at this interface (expressed in mol m<sup>-2</sup>) directly determines the rate of lipolysis. The concentration and physical state of lipid molecules in bulk phases (i.e. the soluble monomer concentration, expressed in mol m<sup>-3</sup>) are important in that they affect the surface phase via bulk surface phase equilibria. Therefore, it is insufficient to simply define the interfacial concentration of a substrate. Different molecular states may exist in different phases or even within a single phase.

#### *Kinetics and interfacial activation*

The physical properties of lipids in general have caused many difficulties in studying the properties of lipolytic enzymes. Sarda and Desnuelle [16,17] clearly demonstrated a fundamental difference between esterase and lipase activity based upon their ability to be activated by interfaces. Esterase activity is a function of substrate concentration as described by Michaelis-Menten kinetics with the maximal reaction rate being reached long before the solution becomes substrate-saturated; the formation of a substrate/water emulsion does not change the reaction rate. In contrast, lipases show almost no activity with the same substrate as long as it is in its monomeric state. However, when the solubility limit of the substrate is exceeded, there is a sharp increase in enzyme activity as the substrate forms an emulsion. This is illustrated in Fig. 2A where the vertical broken line represents the substrate saturation. To the left of this line, the substrate triacetin is dissolved in water; to the right, the substrate forms an emulsion with an increasing interfacial area.

These experiments demonstrated that lipase activity depends on the presence of an interface. They led to the definition of lipases as carboxylesterases acting on emulsified substrates.

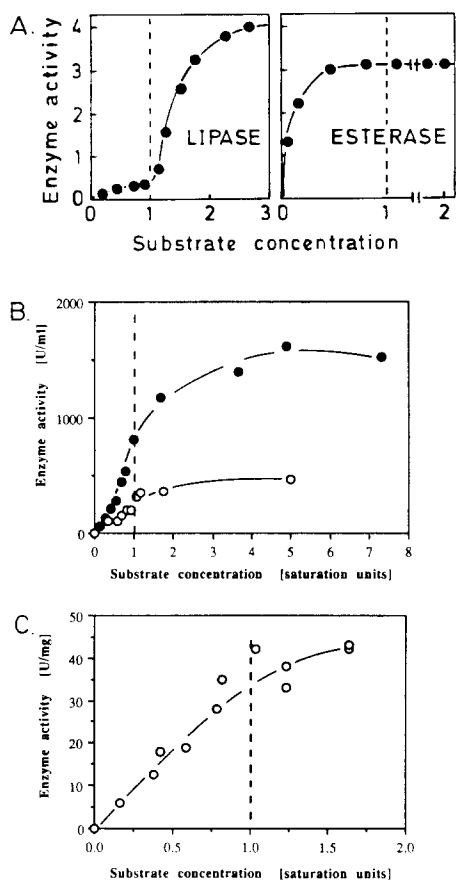


Fig. 2. Interfacial activation of lipases. (A) Classical activity profile of a pancreatic lipase and a horse liver esterase at different substrate concentrations exceeding the saturation point (modified from [16]). The dashed lines indicate the point of substrate saturation. (B) Activity of *P. aeruginosa* lipase at different substrate concentrations of triacetin (○, saturation concentration 306 mM) and tripropionin (●, saturation concentration 15 mM). (reproduced from [18]). (C) Activity of *B. subtilis* lipase at different substrate concentrations of triacetin (modified from [19]).

This property found an elegant explanation when the first three-dimensional structures of lipases had been elucidated. It was found that the active site of lipase was covered by a lid-like polypeptide chain which rendered the active site inaccessible to substrate molecules, thereby causing the enzyme to be inactive on monomeric substrate molecules [20,21]. However, when a lipase was bound to a lipid interface, a conformational change took place causing the lid to move away

whereby the active site of the lipase became fully accessible. As a result, the hydrophobic side of the lid became exposed to the lipid phase, thus enhancing hydrophobic interactions between the enzyme and the lipid surface [22,23]. This observation explains the interfacial activation phenomenon with the lid causing inactivation if no lipid interface is present and has been used to discriminate between 'true' lipases and esterases [16] by defining a lipase as an enzyme which shows interfacial activation in the presence of long-chain triacylglycerols as substrates. If an enzyme hydrolyzing these substrates does not show interfacial activation it should be called an esterase. However, this definition should be used with care for several reasons: (i) the detection of interfacial activation requires pure lipase enzyme to avoid potential effects of other carboxyl hydrolases; (ii) the same lipase may show a distinctly different behaviour depending on the 'quality' of the interface. An example is *S. hyicus* lipase which is able to degrade acylglycerols as well as phospholipids. It is activated in the presence of a tributyrin interface, but not in the presence of an interface composed of diheptanoyl-phosphocholine [24]. (iii) Lipases from *P. aeruginosa* [18] (see Fig. 2B), and *B. subtilis* [19] (see Fig. 2C) do not show activation in the presence of emulsified substrates; instead, their activity continuously increases indicating that these enzymes are able to degrade both emulsions and monomeric substrates, whereas true esterases degrade only monomeric substrates. Therefore, a lipase should not be defined solely according to its interfacial activation behaviour, but also according to its capability to hydrolyse emulsions of long-chain acylglycerols.

Kinetics of lipases cannot be described with the Michaelis-Menten model since this model is valid only in the case of one homogenous phase, i.e. for soluble enzymes and substrates. Therefore, a new model has been proposed to describe the kinetics of catalysis by lipolytic enzymes [25] which consists of two steps (Fig. 3): (1) the physical adsorption of the enzyme at the lipid interface may include an activation of the enzyme (opening of the lid which blocks the active site) [22,23]; (2) the formation of the enzyme/substrate complex

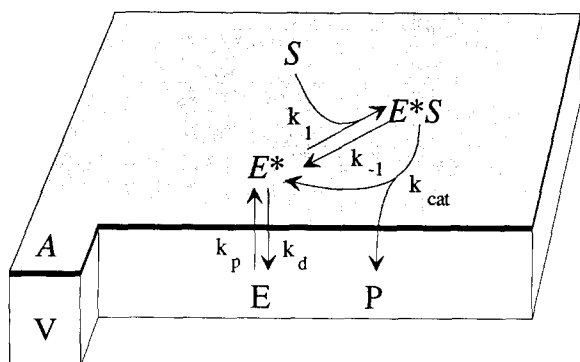


Fig. 3. Model for description of interfacial kinetics with a water-soluble lipase enzyme acting on insoluble substrate. For details, see text and [25].

which can be hydrolysed to give the product and regenerate the adsorbed enzyme. This second step may be described by an 'interfacial' Michaelis-Menten model with the substrate concentrations expressed in [mol/surface] instead of [mol/volume]. Equations have been reported which perfectly describe the experimental results [25]. Additionally, models to describe the kinetics

of competitive inhibition of lipases in the presence and absence of detergents as well as for interfacial inactivation have been proposed [26,27].

#### Substrate specificity of lipases

The glycerol molecule as the basic building block of the lipase substrate triacylglycerol contains two primary and one secondary hydroxyl groups. Although the molecule has plane symmetry, the two primary groups are sterically distinct. Substitution of these hydroxyl groups with two different substituents will lead to optically active derivatives. In a generally adopted nomenclature (IUPAC-IUB Commission on Biochemical Nomenclature, 1967), glycerol is written in a Fisher projection with the secondary hydroxyl group to the left, and the carbon atoms numbered 1, 2, and 3 from top to bottom (*sn*-, i.e. stereospecifically numbered glycerol), thereby allowing the unambiguous description of isomeric glycerides. Lipases can be classified into three groups ac-

Plate assays				
Substrate	Reaction product	Method		
Glycerides (triolein)	Free fatty acids	Coloured indicators [Victoria blue, methyl red, phenol red, rhodamine B]		
Spectroscopic				
Substrate	Reaction product	Method	Final product	Wavelength
1,2-diglycerides	Glycerol	Enzymatic conversion	Quinone	550 nm
Glycerides (triolein)	Free fatty acids	Enzymatic conversion	NAD	340 nm
Glycerides	Free fatty acids	Complex formation	Rhodamine 6G	513 nm
Glycerides (triolein)	Free fatty acids	Negative charge	Safranine	520 / 560 nm
Glycerides	Free fatty acids	Complex formation	Cu(II) salt	715 nm
<i>p</i> -nitro-phenyl esters	<i>p</i> -nitro-phenol	Product is coloured		410 nm
2,3-dimercaptopropan-1-ol tributyroate	Glycerol analogue (2 over 3 positions)	Reduction with DTNB	TNB	412 nm
Arylethene derivatives	Hydrolysis products are coloured			Variable
Fluorescence				
Substrate	Reaction product	Method	Final product	Wavelength
Glycerides (triolein)	Free fatty acid	Complex formation	11-(dansylamino)undecanoic acid	ex. 350 nm, em. 500 nm
Glycerides containing pyrene ring	Free fatty acid analogues or Aggregated substrate	Fluorescence shift	Free fatty acid analogues or glyceride analogues	ex. 340 nm, em. 400 nm 450 nm
Titrimetric				
Substrate	Reaction product	Method		
Glycerides (Tributyrin)	Free fatty acids	pH - determination		
Surface pressure				
Substrate	Reaction product	Method		
Dicaprin	Free fatty acids	Measurement of barrier movement		
Long chains triglycerides	Free fatty acids	Measurement of drop volume or decrease in surface tension		

Fig. 4. Assays for determination of lipase activity.

cording to their substrate specificity. The first group shows no positional and no specificity with respect to the chemical structure of the fatty-acid. Examples are the lipases from *S. aureus* [28,29], *S. hyicus* [24], *Corynebacterium acnes* [30], and *Chromobacterium viscosum* [31]. The second group hydrolyzes only primary ester bonds (i.e. ester bonds at atoms C1 and C3 of glycerol). The lipases of *P. fragi*, *P. fluorescens* and *P. geniculata* were previously found to be members of this group [28,32]. More recently, however, the lipases of *P. glumae* [33,34] and of *P. fluorescens* [35] were also shown to have a low activity towards the secondary ester bond. In fact, it seems that most bacterial lipases have a certain preference for the primary ester bonds but are also capable to hydrolyze the secondary ester bond, and thus belong to the first group. The third group exhibits a pronounced fatty-acid preference; an example is lipase B from *Geotrichum candidum* which is specific for fatty acids with a double bond between C9 and C10 [36]. According to present knowledge, no bacterial lipase belongs to this group. Recently, it was demonstrated that the stereospecificity of lipases is strictly dependent on the surface pressure of the substrate [37,38]. An increase of the lipid density at the air/water interface decreased the stereospecificity of several lipases. The stereospecificity of lipases may also depend on the fatty acid chain length of the substrate: the relative stereopreference of several microbial and non-microbial lipases appeared to be different on trioctanoin and triolein as substrates. For some lipases, the stereospecificity even changed from one to the other enantiomer of the substrate [39].

### Lipase assay systems

A number of assays to determine lipase activity have been developed, some of them for the determination of mammalian lipase activity for diagnostic purposes. These procedures could at least partly be adapted to determine the activity of microbial lipases. A summary of currently used methods is given in Fig. 4.

