GENERAL INTRODUCTION

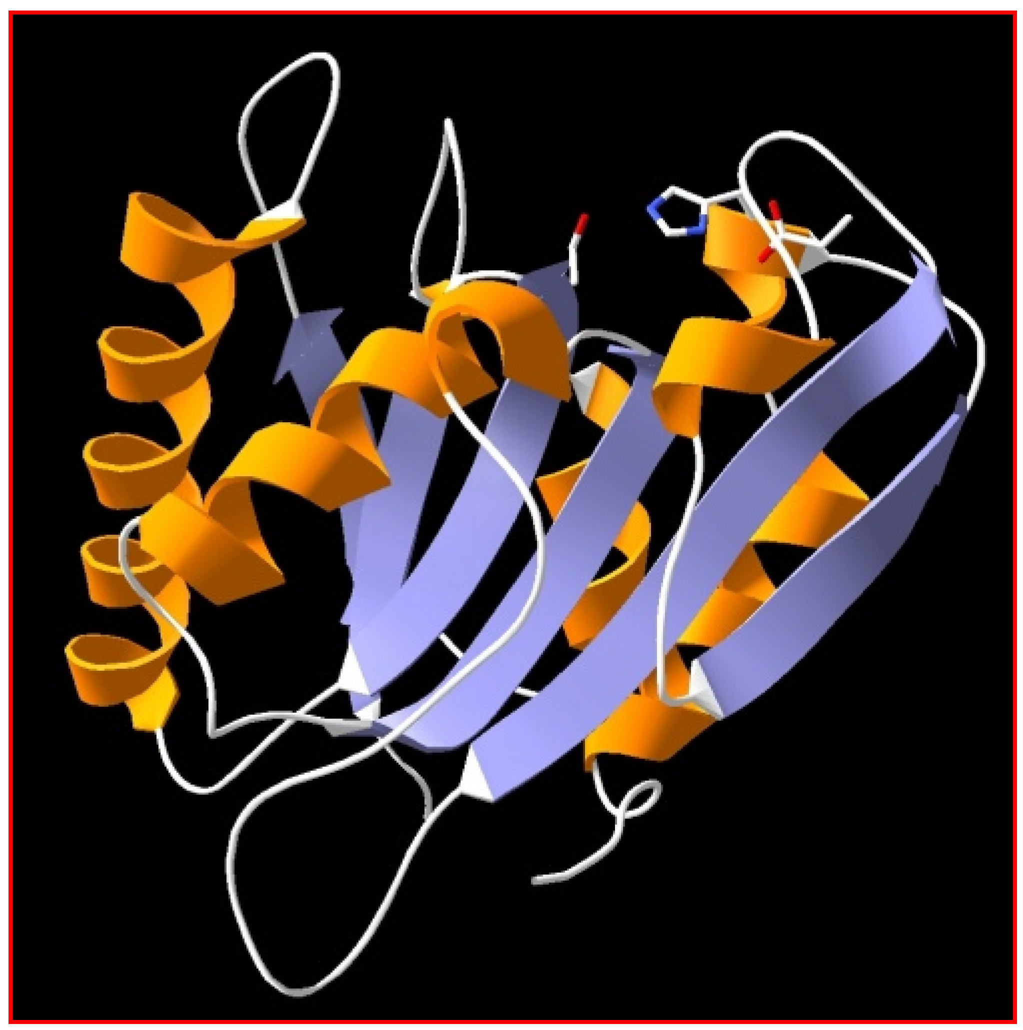


Figure I.1 Three-dimensional structure of *B. subtilis* LipA (van Pouderoyen *et al*., 2001).

1 LIPIDS: CLASSIFICATION AND PROPERTIES

1.1 DEFINITION AND PROPERTIES OF LIPIDS

No precise definition of the term “lipid” (Greek: *lipos*, fat) exists because they are better defined by their physical behaviour than by their chemical structure, which is very heterogeneous. In its broadest sense, "lipid" defines substances of biological origin as oils, fats and waxes that are soluble in organic solvents such as chloroform, but only sparingly soluble, if at all, in water. This definition covers a wide range of substances which contain moieties belonging chemically to hydrocarbons, alcohols, aldehydes, acids, amines and glucides. Most of them form molecules such as waxes, triglycerides, phospholipids, etc, whereas other substances such as fat soluble vitamins, coenzymes, pigments, terpenes, sterols and phenolics are also considered as lipids because they are extracted with “fat” solvents (Boyer, 2000; http://www.cyberlipid.org).

Lipids are mainly composed by carbon and hydrogen, elements that confer a non-polar behaviour, although they can also have polar groups containing oxygen, nitrogen, and phosphorous. The most common functional chemical groups present in lipids are simple or double carbon-carbon bonds, carboxylate esters, phosphate esters, and amides (Boyer, 2000). Therefore, lipids are hydrophobic or amphipathic (usually with a polar head connected to a non-polar structure), and they have a strong tendency to associate through non-covalent forces via an entropy-driven hydrophobic effect. This association is stabilized by van der Waals interactions between the hydrocarbon regions of the molecules. As a result, hydrophobic lipids are insoluble in aqueous environments and do not even form micelles very effectively, thus they form oily droplets. On the contrary, amphipathic lipids tend to form surface monolayers, bilayers, or micelles (Figure I.2) in contact with water (Mathews *et al*., 2000). The maximum concentration of monomers in aqueous solution is called “saturation value” (for hydrophobic molecules) or “critical micelle concentration” (CMC; for amphipathic compounds), and it corresponds to the concentration at which these molecules start to form emulsions of oil droplets or micelles, respectively (Jaeger *et al*., 1994).