### Spectrophotometry and fluorimetry

Several assays for lipase activity are based on spectroscopic measurements. Some of them make use of natural substrates yielding products that react with other compounds or may be used as substrates by other enzymes. In a colorimetric assay using long-chain fatty acid 1,2-diglycerides, the lipase produces a 2-monoglyceride from which glycerol is released by the action of a 2-monoglyceride lipase. Glycerol concentration is determined by a sequence of enzymatic reactions with glycerol kinase, glycerol phosphate oxidase, and peroxidase that produce a violet quinone monoimine dye with a peak absorption at 550 nm [40]. Another series of coupled enzymatic reactions used the oxidation of NADH as the final step [41]. Rhodamine 6G was used for complexation with free fatty acids liberated during lipolysis. A pink colour appears and absorbance was monitored at 513 nm [42]. The metachromatic properties of the cationic dye safranin were used to detect a change in the net negative charge at the lipid/water interface, which was monitored by the change in absorbance of safranin. Very low amounts of lipolytic enzyme can be detected using this method [43]. Immobilized triacylglycerols were hydrolyzed and the released fatty acids were extracted with benzene and converted to the corresponding Cu(II) salts which were measured spectrophotometrically [44]. Other spectroscopic assays used substrate derivatives as  $\beta$ -naphthyl caprylate [45] or 2,3-dimercaptopropane-1-ol tributuroate as substrate and 5,5'-dithiobis(2-nitrobenzoic acid) as chromogenic reagent [46]. Other substrates were substituted arylethene derivatives; the hydrolysis products of these compounds are coloured and many of them are water-soluble making them suitable precursors for chromogenic enzyme substrates [47]. Para-nitrophenyl-esters of various chain-length fatty acids are also used as substrates. However, these compounds are not suitable for specific lipase assays because they are also cleaved by esterases [48]. Some of the spectrophotometric methods can be used in the presence of organic solvents. This is useful during lipase purification with the reversed micelle method [49–54].

Fluorescent compounds have also been used for lipase assays. In a continuous assay procedure the displacement of the fluorescent fatty acid probe 11-(dansylamino)undecanoic acid from a fatty acid binding protein was measured which is caused by long-chain fatty acids released as a result of lipase activity [55,56]. It is also possible to use triacylglycerols having one of the alkyl groups substituted with a fluorescent group (e.g. pyrenyl) [57,58]. In an aggregated substrate the pyrene groups are close to each other and fluoresce at 450 nm. When fatty acids are cleaved, the pyrene group's fluorescence shifts to 400 nm. Plate assays have been described to screen for lipase-producing microorganisms using either Victoria blue B, Methyl red, Phenol red or Rhodamine B as indicators [59–61]. Substrate hydrolysis causes the formation of colour or fluorescent halos around bacterial colonies.

#### *Titrimetry*

The lipolytic reaction liberates an acid which can be titrimetrically assayed. Coloured indicator reagents have been used for plate assays, but a very useful quantitative technique is to measure the pH during the reaction course. Since the pH is an important parameter for enzyme catalysis, it should be kept constant by continuously adding NaOH solution the volume of which is monitored as a function of time. This method is called the pH-stat method [62–64]. The reaction rate obtained is a linear function of the lipase concentration and of the 'substrate concentration'. As mentioned above substrate concentration should be expressed in [mol/surface] since the substrate is insoluble and thus forms an emulsion. Measurements should always be done under carefully controlled conditions, i.e. at a given volume of substrate and a given stirring rate, to ensure a reproducible quality of the interface.

#### *Controlled surface pressure*

Lipases act at the interface between a hydrophobic substrate and a hydrophilic water phase, and hence the surface pressure is a very important parameter which has often been neglected. For an assay of lipolytic enzymes, this

parameter is at least as important as pH or temperature. The effect of the surface pressure can be studied by the monolayer technique (see e.g. [25,65]). A monomolecular substrate film is spread at the air-water interface which can be compressed with a surface barrier, changing the surface density of the substrate and thus the interfacial tension. The lipase injected into the water subphase will bind to the film and hydrolyze the substrate. The easiest way is to choose a substrate (e.g. trioctanoin, didecanoin or didodecanoin) which itself is insoluble in water, but which will generate soluble products. It is also possible to use substrates with longer acyl-chains under conditions where the surface pressure is above  $23 \text{ mN m}^{-1}$  and albumin is present in the subphase as a product-acceptor [66]. When the substrate is hydrolyzed, it will leave the interface, thereby decreasing the surface density and surface pressure which is then compensated by compression of the film by the mobile surface barrier. The barrier movement is monitored as a function of time. There are at least five major reasons for using lipid monolayers as substrates for lipolytic enzymes. (i) The monolayer technique is highly sensitive, and only small amounts of lipid are needed for kinetic measurements. (ii) During the course of the reaction, it is possible to monitor several physicochemical parameters characteristic of the monolayer film, e.g. surface pressure, potential, or radioactivity. (iii) The lipid packing in a monomolecular film of substrate is kept constant during the course of hydrolysis, and it is therefore possible to obtain accurate presteady-state kinetic measurements with minimal perturbation caused by increasing amounts of reaction products. (iv) The 'interfacial quality' can be modulated. It depends on the nature of the lipids forming the monolayer, their orientation and conformation, their molecular and charge densities, the water structure and the viscosity. (v) Inhibition of lipolytic enzyme activities by water-insoluble inhibitors can be precisely measured using a 'zero-order' trough and mixed monomolecular films in the absence of any synthetic, non-physiological detergent. The monolayer technique is therefore suitable for modeling *in vivo* situations.

Another method to monitor the interfacial



tension during lipase assays is the 'oil-drop method' [67]. This method consists of forming an oil-drop in a water solution with the drop connected to a syringe containing the oil. The shape of the drop is directly correlated to the interfacial tension of oil/water. When no detergent or fatty acid is present in the medium, the drop is shaped like an apple. When a lipase is added to the water phase, it binds to the oil/water interface and hydrolyzes the substrate. The released products remain in the interface, and consequently, the interfacial tension decreases. The shape of the drop changes to a pear form and, at a certain point, it will leave the support. A computer-controlled device called 'oil drop tensiometer' has been developed to automatically perform this type of lipase assay [68].

#### *Other assays*

Other methods to determine lipase activity include a high-performance liquid chromatographic assay [69,70]. This assay involves incubation of  $\beta$ -naphthyl laurate with enzyme followed by the quantification of naphthol after separating it from the assay solution by reversed phase HPLC. It is also possible to use NMR for quantitating lipase activity in biphasic macroemulsions [71], or infrared spectroscopy for measuring lipase-catalyzed hydrolysis of triglycerides in reverse micelles [72]. Finally, a conductometric method has been described using the short-chain substrate triacetin [73].

#### **Physiology and regulation of lipase production**

Extracellular lipases normally appear in the culture medium when the bacterial cells reach the end of the logarithmic growth phase. Regulation could generally affect every step involved in directing lipases to their extracellular destination, starting with transcription of the lipase structural genes, proceeding with the translation of the respective m-RNAs and the subsequent secretion of the protein through both inner and outer membranes. Regulation mechanisms involving stationary-phase-specific promoters [74] preceding lipase structural genes or consensus DNA-se-

quences pointing to an involvement of specialized  $\sigma$ -factors as the *rpoN* gene product in *Escherichia coli* [75] have not been described. Recently, it was shown that the production of various extracellular proteins was regulated by a transcriptional activator–autoinducer complex similar to the LuxR–LuxI system regulating the expression of the *lux* regulon in bioluminescent bacteria [76]. In *P. aeruginosa*, an autoinducer (*N*-(3-oxododecanoyl)-L-homoserine lactone; PAI) and a transcriptional activator (LasR) regulate the expression of genes *lasB*, *lasA*, and *aprA* coding for extracellular proteases [77,78]. In *P. aeruginosa* LasR mutants extracellular lipase activity is significantly lowered. At present, experiments are carried out to determine whether the expression of the lipase gene of *P. aeruginosa* is directly regulated by the LasR-PAI system (B. Iglewski and K.-E. Jaeger, in preparation).

#### *Factors affecting lipase production*

A variety of conditions have been described which stimulate or repress the production of lipases by bacteria. The *S. hyicus* lipase, which was cloned and expressed in *S. carnosus*, has been produced in a one-vessel dialysis fermentor by increasing the cell mass yielding up to 230 mg of lipase per liter of culture supernatant [79]. Production of *P. fluorescens* lipase was influenced by the concentration of iron(III) in the medium with high iron concentrations repressing lipase and pyoverdine synthesis [80,81]. However, direct evidence for the existence of an iron-repressor complex was not described. Carefully controlled automatic feeding of both olive oil as a carbon source and iron in a fed-batch culture of *P. fluorescens* led to mass production of lipase up to 200 mg l<sup>-1</sup> [82,83]. A systematic study on the regulation of lipase production by *P. aeruginosa* revealed that limitation of carbon and/or energy sources increased lipase production which was strongly induced by triglycerides and detergents like Tweens or Spans. Long-chain fatty acids (e.g. oleic acid) repressed lipase production. Optimum conditions were achieved in a Tween 80-limited continuous culture grown at pH 6.5, 35.5°C at a dilution rate of 0.04 h<sup>-1</sup> [84].

In certain Gram-negative bacteria, the last step of the secretion process, i.e. the release of lipase from the bacterial outer membrane could be influenced by treating the bacterial cells with non-metabolizable polysaccharides like glycogen and hyaluronate for *S. marcescens* [85] and, in addition, alginate for *P. aeruginosa* [86–89]. Presumably, cell-bound lipase was detached from binding sites at the outer membrane via direct interaction with the polysaccharides [85,88]. Alginate, which forms the mucoid slime layer produced by clinical *P. aeruginosa* strains, might additionally serve as a temporary reservoir for lipase [90]. The affinity of alginate to lipase has been used to develop a purification protocol based on co-precipitation of alginate and lipases from culture supernatants of *P. aeruginosa*, *C. viscosum*, and *Rhizopus delemar* [91].

In summary, the studies described above aimed at defining conditions of optimum lipase production rather than elucidating the mechanisms responsible for the observed effects. Although it is possible to produce substantial amounts of lipase protein from both Gram-positive and Gram-negative bacteria, no clear general picture is emerging so far from the large amount of experimental data concerning the physiology of lipase biosynthesis and its regulation. Recently, evidence was presented for a complex mechanism of regulation of exoprotein synthesis in *S. aureus*. A mutation caused by a chromosomal insertion of transposon Tn551 in *S. aureus* resulted in sharply reduced extracellular lipase activity, presumably by inactivating a transcriptional activator (*xpr*) of the lipase structural gene [92]. The synthesis of different exoproteins including lipase appeared to be regulated by three genetic loci, *agr* and *xpr* and *sar*, interacting at the genotypic level. At least one of the proteins encoded for by these loci is assumed to be a sensory protein responding to environmental stimuli as pH or glucose concentration [93] suggesting an elegant explanation of a variety of effects which have been observed to influence the level of lipase production.

#### *Mechanisms of secretion*

The secretion of lipases is mediated by different secretion systems used by the particular bac-

terial cells. Among the Gram-positive bacteria, protein secretion has been studied in some detail only in *Bacillus* species where the counterparts of the *E. coli* genes *secA*, *Y*, and *E* have been identified together with chaperones DnaK, GroEL, and GroES. However, knowledge of the function of the *Bacillus* export machinery is still very limited [94], particularly the secretion of *Bacillus* lipases has not yet been addressed. *S. aureus* lipase is synthesized as a prepropeptide which is processed to form a 82-kDa prolipase by cleavage of a 46-amino acid signal peptide [95] also suggesting a *sec* gene-dependent mechanism. This prolipase is further cleaved to yield a mature 46-kDa lipase which retains full enzymatic activity. Proteolytic processing is mediated by a metallocysteine protease which cleaves off both N- and C-terminal moieties [95]. The lipase of *S. hyicus* is organized as a prepro-enzyme composed of a 38-residue signal peptide, a 207-residue propeptide and a 396-residue mature lipase protein [24,96]. There is good evidence that the propeptide is essential for efficient secretion and may function as an intramolecular chaperon [97]. While in *S. hyicus* the prolipase is processed to yield the mature 46-kDa form, an *S. carnosus* clone does not process the prolipase because it lacks the corresponding protease(s). In *S. hyicus*, processing of the prolipase is carried out by an extracellular, neutral metalloprotease which has recently been characterized [98].

The lipases of the species *Pseudomonas* are secreted using at least two different pathways. The two-step pathway which requires an export machinery of at least 12 different proteins (products of the *xcp* genes) is used by the signal sequence-containing lipase of *P. aeruginosa* [99]. This lipase is not transported into the extracellular medium in *P. aeruginosa pilD* mutants [Lory, S. and Jaeger, K.-E., unpublished observations] which lack a peptidase (PilD or XcpA) that specifically processes proteins Xcp T, U, V, and W which mediate secretion through the outer membrane [99]. Furthermore, periplasmic intermediates of *P. aeruginosa* lipase have been demonstrated in wild-type strains *P. aeruginosa* PAC 1R, PAO1, and PAK (Jaeger, K.E. and Dankert, W., unpublished observations). The

two-step pathway of secretion requires the presence of those periplasmic intermediates which are not detectable when proteins use the one-step pathway of secretion [100]. The prototype enzyme using this unspecific pathway is *E. coli* haemolysin which is secreted through a pore-like structure formed by three proteins HlyB, HlyD, and TolC with one of them belonging to the ATP-binding-cassette family of transport proteins. Haemolysin is synthesized without a signal sequence, but contains an amphiphilic helix and a so-called aspartate box as C-terminally located secretion signals [101]. The lipase of *P. fluorescens* also contains such carboxy-terminal secretion signals already pointing to a secretion by the one-step pathway

[102]. Recently, it was demonstrated that the three-component *Apr* DEF system which directs *P. aeruginosa* alkaline protease into the culture medium can also efficiently mediate secretion of *P. fluorescens* lipase in both *P. aeruginosa* and *E. coli* [103]. The lipase from *S. marcescens* was efficiently secreted by *E. coli* cells containing a second plasmid with three genes building the one-step transport apparatus for protease secretion in *Erwinia chrysanthemi* (Benedik, M., Li, X., Tetling, S. and Jaeger, K.-E., manuscript submitted). The results obtained so far clearly show that the two-step pathway of secretion used by lipases of *P. aeruginosa* or *P. glumae* is highly specific, giving rise to difficulties in obtaining

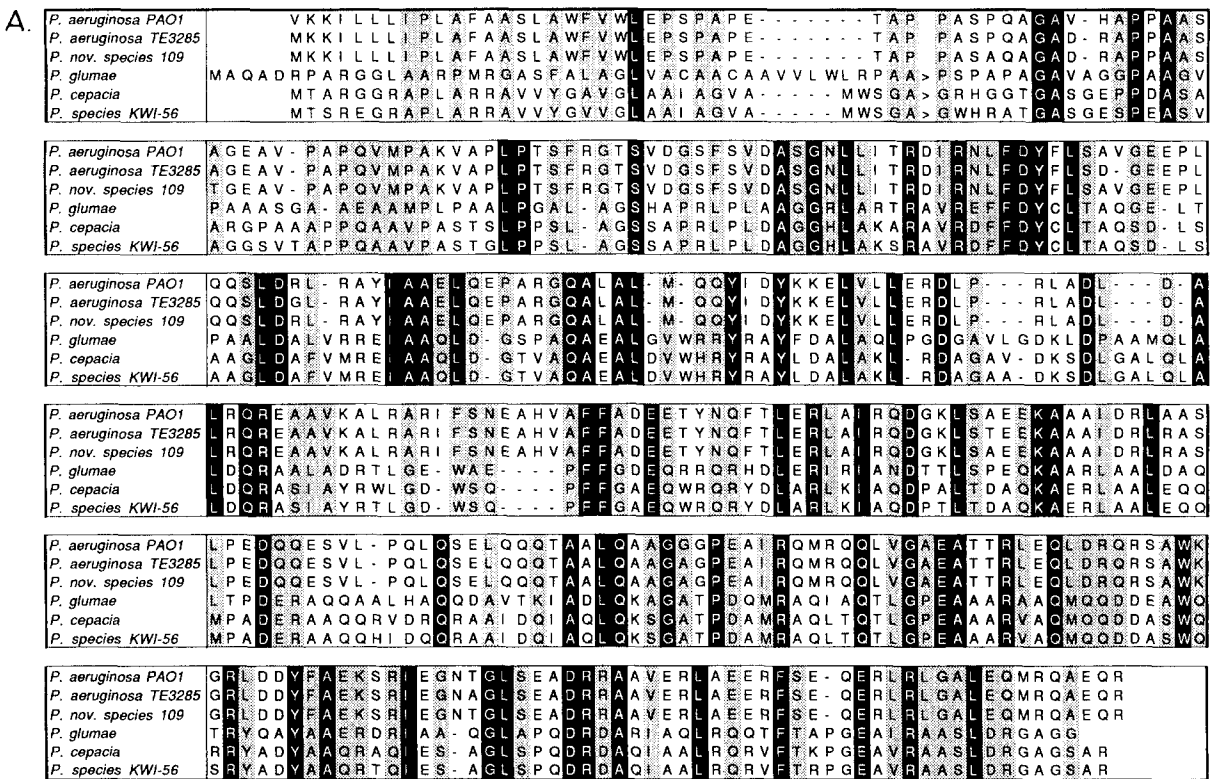


Fig. 5. Lif-proteins of *Pseudomonas*. (A) Comparison of amino acid sequences of Lif proteins from *P. aeruginosa* strains PAO1 ([108]; Schneidinger et al., in preparation), and TE3285 [109], *Pseudomonas* nov. species 109 [110], *P. glumae* [105,106], *P. cepacia* [104], and *P. species* KWI-56 [107]. (B) Model for the function of Lif-proteins. A lipase (Lip) is shown to be transported by the two-step pathway. After being secreted through the bacterial inner membrane (im) by a Sec-dependent mechanism, folding of lipase in the periplasm (p) is assisted by a lipase-specific foldase (Lif) which is anchored to the im via its N-terminal part. After being folded the lipase is further secreted through the outer membrane (om) to reach the extracellular medium (em). The two-step secretion pathway including gene products Xcp Q, T, U, V, W, P, X, Y, Z and Sec is drawn essentially as described by Tommassen et al. [99]; the location and function of the Lif protein is as proposed for *P. glumae* LipB protein by Frenken [102,105,106].

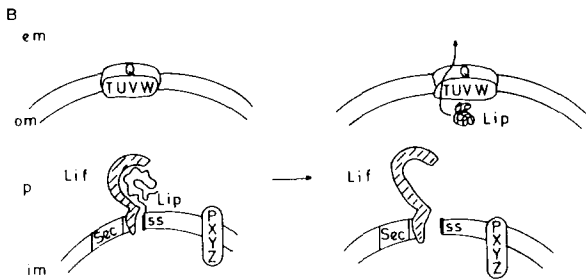


Fig. 5 (continued).

enzymatically active lipases from heterologous hosts. On the other hand, it may cause much fewer problems to isolate active enzyme from the culture medium if lipase genes are expressed which originate from strains as *P. fluorescens* or *S. marcescens* using the one-step pathway of secretion.

#### Role of accessory proteins

*Pseudomonas* strains producing lipases which belong to homology groups I and II (see below) are unique in that they contain additional genes coding for so-called helper proteins located immediately downstream the lipase structural genes. Helper proteins have been described for lipases of *P. cepacia* (LimA, [104]), *P. glumae* (LipB, [105,106]), *P. aeruginosa* (Act, [107], LipH, [108], LipB, [109], and LimL, [110]). Fig. 5A shows the amino acid sequences of these proteins which share a high degree of homology (about 75%). The *P. aeruginosa* protein LipH, in contrast to the published amino acid sequence [108], also shared this homology as already suggested by Frenken [102]. A PCR-amplified *lipH* gene was cloned, sequenced, subsequently overexpressed and the N-terminal amino acid sequence was determined (Schneidinger, B. and Jaeger, K.-E., manuscript in preparation). The amino acid sequence of the *P. aeruginosa* LipH protein shown in Fig. 5A was completed accordingly.

The helper proteins seem to act as molecular chaperones in that they assist lipase proteins in proper folding to achieve their native conformation. By using recombinant helper proteins, dena-

tured lipases could be renatured to yield enzymatically active lipase [111,112]. Furthermore, a complex formed by lipase and its helper protein could be precipitated by using antibodies raised against either lipase or the helper protein [112]. The most detailed analysis of the role of a helper protein has been performed with LipB protein from *P. glumae* [105,106]. This protein is anchored to the bacterial inner membrane by an N-terminal transmembrane helix and acts as a lipase-specific foldase, i.e. a chaperone-like protein which assists lipase in obtaining its enzymatically active conformation without requiring ATP-hydrolysis. Therefore, in order to avoid further confusion with the nomenclature of these proteins we suggest to name them using the first letter each of the genus and species name of the bacterium followed by the letters Lif for lipase-specific foldase (e.g. *PaLif* for *P. aeruginosa* lipase-foldase, formerly LipH). Fig. 5B schematically summarizes the present knowledge on the role of these proteins which is mainly based on the studies by Leon Frenken and co-workers [102,105,106] of *PgLif* (formerly *P. glumae* LipB protein). The two-step secretion pathway is depicted as described for *P. aeruginosa* enzymes [99] with lipase being anchored to the cytoplasmic membrane via its signal peptide. The correct folding which takes place in the periplasm is somehow mediated by the Lif protein which is also anchored to the cytoplasmic membrane. Finally, the correctly folded lipase is secreted through the outer membrane mediated by one or more of XcpQ, T, U, V, and W proteins [99]. It is evident from this figure that there remain many open questions concerning both the secretion and localization of the Lif proteins themselves and the mode and specificity of their interaction with the lipase enzymes.

In Gram-positive bacteria, no accessory proteins have been described so far. The N-terminal pro-parts of the Gram-positive lipases may function as intramolecular lipase-specific foldases which are cleaved off after secretion of the corresponding lipase protein is completed. A similar folding mechanism assisted by intramolecular chaperones has been proposed for proteases of Gram-positive bacteria [113].