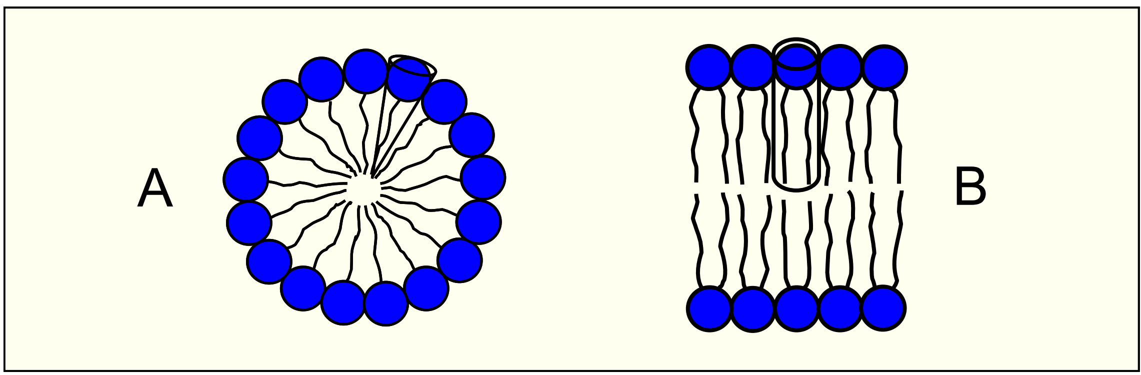


Figure I.2 Micelles and bilayers formed by amphipathic lipids.

Wedge-shaped lipids such as fatty acids tend to form spherical micelles in water (A), whereas cylindrical lipids such as phospholipids pack together better and form lipid bilayers (B) (Mathews *et al*., 2000).

Lipids are found in all living beings, where they carry out a wide range of functions due to their high chemical variability. They are involved in forming biological membranes, energy storage, heat, water or electric insulation, heat production, and intracellular or intercellular signalling. Moreover, they act as hormones, pigments, vitamins, enzymatic cofactors, electron transporters, and detergents (Boyer, 2000).

1.2 CLASSIFICATION OF LIPIDS

Previous classifications of lipids were founded on the well known ability of some lipids, said "saponificable", to be hydrolyzed by basic solutions into compounds such as glycerol and fatty acids. Lipids resistant to this type of treatment were named "unsaponificable". However, there are several crossing-overs between the two classifications, since polar and neutral lipids can be saponificable or not. This fact, together with the variability of the new types of lipids found during the last years, have led to the elaboration of a new classification based on the chemical composition of lipids (Boyer, 2000; http://www.cyberlipid.org). Thus, lipids can be divided into:

● **Simple lipids**: they contain one or two different types of compounds. However, lipids containing sugars (glycolipids) are excluded form this group, and are classified as complex lipids.

● **Complex lipids**: they are frequently constituted by three or more chemical identities (e.g. glycerol, fatty acids, and sugar), and they are usually amphipathic.