### Biochemistry and molecular genetics of lipases

A variety of lipases from both Gram-positive and Gram-negative bacteria have been purified, biochemically characterized and the respective genes cloned and sequenced. Table 1 lists 24

different lipases with the majority of them (19 lipases) being produced by Gram-negative bacteria. The most important Gram-negative genus is *Pseudomonas* with at least seven different lipase-producing species and five lipases characterized originating from *P. aeruginosa* alone. The charac-

Table 1

#### Properties of bacterial lipases

Source of lipase	Gene cloned and sequenced	Signal sequence (aa)	Helper protein	Molecular mass (kDa)	Substrate specificity	Specific features	References
<i>S. aureus</i>	yes	yes 37	no	76	broad	synthesized as preproprotein	[114–117]
<i>S. hyicus</i>	yes	yes 38	no	71.4	broad	synthesized as preproprotein	[24,118]
<i>S. epidermidis</i>	yes	yes 31	no	77	n.d.	synthesized as preproprotein	[119]
<i>B. subtilis</i>	yes	yes 31	no	19.4	1,3 position and C8-FA	stable at pH 12	[19,120]
<i>Streptomyces</i> species	yes	yes 48	no	27.9	n.d.	no	[121]
<i>Aeromonas hydrophila</i>	yes	yes 48	no	71.8	preference for C6–C8-FA	no	[122]
<i>Xenorhabdus luminescens</i>	yes	yes 24	no	68.1	n.d.	no	[123]
<i>Moraxella</i> species	yes	no	no	34.7	n.d.	active at 4°C	[124–126]
<i>Propionibacterium acnes</i>	no	n.d.	no	41.2	broad	forming high $M_r$ aggregates	[127]
<i>Chromobacterium viscosum</i>	yes	n.d.	no	33	broad	active in aqueous and organic solvents	[128,129]
<i>Pseudomonas aeruginosa</i> PAO1/PAC1R	yes	yes 26	yes	30	broad	forming high $M_r$ aggregates with LPS	[48,108,130]
<i>P. aeruginosa</i> TE3285	yes	yes 26	yes	30	n.d.	no	[109,111]
<i>P. species</i> 109	yes	yes 26	n.d.	30	preference for C4–C6-FA	catalyzes formation of lactones	[131]
<i>P. aeruginosa</i> EF2	no	n.d.	n.d.	29	1,3 position and C18-FA	forming high $M_r$ aggregates	[132]
<i>P. aeruginosa</i> YS7	no	n.d.	n.d.	40	n.d.	active in 99.5% DMSO	[133]
<i>P. alcaligenes</i> 24	yes	yes	yes	30	1,3 position and C12–C18-FA	no	[134,135]
<i>P. fragi</i>	yes	no	n.d.	30	broad	stable at pH 9 and 50°C	[136–138]
<i>P. glumae</i>	yes	yes 39	yes	33	broad	contains $Ca^{2+}$ -binding site	[34,139]
<i>P. cepacia</i>	yes	yes 44	yes	33	broad	no	[104]
<i>P. species</i> KWI56	yes	yes 44	yes	33	n.d.	no	[107]

Table 1 (continued)

Source of lipase	Gene cloned and sequenced	Signal sequence (aa)	Helper protein	Molecular mass (kDa)	Substrate specificity	Specific features	References
<i>P. species</i> ATCC 21808	no	n.d.	n.d.	35	preference for C8-C10-FA	very hydrophobic	[140]
<i>P. fluorescens</i> B52	yes	no	no	50.2	n.d.	no	[141]
<i>P. fluorescens</i> SIKW1	yes	yes 23	no	48	1,3 position and C6-C8-FA	activation by Ca <sup>++</sup>	[142-144]
<i>P. putida</i>	no	n.d.	n.d.	45	n.d.	stable at 75°C	[145]

Abbreviations: aa, amino acids; C8-FA, fatty acid with a chain length of eight carbon atoms.

teristics shown in Table 1 and discussed below clearly indicate that these lipases represent a remarkably versatile group of enzymes, which share only little homology on the basis of amino acid sequences. Probably the most important criterion to compare the biochemical properties of lipases and to judge their commercial usefulness is the determination of their specific activities and substrate specificities which show extensive variation (Table 1). Although researchers try to experimentally address this point as careful as possible there remain doubts as to the comparability of the results. Difficulties arise from the fact that no standard assay system is available and, in addition, the proper performance of these assays requires not only sophisticated technical equipment (see above) but also a considerable degree of experience by the experimenter. We therefore strongly recommend to initiate a joint study to determine substrate specificities of all available bacterial lipases<sup>1</sup>.

#### Gram-positive bacteria

*S. aureus* and *S. hyicus* produce lipases which were subject of a number of independent studies [24,114-118] and their biochemical properties have been reviewed in detail [5]. These lipases have a broad substrate specificity, including the

ability to hydrolyze water-soluble substrates; the *S. hyicus* enzyme also exhibited phospholipase A and lysophospholipase activity [24]. The genes have both been sequenced revealing proteins of molecular masses 76 and 71.4 kDa, respectively [117,118]. These primary translation products were proteolytically processed to yield extracellular 46-kDa lipases which retained full enzymatic activity [24,95,96,98]. The lipase of *S. epidermidis* shares homology with the *S. aureus* and *S. hyicus* enzymes with a 77-kDa preproprotein which is processed to yield a 43-kDa mature extracellular lipase [119]. The lipase from *Streptomyces* species is a small protein of molecular mass 27.9 kDa which may require a second gene product for high level expression [121].

Probably the most interesting of the Gram-positive lipases is the enzyme of *B. subtilis*, which was already biochemically characterized 15 years ago [146]. Recently, the gene has been cloned and sequenced, revealing an exceptionally small protein of  $M_r$  19400. The first Gly-residue in the lipase-specific consensus sequence Gly-X<sub>1</sub>-Ser-X<sub>2</sub>-Gly was found to be altered into Ala [120] and biochemical characterization revealed an extremely alkaline optimum of pH 10 [19].

#### Gram-negative bacteria

Most of the Gram-negative lipases described so far belong to the genus *Pseudomonas* and have recently been reviewed in greater detail [8]. A comparison of the amino acid sequences suggests to divide the *Pseudomonas* lipases into three groups [102]. If the ordering starts with the smallest lipases following increasing  $M_r$ , group I com-

<sup>1</sup> Such a study could be performed by an experienced and well equipped laboratory, e.g. that headed by Robert Verger in Marseille, France. This group has published a comparative study of 25 different lipases; however, only five of them were of bacterial origin [39].

prises lipases from *P. aeruginosa*, *P. alcaligenes*, *P. fragi*, and *Pseudomonas* species 109. The prototype enzyme of this group is the lipase from *P. aeruginosa* PAO1 which is a reference wild-type strain. The corresponding lipase gene has been cloned and sequenced [108], this lipase is probably identical to the one from *P. aeruginosa* PAC1R which has been extensively characterized on the basis of N-terminal amino acid sequence,  $M_r$ , biochemical properties and immunological crossreaction with the *P. aeruginosa* PAO1 enzyme ([48,130,147]; and Jaeger, K.-E., unpublished results). The group I lipases have about 285 amino acids corresponding to a  $M_r$  of 30 000, they contain two Cys-residues forming a disulfide bond and require an additional gene product for correct folding and secretion (lipase-specific foldase, see above). The lipase from *P. aeruginosa* strain EF2 had an acid  $pI = 4.9$  and showed a marked regiospecificity for 1,3-oleoyl residues of triolein [132], whereas the other *P. aeruginosa* lipases had a  $pI = 5.9$  and did not show positional specificity. The enzyme from *P. aeruginosa* PAC1R, however, was absolutely stereoselective towards the sn-1 position of the triglyceride substrate trioctanoin [39]. Another interesting observation was the association of the *P. aeruginosa* PAC1R lipase with lipopolysaccharide (LPS) [48] which is the major lipid component of the outer membrane of Gram-negative bacteria. Native lipase was shown to be an amphiphilic protein forming large micellar aggregates with LPS which could be isolated from bacterial culture medium [148]. The occurrence of lipase in the form of lipase-LPS micelles could explain some properties that have frequently been observed upon purification and characterization of *Pseudomonas* lipases: (i) hydrophobic properties and the presence of high  $M_r$  aggregates [131,132,148], and (ii) localization of the enzyme at the outer site of the bacterial outer membrane [133,135]. The presence of LPS in purified *Pseudomonas* lipases may easily be overlooked, e.g. if SDS-polyacrylamide gels, used to monitor purification progress, are stained with Coomassie brilliant blue, which, in contrast to silver nitrate, will not stain LPS. In the case of *P. aeruginosa* lipase, trace amounts of residual LPS have been detected even in highly

purified lipase samples on Western blots using monoclonal antibodies directed against *P. aeruginosa* LPS (Jaeger, K.-E. and Kinscher, D.A., unpublished results).

*Pseudomonas* group II lipases which show about 60% amino acid homology to those of group I consist of about 320 amino acid residues having a  $M_r$  of about 33 000 and contain one disulfide bridge. The prototype lipases are those from *P. cepacia* and *P. glumae* which have been extensively characterized both biochemically and genetically by researchers from Novo-Nordisk (Denmark) and Unilever (the Netherlands), respectively [104,139]. They exhibit a broad substrate specificity making them suitable candidates for biotechnological applications, e.g. as an additive to household detergents. The stability of *P. glumae* lipase has been improved with respect to proteolytic degradation by genetic engineering via site-directed mutagenesis of residues forming a primary proteolytic cleavage site [149]. Expression of both genes in different heterologous hosts clearly demonstrated the absolute requirement of an additional gene product (Lif-protein) to achieve the enzymatically active conformation [104–106,112]. Within group II, the lipase from *C. viscosum* should be mentioned which is commercially available (e.g. from Toyo Jozo, Japan) and has been extensively characterized and found useful for industrial applications [53,128]. Recently, a comparison of the three-dimensional structures of lipases from *P. glumae* [150] and *C. viscosum* [151] revealed similarities. These results were confirmed by comparison of both lipases with respect to their biochemical properties and N-terminal amino acid sequences, suggesting that *C. viscosum* lipase is closely related or identical to the *P. glumae* enzyme [152].

Group III lipases of *Pseudomonas* are considerably larger, with about 475 amino acids and a  $M_r$  of 50 000. The prototype organism producing this type of lipase is *P. fluorescens* which belongs to rRNA homology group I as do the members producing group I lipases like *P. aeruginosa* [153]. These lipases are clearly different from group I and II lipases in that they (i) do not contain a typical signal sequence, (ii) do not contain Cys-residues, and (iii) do not require any Lif-like pro-

teins. These characteristics point to a different mechanism of secretion for these lipases as was recently demonstrated for *P. fluorescens* lipase which is secreted by the one-step pathway ([103]; see above). *P. fluorescens* lipase was overexpressed in *E. coli* forming inclusion bodies which were solubilized and subsequently refolded to yield enzymatically active enzyme which was stable at 50°C and preferentially hydrolyzed 1,3 positions of triglyceride substrates [144].

A lipase from *P. putida* was recently purified with a  $M_r = 45\,000$  which proved suitable to hydrolyze fats in isooctane/water two phase systems [145]. The determination of the amino acid sequence has to be awaited to decide whether this lipase belongs to one of the three *Pseudomonas* groups described above.

An interesting type of regulation of lipase activity was described for the lipase of the entomopathogenic bacterium *Xenorhabdus luminescens* which lives in symbiosis with insect parasitic nematodes and exists in a primary and secondary form with the former having about six times more lipase activity in the culture supernatant than the latter. However, both forms of the bacterium showed a similar level of transcription of the lipase gene and translation as judged from Northern and Western blots; therefore, a so far unknown post-translational regulation of lipase activity was assumed [123].

#### *Bacteria from extreme environments*

*Moraxella* strains were isolated from antarctic sea [124]. Their lipases ( $M_r$  35 000) are still active at 4°C. A striking homology was found in a region spanning 89 amino acids around the catalytic Ser of *Moraxella* lipase and human hormone-sensitive lipase which is also active at low temperature. The homologous region flanking the catalytic site should render both lipases more flexible, thereby facilitating substrate hydrolysis at low temperatures, giving rise to an increased cold-adaptability of both organisms [154]. Recently, a lipase gene from the antarctic facultative psychrophile strain *Psychrobacter immobilis* was cloned and sequenced revealing a 317-amino acid prolipase protein of  $M_r$  35 200 which also showed

the characteristic homology around the catalytic site described for *Moraxella* lipase [155]. From Icelandic hot springs, thermophilic bacteria have been isolated which belong to the genera *Thermus* and *Bacillus* which produce lipases active at 80°C, and in a cell-bound form even at 100°C [156]. Further biochemical characterization yielding structural information on these lipases is eagerly awaited.

### **Three-dimensional structure of lipases**

#### *Amino acid sequence comparison*

Bacterial lipases vary considerably in size (20–60 kDa), and, although the alignment of their secondary structural elements reveals a certain degree of variation, they presumably all have a similar overall three-dimensional structure. Alignment of several bacterial lipases (Fig. 6) revealed only little sequence homology. Lipases have been ordered into four groups with the first one containing four lipases of *Pseudomonas* strains which can be divided into two sub-groups. Subgroup I contains the lipases of *P. aeruginosa* and *P. alcaligenes* with a length of 285 amino acid residues. The second sub-group contains the lipases from *P. glumae* and *P. cepacia* with a length of 320 residues. A smaller lipase of *P. fragi* with 277 residues more likely belongs to the first sub-group. The third group of *Pseudomonas* lipases contains the lipases of *P. fluorescens* which are much larger than those of the two previous sub-groups, and show only limited sequence homology with subgroups I and II. They are related to the lipase from the enterobacterium *S. marcescens*, although this lipase has a large extension at the C-terminal part. Two other bacterial lipase groups defined in Fig. 6 are the one containing *Bacillus* lipases, and another one containing *Staphylococcus* lipases. Comparison of lipases from these four groups reveals that the only obvious sequence homologies are located at the N-terminal part with strand number 4 containing the oxyanion hole, the sequence around the active site serine residue with strand 5 and the following helix, strand number 6 next to this



**I**

		1	5	10	15	20	25	30	35	40	45	
<i>P. glumae</i>		A	D	Y	A	A	T	R	<b>P</b>	<b>V</b>	<b>L</b>	<b>V</b>
<i>P. cepacia</i>		A	A	G	Y	A	A	T	<b>P</b>	<b>V</b>	<b>L</b>	<b>V</b>
<i>P. aeruginosa</i>		S	T	Y	T	Q	T	K	<b>P</b>	<b>V</b>	<b>L</b>	<b>V</b>
<i>P. alcaligenes</i>		G	L	F	G	S	T	G	<b>P</b>	<b>V</b>	<b>L</b>	<b>V</b>
<i>B. subtilis</i>		A	E	H	N	P	V	V	<b>V</b>	<b>M</b>	<b>V</b>	<b>H</b>
<i>B. pumilus</i>		A	E	H	N	P	V	V	<b>V</b>	<b>M</b>	<b>V</b>	<b>H</b>
<i>S. aureus</i>		A	N	Q	V	O	P	L	<b>N</b>	<b>K</b>	<b>Y</b>	<b>P</b>
<i>S. hyicus</i>		V	K	A	A	E	A	P	<b>E</b>	<b>A</b>	<b>K</b>	<b>G</b>
<i>P. fluorescens</i>		T	A	Q	A	E	V	L	<b>G</b>	<b>K</b>	<b>Y</b>	<b>D</b>
<i>S. marcescens</i>		T	A	Q	A	E	V	L	<b>G</b>	<b>K</b>	<b>Y</b>	<b>D</b>

		30	35	40	45	50	55	60	65	70	75	80
<i>P. glumae</i>		L	S									
<i>P. cepacia</i>		L	S									
<i>P. aeruginosa</i>		V	S									
<i>P. alcaligenes</i>		V	S									
<i>B. subtilis</i>		A	V									
<i>B. pumilus</i>		A	I									
<i>S. aureus</i>		H	Q	A	S	V	S	A	L	S	N	H
<i>S. hyicus</i>		Y	E	A	S	V	S	A	L	S	N	H
<i>P. fluorescens</i>		C	R									
<i>S. marcescens</i>		T	L									

		85	90	95	100	105	110	115	118	120	122
<i>P. glumae</i>		V	G	H	S	O	G	G	L	S	R
<i>P. cepacia</i>		V	G	H	S	O	G	G	L	S	R
<i>P. aeruginosa</i>		V	G	H	S	O	G	G	L	S	R
<i>P. alcaligenes</i>		V	G	H	S	O	G	G	L	S	R
<i>B. subtilis</i>		V	A	H	S	M	O	G	G	A	N
<i>B. pumilus</i>		V	A	H	S	M	O	G	G	A	N
<i>S. aureus</i>		V	G	H	S	M	O	G	G	A	N
<i>S. hyicus</i>		V	G	H	S	M	O	G	G	A	N
<i>P. fluorescens</i>		S	G	H	S	L	G	L	A	V	N
<i>S. marcescens</i>		S	G	H	S	L	G	L	A	V	N

		120	125	130	135	140	145	150	155	160	165	170
<i>P. glumae</i>		E	F	A	D	F	V	Q	D	V	L	A
<i>P. cepacia</i>		E	F	A	D	F	V	Q	D	V	L	A
<i>P. aeruginosa</i>		D	T	A	D	F	L	R	Q	I	P	
<i>P. alcaligenes</i>		D	T	A	D	F	L	R	Q	I	P	
<i>B. subtilis</i>												
<i>B. pumilus</i>												
<i>S. aureus</i>		Q	A	A	K	F	G	N	T	E	A	V
<i>S. hyicus</i>		H	A	S	D	I	G	N	T	P	I	R
<i>P. fluorescens</i>		I	G	Y	E	N	D	P	V	F	R	A
<i>S. marcescens</i>		I	G	Y	E	N	D	P	V	F	R	A

**II**

		175	180	185	190	195	200	205	210	215	220	225
<i>P. glumae</i>		Y	N	R	N	F	P	S	A	G	L	G
<i>P. cepacia</i>		Y	N	R	N	F	P	S	A	G	L	G
<i>P. aeruginosa</i>		F	N	A	K	Y	P	Q	G	I	P	T
<i>P. alcaligenes</i>		F	N	A	K	Y	P	Q	G	I	P	T
<i>B. subtilis</i>												
<i>B. pumilus</i>												
<i>S. aureus</i>		S	D	D	N	A	A	Y	D	L	T	L
<i>S. hyicus</i>		S	E	D	T	G	L	Y	D	L	T	L
<i>P. fluorescens</i>		V	S	H	L	P	F	F	Y	D	G	M
<i>S. marcescens</i>		L	S	H	L	P	F	F	Y	D	G	M

		235	240	245	250	255	260	265	270	275	280
<i>P. glumae</i>		D	V	A	N	T	D	P	S	T	L
<i>P. cepacia</i>		D	P	A	N	V	L	D	L	S	T
<i>P. aeruginosa</i>		N	F	L	-	D	-	P	S	D	A
<i>P. alcaligenes</i>		N	V	L	-	D	-	V	S	D	L
<i>B. subtilis</i>											
<i>B. pumilus</i>											
<i>S. aureus</i>		-	P	D	L	G	T	F	F	L	M
<i>S. hyicus</i>		-	A	D	L	G	M	E	F	T	K
<i>P. fluorescens</i>		-	-	-	-	V	Q	D	L	N	R
<i>S. marcescens</i>		-	-	-	-	V	Q	D	L	N	R

		285	290	295	300	305	310	315	320
<i>P. glumae</i>		V	I	S	T	S	Y	H	-
<i>P. cepacia</i>		V	L	S	T	S	Y	H	-
<i>P. aeruginosa</i>		V	I	R	D	N	Y	R	-
<i>P. alcaligenes</i>		V	I	R	D	N	Y	R	-
<i>B. subtilis</i>		R	N	V	Q	I	H	G	-
<i>B. pumilus</i>		R	N	V	Q	I	H	G	-
<i>S. aureus</i>		V	K	P	I	Q	G	-	W
<i>S. hyicus</i>		V	M	P	T	M	K	G	-
<i>P. fluorescens</i>		G	H	N	T	F	L	F	-
<i>S. marcescens</i>		G	Y	N	L	I	A	G	-

Table 2

Crystallization conditions and preliminary X-ray analysis of several bacterial lipases

Source	Crystallisation conditions	Space groups	Unit cell parameters	Diffraction limit (Å)	Matthews volume (Å <sup>3</sup> /Da)	Reference
<i>Pseudomonas cepacia</i>	50 mM Hepes, pH 7.5 60% (v/v) methyl-pentane-diol 0.2 M sodium citrate 0.36% β-octyl glucoside, 22°C	<i>P</i> 2 <sub>1</sub>	<i>a</i> = 84.91 Å <i>b</i> = 47.33 Å <i>c</i> = 86.00 Å β = 116.09°	1.6	2.35	[160]
<i>Pseudomonas</i> species	pH 5.0–5.5 8% (w/w) sodium tartrate 4.8% octyltetra- oxyethylene 1.6% β-octyl glucoside	<i>C</i> 2	<i>a</i> = 92.7 Å <i>b</i> = 47.4 Å <i>c</i> = 86.5 Å β = 122.3°	2.5	2.4	[140]
<i>Pseudomonas glumae</i>	0.1 M Tris, pH 9.0 10% (v/v) acetone 27–29% PEG 8000 trace of β-octyl glucoside	<i>P</i> 2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	<i>a</i> = 158.1 Å <i>b</i> = 158.6 Å <i>c</i> = 63.4 Å	3.0	3.0	[150,161]
<i>Pseudomonas putida</i>	pH 4.5 potassium tartrate	<i>P</i> 4 <sub>3</sub> 2 <sub>1</sub> 2	<i>a</i> = 58.5 Å <i>b</i> = 58.5 Å <i>c</i> = 144.8 Å	2.5		[162]
<i>Pseudomonas fluorescens</i>	pH 8.5 35% (v/v) propanol 4°C and 22°C	<i>C</i> 2	<i>a</i> = 92.0 Å <i>b</i> = 47.2 Å <i>c</i> = 85.2 Å β = 121.5°	1.6	2.20	[163]
<i>Chromobacterium viscosum</i>	pH 6.4 10–14% PEG 4000 10–14% methyl- pentanediol 0.25% β-octyl glucoside	<i>P</i> 2 <sub>1</sub> 2 <sub>1</sub> 2	<i>a</i> = 41.1 Å <i>b</i> = 156.8 Å <i>c</i> = 43.6 Å	2.2	2.15	[151]
<i>Bacillus subtilis</i>	0.1 M ethanolamine pH 9–10 38–45% PEG 4000 10–25 mM sodium sulfate 0.7% β-octyl glucoside 16–22°C	<i>C</i> 2	<i>a</i> = 121.2 Å <i>b</i> = 93.2 Å <i>c</i> = 81.0 Å β = 110.7°	2.5	2.9	[164]

Space group numbers are written as defined in the International Tables for Crystallography [165].

Fig. 6. Comparison of amino acid sequences of bacterial lipases. From top to bottom, lipases are from *P. glumae* [139], *P. cepacia* [104], *P. aeruginosa* [108], *P. alcaligenes* [135], *B. subtilis* [120], *B. pumilus* [157], *S. aureus* [117], *S. hyicus* [118] (46-kDa mature lipase), *P. fluorescens* [143] (117 residues at the N-terminal part are not shown) and *S. marcescens* [158] (117 residues at the N-terminal and 151 residues at the C-terminal part are not shown). Black boxes represent amino acid residues highly conserved in at least two of the four groups of lipases; gray boxes represent similar amino acid residues. The numbering of amino acids is given for the *P. glumae* lipase. Secondary structural elements (h: α-helices, s: β-strands) refer to the *P. glumae* lipase and are numbered as defined by Ollis et al. [159].

residue, and a small region around the active site aspartate residue. These small regions of homology, however, permit to postulate that all these lipases will have a similar fold which presumably resembles the so-called  $\alpha/\beta$  hydrolase fold [159].

One of the interesting differences in the sequences of the *Pseudomonas* lipases is the presence of 21 extra amino acids in the sequence of *Pseudomonas* subgroup II (from residue numbers 215–235 in *P. glumae* lipase, see Fig. 6) as compared to those from subgroup I. In the three-dimensional structure, these amino acids are involved in formation of a short  $\beta$ -sheet composed of two antiparallel  $\beta$ -strands pointing into the solvent [150]. The role of this extra structural element of subgroup II *Pseudomonas* lipases is not known.

#### *X-ray structure of lipases*

A number of laboratories are working towards crystallization of bacterial lipases in order to determine their three-dimensional structures. Table 2 lists the different crystallization conditions published for a number of different bacterial lipases.

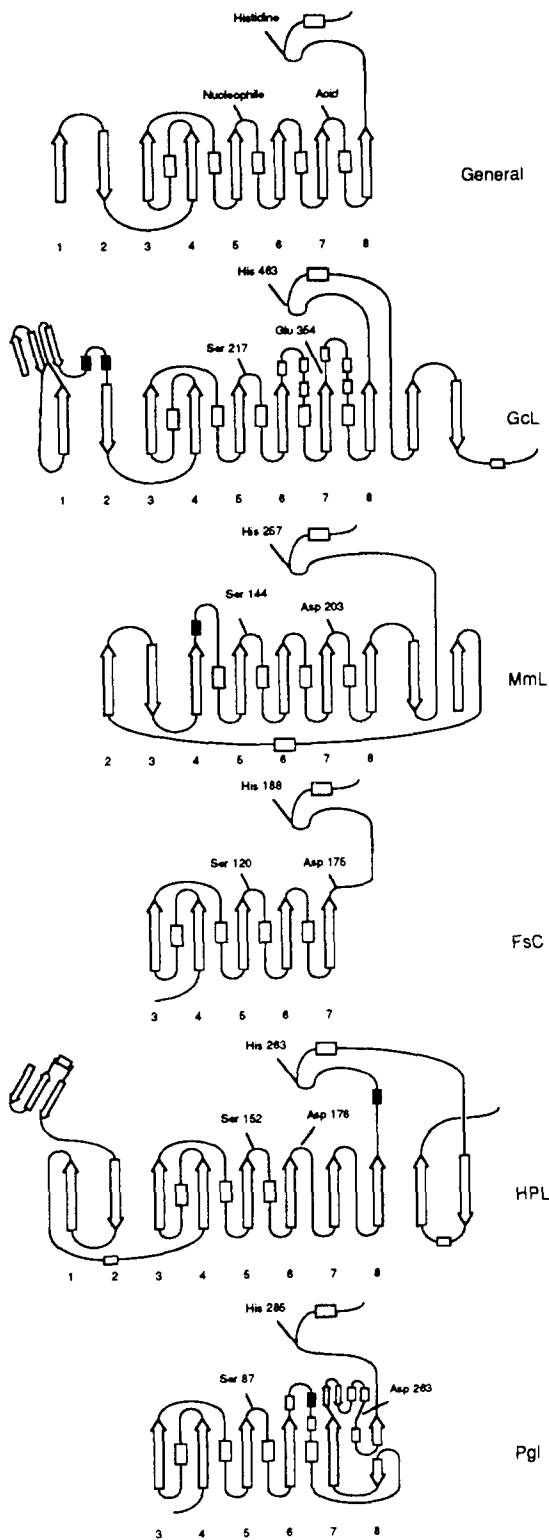
At present, five X-ray structures of microbial lipases have been elucidated. Four of them are fungal lipases from *Rhizomucor miehei* [21], *Geotrichum candidum* [166], *Candida rugosa* [167] and the cutinase of *Fusarium solani* [168]. The structures of the lipases from *Candida rugosa* and *Geotrichum candidum* are very similar since these two lipases share a high level of sequence homology. The only X-ray structure of a bacterial lipase published so far is the one of *P. glumae* [150]. In addition, the structure of lipase from human pancreas has been determined [20].

In spite of the evolutionary distance by which the microbial lipases are separated from the mammalian ones, certain intriguing regularities are observed. The six X-ray structures of lipases revealed an  $\alpha/\beta$  structure with a mixed central  $\beta$ -pleated sheet containing the catalytic residues, although the connections between conserved structural elements varied (see Fig. 7). This special  $\alpha/\beta$ -fold has now been recognized as a general folding pattern for different hydrolases [159] such as acetylcholine esterase [169], serine car-

boxypeptidase [170], haloalkane dehalogenase [171], and diene lactone hydrolase [172]. Based on this knowledge a three-dimensional structural model was built for the *P. aeruginosa* lipase which allowed to successfully predict biochemical properties of this lipase [18].

The catalytic centre of the lipases contains a serine-protease-like catalytic triad consisting of Ser-His-Asp residues, and the active site serine residue is located in a  $\beta$ - $\epsilon$ Ser- $\alpha$  motif. This motif consists of a six-residue  $\beta$ -strand (strand 5 in Fig. 7), a four-residue type II' turn with serine in the  $\epsilon$  conformation, and a buried  $\alpha$ -helix packed parallel against strand 4 and 5 of the central  $\beta$ -sheet. The invariant first and last glycine residues in the consensus sequence Gly-X-Ser-X-Gly (where X represents any amino acid) of this motif are in extended and helical conformations, respectively, which are conserved because of the steric requirements imposed by the packing stereochemistry of the  $\beta$ - $\epsilon$ Ser- $\alpha$  motif. This well conserved 'lipase box' differs in lipases from *Bacillus* strains where the first Gly residue is replaced by an Ala [120,157].

An unusual and interesting feature of the structure of lipases is that the active site is completely buried under a lid-like structure composed of one or two  $\alpha$ -helices (drawn in black in Fig. 7). This finding led to the hypothesis that triacylglycerol lipases undergo a conformational change in response to adsorption at the oil-water interface. The lid moves, thereby allowing the active site to become accessible for the substrate. This hypothesis has been confirmed by X-ray crystallographic studies of *R. miehei* and human pancreas lipases [22,23]. It seems that this important structural change of the protein is accompanied by another movement in a turn following  $\beta$ -strand 4 leading to the correct positioning of the so-called oxyanion hole. Another result of this conformational change is a significant increase in the hydrophobic surface of the enzyme which is involved in the lipid-surface recognition. The bacterial lipase of *P. glumae* also shows a buried active site. Its lid is formed by one  $\alpha$ -helix consisting of 13 residues. This lipase, as well as the one of *P. aeruginosa*, does, however, not show interfacial activation [18,34].



Finally, in contrast to other lipases, the one of *P. glumae* contains a bound calcium ion. Although located close to the active site, its role seems not to be catalytic, but to stabilize the local structure adjacent to the active site. The calcium binding site is expected to be well conserved among all *Pseudomonas* lipases. Fig. 8 displays the active site as well as the potential calcium binding site in a structural model of the lipase from *P. aeruginosa*.

### The catalytic triad

Determination of the three-dimensional structures of different lipases has confirmed their classification as 'serine hydrolases'. Their active site is composed of three residues: a serine residue hydrogen-bonded to a histidine residue, and a carboxylate-residue hydrogen bonded to this same histidine residue. The carboxylate may be either an aspartate or a glutamate residue. For lipases of *P. glumae* and *P. aeruginosa* the residues forming the catalytic triad are known [18,150]; for *S. hyicus* lipase there is good evidence from site-directed mutagenesis and inhibitor studies that residues Ser<sup>369</sup>, His<sup>600</sup>, and Asp<sup>559</sup> form the catalytic triad [173,174]. The architecture of the catalytic triad of lipases is very similar to the one found in serine proteases [20,21] (see also Fig. 8). During the reaction, a tetrahedral intermediate is formed which decomposes into an acyl-enzyme complex. The free lipase is regenerated by a hydrolytic reaction mediated by a water molecule. Fig. 9 describes the reaction mechanism of lipases hydrolyzing an ester bond. First, a nucleophilic attack of the oxygen of the serine side chain on the carbonyl carbon atom of the ester bond leads to the formation of a tetrahedral

Fig. 7. Comparison of secondary structural elements of lipases with known three-dimensional structure. Lipases are from *G. candidum* (GcL) [166], *R. miehei* (MmL) [21], *F. solani* (FsC) [168], human pancreas (HPL) [20] and *P. glumae* (Pgl) [150]. The model structure shown on top is deduced from a comparison of different hydrolase structures [159], numbering of the strands refers to this model. Helices of the lid-structures are drawn in black; amino acid residues forming the catalytic triads are indicated.

intermediate (Fig. 9, reaction 1). The histidine assists in increasing the nucleophilicity of the serine hydroxyl group. The histidine imidazole ring becomes protonated and positively charged. The positive charge is stabilized by the negative charge of the acid residue (Fig. 9, reaction 2). The tetrahedral intermediate is stabilized by two hydrogen bonds formed with amide bonds of residues which belong to the oxyanion hole. Finally, the alcohol is liberated leaving behind the acyl-enzyme complex (Fig. 9, reaction 3). By nucleophilic attack of a hydroxyl ion, the fatty acid is liberated and the enzyme regenerated (Fig. 9, reaction 4).

### Lipases as virulence factors

Most bacterial species investigated for lipase production and used for cloning of lipase genes are non-pathogenic, mainly because these lipases were intended to be used for biotechnological applications. There are, however, a few exceptions of lipases from bacterial strains which are known to play an important role in a variety of diseases. *Propionibacterium (Pb.) acnes* is involved in pathogenesis of acne vulgaris, a disease of the skin which affects close to 100% individuals during puberty differing only in severity of expression [175]. As a normal inhabitant of human skin it resides under micro-aerobic conditions inside the pilosebaceous follicles. Its main source of nutrients is thought to be sebum, a secretion of the sebaceous glands which is mainly composed of diverse lipids. *Pb. acnes* lipase which has been purified and biochemically characterized is considered to cleave part of the sebum triglycerides, thereby producing free fatty acids which may predispose human carriers to acne [127]. In addition, *Pb. acnes* lipase is regarded as a possible colonisation factor because it could be demonstrated that the presence of free fatty acids efficiently increased cell-to-cell adherence within the pilosebaceous follicles thereby promoting colonisation and persistence of *Pb. acnes* [176]. Another potentially pathogenic bacterium prevalent on human skin is *S. epidermidis* which also produces extracellular lipase. The lipase gene has

been cloned and sequenced, but the role of this lipase in pathogenesis has not yet been established [119].

Staphylococci are known to cause a variety of infections like deep and superficial abscesses, endocarditis, meningitis, wound infection and sepsis; they are also involved in food poisoning and toxic shock syndrome. Extracellular enzymes produced by *S. aureus* have been implicated in pathogenicity, especially coagulase, proteases, nucleases, pyrogenic exotoxins, at least four different hemolysins and also a lipase [177]. Anti-lipase IgG antibodies have been demonstrated in patients suffering from *S. aureus* infections [178], thereby proving that *S. aureus* produces lipase when residing in the infected patient. *S. aureus*

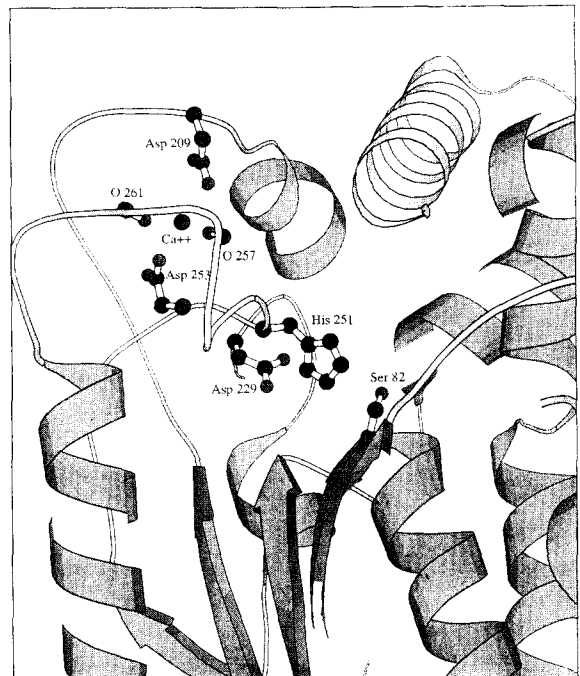


Fig. 8. Three-dimensional structure of the active site of the *P. aeruginosa* lipase in a model built using X-ray coordinates determined for *P. glumae* lipase [150]. The active site residues are Ser<sup>82</sup>, His<sup>251</sup> and Asp<sup>229</sup>; a potential calcium binding site is displayed with Asp<sup>209</sup>, Asp<sup>253</sup> and carbonyl oxygen atoms of residues 261 and 257 as the calcium ligands. The  $\alpha$ -helix shown on top (light print) could represent a lid-like structure as deduced from the X-ray structure of *P. glumae* lipase.

lipase could block phagocytic killing of bacterial cells by granulocytes. This effect was accompanied by a marked change of granulocyte surface structure which may indicate a severely impaired host defence [179].

Probably the most important pathogen known to produce a lipase is *P. aeruginosa* which causes a variety of infectious diseases in immunocompromised patients like those suffering from cancer, burn wounds or cystic fibrosis (CF) where *P. aeruginosa* infection of the lungs is still regarded as the main cause of death [180]. Clinical *P. aeruginosa* isolates from CF patients produced both lipase and phospholipase C [181]. Antisera obtained from CF patients with increasing duration of *P. aeruginosa* infection contained increasing amounts of anti-lipase antibodies indicating the presence of lipase in the *P. aeruginosa*-infected patient [182]. In vitro studies of the function of cells mediating the immune response revealed that lipase significantly inhibited monocyte chemotaxis and chemiluminescence [148]. Fur-

thermore, lipase triggered the release of 12-hydroxyeicosatetraenoic acid (12-HETE) from human platelets which are involved in inflammatory processes. Whereas *P. aeruginosa* lipase itself caused only moderate release of 12-HETE, a combination of lipase with phospholipase C yielded in a dramatic increase in the formation of this compound [183]. These results suggested a synergistic action of at least two lipolytic enzymes synthesized by *P. aeruginosa*. Further evidence supporting this concept came from in vitro studies demonstrating that the major lung surfactant lipid, i.e. dipalmitoylphosphatidyl-choline, was completely degraded only in the presence of both lipase and phospholipase C [181]. It is interesting to note that the bacterial phospholipases C have been studied in greater detail with respect to their role as potential virulence factors, e.g. the *S. aureus*  $\beta$ -toxin and the heat-labile toxin of *P. aeruginosa* [184]. There is no doubt that the infection process is a multifactorial event and various compounds synthesized and released by the bac-

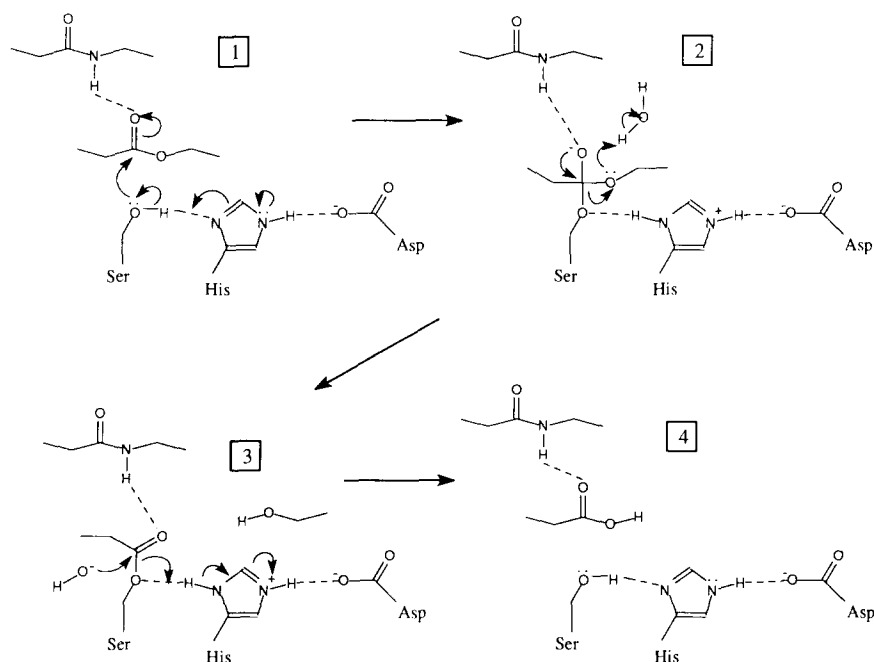


Fig. 9. Detailed mechanism of hydrolysis of an ester bond by a lipase (see text for detailed explanation).

terial cells may equally contribute to the establishment and persistence of the infecting organisms. Obviously, the existing data concerning the role of lipases as virulence factors are rather preliminary and many more studies are needed. The above-described findings clearly suggest that lipases should be studied in combination with other extracellular enzymes, especially phospholipases, which could provide reasonable explanations of a variety of effects related to membrane damage which are frequently observed in patients infected with lipase-producing bacteria.

### Lipases in biotechnology

For several decades, lipases are already used in industry although the number of applications and therefore their importance to the enzyme manufacturing industry was rather small [185]. It has been estimated earlier that from the worldwide enzyme market of 600 million US \$, only some 20 million is accounted for by lipases [186]. The major application of lipase was for flavour development in food such as Italian cheeses [187]. The reason for this low interest is most likely the limited availability and relatively high costs of these enzymes, especially for the potential larger applications such as the detergent industry. However, the production technology of lipases has made great progress in the last 5 years, mainly as a result of recombinant DNA technology by which it is possible to construct microbial strains which produce different types of lipases in an economically attractive way. As a consequence of this development, many different lipases from as many different microorganisms are now available and especially the detergent industry did benefit from this development and is by now the largest application area of industrial lipases.

Remarkably, all commercial applications of lipases thusfar concern enzymes of fungal or yeast origin. This does not imply inferior characteristics of bacterial lipases, but can be explained by the fact that the food applications, which until recently exceeded the number of non-food applications, prefer the use of lipases from fungal origin because of their proven GRAS-status (=

Generally Regarded As Safe). However, as will be shown in the following paragraphs, for various applications bacterial lipases are as good as, or sometimes to be preferred to, their eukaryotic counterparts.

### *Hydrolysis versus synthesis*

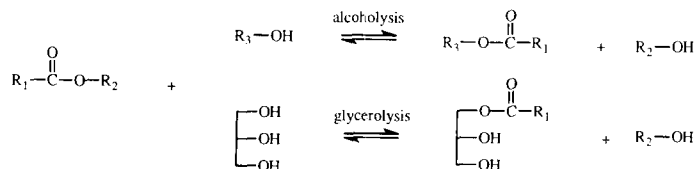
The hydrolysis of fats and oils (triacylglycerols) is an equilibrium reaction and therefore it is possible to change the direction of the reaction to ester synthesis by modifying the reaction conditions (Fig. 1). The equilibrium between forward and reverse reactions in this case is controlled by the water content of the reaction mixture, so that in a non-aqueous environment lipases catalyze ester synthesis reactions. Different types of synthesis reactions can be distinguished: common ester synthesis from glycerol and fatty acids and the biotechnologically more important transesterification reactions in which the acyl donor is an ester (Fig. 10). Transesterifications involving fats and oils can further be specified depending on the type of acyl acceptor. Glycerolysis and alcoholysis refer to the transfer of an acyl group from a triglyceride to either an alcohol or glycerol. In interesterifications (Fig. 10), the acyl group is exchanged between a (tri)glyceride and either a fatty acid (also called acidolysis) or a fatty acid ester (more specifically another (tri)glyceride). Interesterifications require a small amount of water, in addition to the amount needed for the enzyme to maintain an active hydrated state. As the presence of (too much) water will decrease the amount of ester synthesis products, the water content should be carefully adjusted to achieve accumulation of desired reaction products.

In the following paragraphs, the actual application of lipases as well as promising new developments will be summarized. In detergents, only the hydrolyzing capability of lipase is relevant, whereas in the processing of fats and oils as well as in organic synthesis both hydrolysis and synthesis reactions are of importance.

### *Detergents*

The great breakthrough in the application of lipase in household detergents came when

### Transesterification



### Interesterification

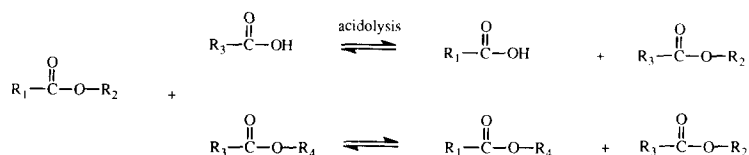


Fig. 10. Industrially important reactions catalyzed by a lipase. Transesterification involves the transfer of an acyl group to an alcohol (alcoholysis) or glycerol (glycerolysis); interesterification describes the transfer of an acyl group to a fatty acid (acidolysis) or a fatty acid ester.

NOVO/Nordisk launched the product Lipolase<sup>TM</sup> in 1988 (Table 3). This product contains the extracellular lipase from the fungus *Humicola lanuginosa* which is produced on industrial scale using *Aspergillus niger* as a host organism. Presently, this product is globally used by at least the two largest detergent manufacturers Procter & Gamble [188] and Unilever.

The second commercially available product is Lumafast<sup>TM</sup> (Genencor International) and contains a bacterial lipase from *P. mendocina*

(formerly known as *P. putida*). It differs from other known *Pseudomonas* enzymes as suggested by the lack of any amino acid sequence homology (O. Misset, unpublished results), but seems to be related to the cutinases [162] and hydrolyzes short-chain triglycerides [189].

Another lipase product for detergents was developed by Gist-brocades (Lipomax<sup>TM</sup>) and is expected to enter the enzyme market in 1995. This product contains the extracellular lipase from *P. alcaligenes* (formerly designated *P. pseudoalcali-*

Table 3

Microbial lipases used as additives in household detergents

Origin of lipase	Product name	Year of introduction	Company (location)
<b>Fungal</b>			
<i>Humicola lanuginosa</i>	Lipolase	1988	NOVO-Nordisk (Denmark)
<b>Bacterial</b>			
<i>Pseudomonas mendocina</i>	Lumafast	1992	Genencor (USA)
<i>Pseudomonas alcaligenes</i>	Lipomax	1995	Gist-brocades (The Netherlands)
<i>Pseudomonas glumae</i>	n.a.	n.a.	Unilever (The Netherlands)
<i>Pseudomonas species</i>	n.a.	n.a.	Solvay (Belgium)
<i>Bacillus pumilus</i>	n.a.	n.a.	Solvay (Belgium)

n.a., no annotation.



genes), the properties of which perfectly match with the conditions of the washing process [134]. The enzyme is active at pH 7–11 and at temperatures up to 60°C hydrolyzing triglycerides with chain lengths varying from C2 to C18. However, the highest activity is observed with longer chains (> C12).

Finally, the patent literature describes several other bacterial lipases which were screened for detergent applications such as those from *Pseudomonas* and *Bacillus* species.

### Processing of fats and oils

In the processing of fats and oils, both hydrolysis and synthesis take place. The number of applications in which lipase is used (see Table 4), however, is still limited when compared with the cheaper chemical processes.

### Hydrolysis

The conventional chemical fat-splitting processes require rather harsh conditions with re-

spect to temperature (240–260°C) and pressure (60 bar). This inevitably produces undesirable side effects, like product discoloration and degradation of some fatty acids. However, due to the cheapness and efficiency of the chemical process, enzyme applications may be economically competitive only in some special cases. Thusfar, only in one case application of lipase for common fat or oil hydrolysis on small industrial scale has been reported. The Japanese company Miyoshi Oil and Fat Co. has used a fungal lipase for the manufacturing of soap (Jap. Chem. Week, page 2, May 14, 198). However, fungal lipases are not necessarily the best choice, since *Pseudomonas* lipase(s) were shown to be superior to various fungal lipases in lab-scale hydrolyses of beef tallow [190] and castor oil [191].

### Glycerolysis

Chemical glycerolysis of fats and oils is used for the commercial production of monoglycerides, which are applied as emulsifiers in a wide range of foods and cosmetic and pharmaceutical

Table 4  
Biotechnological applications of bacterial lipases

Type of reaction	Origin of lipase	Product (application)	References
Hydrolysis of fats and oils	<i>Pseudomonas</i>		[190,191]
Glycerolysis of fats and oils	<i>Pseudomonas</i>	Monoacylglycerols (surfactants)	[192–197]
Esterification to glycerol	<i>Chromobacterium viscosum</i>		[198,199]
	<i>Pseudomonas fluorescens</i>		[198]
(Trans)esterification to immobilized glycerol	<i>Chromobacterium viscosum</i>		[200,201]
Acylation of sugar alcohols	<i>Chromobacterium viscosum</i>	Sugar monoacylestere (surfactants) Enrichment of PUFAs	[202]
Acidolysis/Alcoholysis of fish oils	<i>Pseudomonas</i>		[203,204]
Resolution of racemic alcohols/esters	<i>Arthrobacter</i> <i>Pseudomonas cepacia</i>	Building blocks for insecticides/chiral drugs	[12,206]
Polytranssterification of diesters with diols	<i>Chromobacterium</i>	Oligomers	[207]
	<i>Pseudomonas</i>	Alkyds (polyester intermediates)	[208,209]
	<i>Pseudomonas</i>	Macrocyclic lactones	[215]
Transesterification of monosaccharides	<i>Pseudomonas (cepacia)</i>	Acrylate esters (polyacrylate intermediates)	[210–212]
Intramolecular esterification	<i>Pseudomonas</i>	Macrocyclic lactones	[213,214]

products. It is not likely that this process will be replaced by an enzymatic process in the near future, although lab-scale enzymatic glycerolysis of various fats and oils has been described [192–197]. Mixtures of mono- and diacylglycerols were formed; high yields of monoglyceride were obtained with *Pseudomonas* lipases [193,197]. The reaction temperature should be below a certain critical value [192,195,196], which was also dependent on the type of fat or oil [192]. In addition, the water content of the reaction mixture was shown to be a critical factor [195,196]. Several fungal lipases were shown to be inactive [193].

#### *Esterification*

Glycerides can also be obtained by direct esterification of free fatty acids to glycerol (Fig. 1). However, esterification catalyzed by various microbial lipases always resulted in mixtures of glycerides, with yield and composition of the mixture depending on the source of lipase [198,199]. A process resulting in regioisomerically pure glycerides has been developed comprising as an essential step the adsorption of glycerol onto a solid support. Lipase-catalyzed glyceride synthesis with the immobilized glycerol and various acyl donors (e.g. free fatty acids, fatty acid alkylesters, natural fats and oils) yielded multigram quantities of regio-isomerically pure di- [200] and monoacylglycerols [201]. *C. viscosum* was one of the 1,3-selective lipases producing the desired glycerides with high yield. Monoglycerides were separated from the other reactants in a separate vessel and the undesired products were fed back to the reactor [201].

#### *Alcoholysis / acidolysis*

Using acylacceptors other than glycerol additional mono-acylcompounds can be synthesized. For example, alcoholysis of sugar alcohols with various plant and animal oils has been shown to yield sugar monoesters of fatty acids. Among various lipases, the enzyme from *C. viscosum* showed good catalytic properties [202]. However, as is the case for fat hydrolysis and glycerolysis, chemical synthesis of sugar esters is far cheaper than enzyme technology, hampering commercial

application of enzymes also in this field. On the contrary, refinement of oils containing highly unsaturated fatty acids (PUFAs, poly-unsaturated fatty acids) may be a process with prospects for enzyme application on a commercial scale, because PUFAs are easily subject to decomposition in the chemical process, yielding undesirable oxidation products and polymers. Since fish oils possess poly-unsaturated fatty acids predominantly at the 2-position and this bond is relatively resistant to lipase attack, 1,3-specific lipases can be particularly useful in the concentration of poly-unsaturated fatty acids in monoglycerides. This has been shown in the case of enzymatic alcoholysis of cod liver oil [203] and acidolysis of sardine oil [204], using various microbial lipases. In both cases, *Pseudomonas* lipase gave the best results.

#### *Interesterification*

In addition to the Miyoshi enzymatic oil hydrolysis process, a few lipase-catalyzed synthesis reactions in low-water environment have found (limited) application on commercial scale. An example is the transformation of low-value oils, like the palm-oil mid fraction, into high-value cocoa-butter triglycerides by interesterification. However, since this process is carried out using the immobilized lipase from *Rhizomucor miehei* it will not be further discussed here. Another class of so-called structured triglycerides which may be a potential product of a lipase-catalyzed interesterification reaction are glycerides containing medium-chain fatty acids on the 1- and 3-positions and an essential fatty acid on the 2-position. They can form an alternative for the medium-chain triglycerides which are currently used to meet the nutritional needs of patients with mal-absorption problems, because shortage of essential fatty acids in these patients can easily occur due to sole consumption of medium-chain lipids [205].

#### *Application in organic synthesis*

A literature survey on the application of lipases in organic synthesis reactions reveals an enormous increase in publications during the past few years, especially reactions in non-aqueous

media. In nearly all cases, reactions were described on laboratory scale, with commercial applications seldomly mentioned. It is not within the scope of this review to mention all these reactions, but the main areas of interest will be discussed.

#### *Biocatalytic resolution*

By far the most important application of lipases in organic chemistry is the production of optically active compounds. Most frequently, these compounds are produced through the resolution of racemic mixtures of alcohols or carboxylic esters, although stereospecific synthesis reactions are employed as well. Lipase-catalyzed resolution of racemic mixtures can occur through asymmetric hydrolysis of the corresponding esters, while in non-aqueous media this approach can be extended to stereospecific (trans)esterification reactions. In this way, optically active building blocks for insecticides have been obtained by an ester-hydrolysis reaction using *Arthrobacter* lipase [206]. In the synthesis of various chiral drugs such as  $\alpha$ -blockers, *P. cepacia* lipase (Lipase PS from Amano) is a frequently used enzyme for racemic mixture resolution, via both hydrolysis and acylation reactions (see [12] for a review). An important factor for the economic feasibility of biocatalytic resolution of racemic mixtures is the recovery of the unwanted enantiomer. Although enantiomer recovery is a commonly applied step after classical racemate separation, in lipase catalytic processes this issue has been addressed by only one group [206], who described chemical inversion of the unwanted (R)-alcohol into the (S)-form.

#### *Polymer synthesis*

If, instead of a racemic ester and alcohol (or vice versa), a diester and a diol are used, stereoselective polycondensations occur in organic media. In this way, the formation of optically active trimers and pentamers was observed, using among others a lipase from *Chromobacterium* species [207].

For the enzymatic synthesis of alkyds, unsaturated diesters are combined with aliphatic or aromatic diols in a polytransesterification reac-

tion using a *Pseudomonas* lipase. No isomerization of the double bond was observed under the mild conditions of the lipase-catalyzed reaction, in contrast to the extensive isomerization found during chemical polycondensation [208,209]. In a subsequent cross-linking reaction, alkyds can be polymerized to industrially applicable 'general-purpose polyesters'. Several chemoenzymatic processes have been described for the preparation of various polyacrylates. After a stereoselective reaction of a racemic alcohol with a (meth)acrylate ester as acylating agent using a *Pseudomonas* lipase, (meth)acrylate polymers of higher molecular mass could be obtained employing an additional chemical polymerization step [210]. *P. cepacia* lipase [211] or a lipoprotein lipase from *P. species* [212] catalyzed the transesterification of various monosaccharides with vinylacrylates, whereupon the resulting sugar-acrylate esters were chemically polymerized. The use of the resulting polymers for biomedical applications and membranes was suggested [211].

#### *Intramolecular esterification*

If hydroxyl and ester moieties are present in one molecule, intramolecular esterification occurs, resulting in the synthesis of macrocyclic lactones. C14–C16 macrocyclic lactones are high-grade and expensive substances with a musky fragrance, which are used in perfumes. Upon intramolecular esterification of several hydroxy acids, the yield and ratio of mono- to oligolactone was found to depend on the lipase and on the chain length of the substrate used [213,214]. In addition, macrocyclic lactones can be synthesized by direct condensation of diacids with diols [215].

#### *Flavour development in food*

Traditionally, bacterial lipases produced in situ in various food systems have been involved in development of flavour. Lipases from several bacterial species present in raw milk (mainly *Pseudomonas*, but also some others like *Alcaligenes* and *Achromobacter*) are known to withstand the pasteurization process and affect flavour development during cheese ripening. In addition, lipases produced by bacterial starters play a role in this

process [187]. Other examples of the involvement of lipolytic lactic acid bacteria in flavour development are vegetable fermentations and ripening of some Italian sausages.

## Conclusions

A steadily increasing number of bacterial lipase genes were cloned, sequenced and the corresponding proteins were biochemically characterized with respect to determination of  $M_r$ , pI, pH optimum, and substrate specificities (see Table 1). Furthermore, several environmental factors have been described to influence or regulate the synthesis and release of lipases. In Gram-negative bacteria, lipases may use both major pathways of secretion, depending on the strain studied. An exciting observation was the presence in the *Pseudomonas* family of lipase-specific foldases (Lif proteins) which seem to represent a unique class of chaperone-like proteins assisting lipases in correct folding. Hypothetical counterparts in Gram-positive bacteria may be the intramolecular pro-enzymes, i.e. N-terminally located peptides which are finally cleaved off to yield the mature enzymatically active extracellular lipases.

At present, no unambiguous evidence has been obtained as to the physiological role of lipases. The simple question: "Why do bacteria need lipases?" is commonly answered by referring to the potential of most bacteria to hydrolyze extracellular macromolecules as polysaccharides, proteins or fats by producing and secreting the corresponding hydrolytic enzymes. However, lipases may also exert physiological functions inside the cells or cellular membranes where they could be involved in the metabolism of lipids, perhaps including lipopolysaccharides. At least some clues may be expected from physiological studies with lipase-negative mutants obtained by replacement or inactivation of lipase structural genes.

The three-dimensional structures of bacterial lipases are expected to reveal the  $\alpha/\beta$  hydrolase fold found for lipases of eukaryotic origin, and also for the only known structure of a bacterial lipase. However, more three-dimensional structures of bacterial lipases are urgently needed in

order to understand some of their unique and surprising properties as (i) the variation of substrate specificity caused by a change of the chemical environment, (ii) the general occurrence and the importance for interfacial activation of the lid-like helical structures covering the active site and (iii) the role of ions, particularly  $\text{Ca}^{2+}$ , in stabilizing the three-dimensional structure and influencing enzyme activity. A more extended knowledge of three-dimensional structures of bacterial lipases would greatly facilitate to tailor lipases for industrial applications, thereby further promoting their role as important biotechnological tools.

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## Note added in proof

After submission of the manuscript it came to our knowledge that a bacterial lipase originating from *Pseudomonas aeruginosa* MB 5001 is also used for biotechnological applications by Merck Research Laboratories, Rahway, New Jersey, USA. This lipase catalyzes the formation of a precursor in the synthesis of a leukotriene antagonist (L. Katz et al. (1993), J. Industr. Microbiol. 11, 89–94), has been purified (M. Chartrain et al. (1993), Enz. Microbiol. Technol. 15, 575–580), and is produced during fed-batch cultivation in 2000-l bioreactors (M. Chartrain et al. (1993), J. Ferment. Bioeng. 76, 487–492).

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