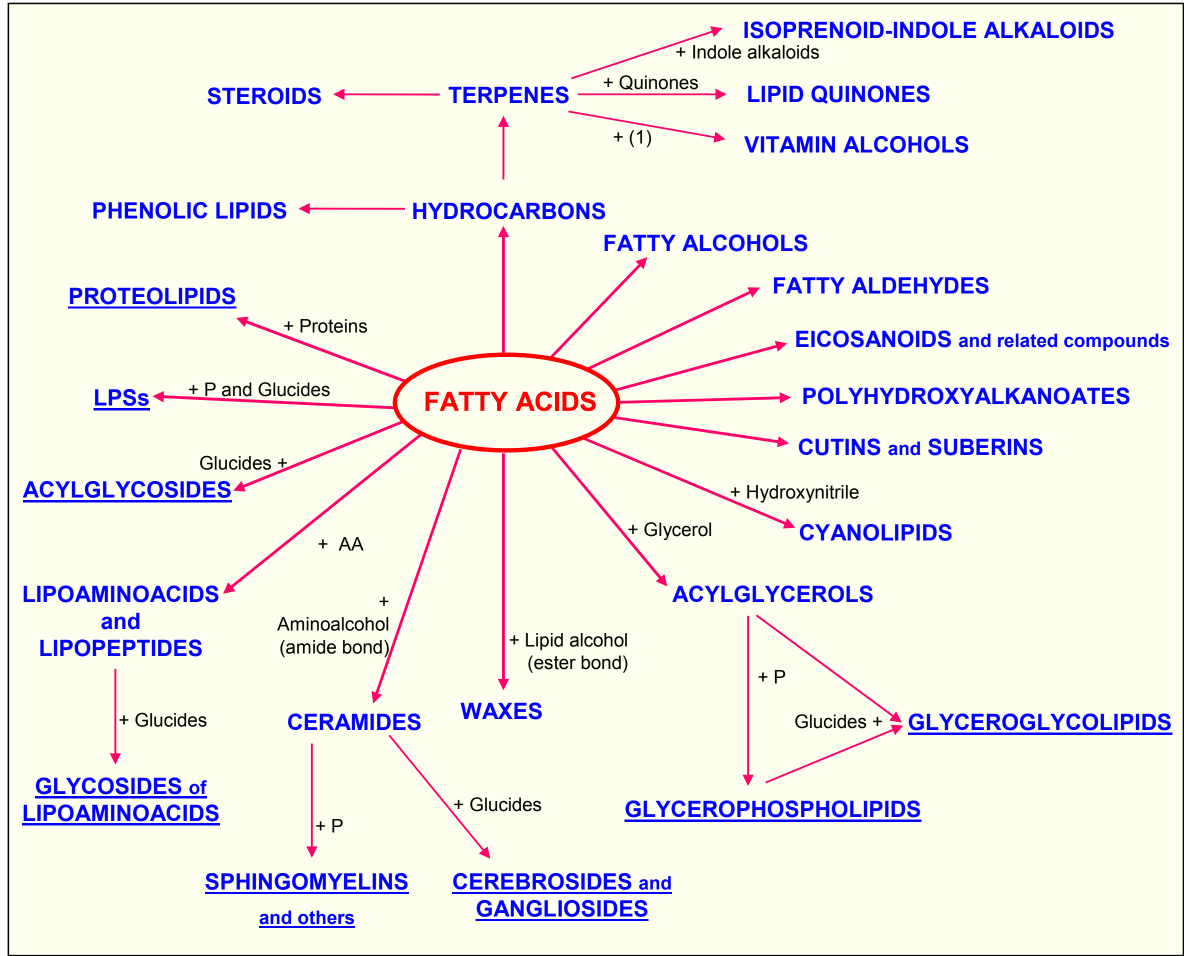


Figure I.3 General classification of lipids.

The scheme represents the main groups of lipids and their relationship with fatty acids, the biosynthetic precursors of almost all lipids found in living beings. Simple lipids are in bold, whereas complex lipids are in bold and underlined. Abbreviations: AA, amino acid(s); P, phosphoric acid group, frequently esterified to a polar head such as choline or ethanolamine; (1), diverse groups such as alcohols, aldehydes, hydroxychroman rings, etc; LPSs, lipopolysaccharides.

This classification (Figure I.3) is only a guideline, as some lipids are grouped in class 1 (simple lipids) by some authors and in class 2 (complex lipids) by others

(http://www.cyberlipid.org). A brief explanation of each group is included in the next pages with special emphasis on acylglycerols and fatty acids because they are directly related with the reactions of hydrolysis and synthesis carried out by lipases.

1.2.1 Fatty acids

The general structure of fatty acids (FAs) is made up of a long and straight aliphatic chain with a hydrophilic carboxylate group attached to one end: $CH_3(CH_2)_nCOOH$, although some of them are dicarboxylic. The most common chain length of FAs is from C_{12} to C_{24}, although it can range from C_2 to C_{80}. According to the chain length, FAs are called short-chain ($C_{2–6}$), medium-chain ($C_{8–12}$), and long-chain ($C_{14–24}$). They have usually an even number of carbon atoms because they are synthesized from acetic acid (C_2) units (Mathews *et al*., 2000).

The simplest FAs are named saturated fatty acids (SFAs), which have no unsaturated linkages. They are highly flexible because they have full rotation around each carbon bond. When double bonds are present, FAs are defined as unsaturated: monounsaturated (MUFAs) if only one double bond is present, and polyenoic (or polyunsaturated fatty acids, PUFAs) if they contain two or more double bonds. The orientation of double bonds is *cis* (Z) rather than *trans* (E). Double bonds of PUFAs are generally separated by a single methylene group, although they can also be polymethylene-interrupted, conjugated, or form a –CH=C=CH– structure (allenic acids). Each *cis* double bond inserts a rigid 30 º bend into the hydrocarbon chain, which affects to the molecular structure (see Figure I.4B), and reduces the flexibility of the chain, the molecular packaging, and the melting point of these compounds. Moreover, double bonds increase the reactivity of the molecule because they can be altered by hydrogenation, oxidation, or halogenation (Boyer, 2000).

In some animals, plants or bacteria, FAs may be more complex since they can have an odd number of carbon atoms and branched chains. They may also contain a variety of other functional groups such as epoxy, hydroxy, keto, methoxy, amine, and amide groups, sulphur or halogen (F, Cl, Br) atoms, acetylenic bonds, or even ring structures (cyclopropane, cyclopropene, cyclopentene, furan, cyclohexyl, phenlylalkyl,

and lipoic acids). Moreover, some multibranched FAs have isoprenoid structures derived from the diterpene phytyl moiety of chlorophyll. The groups mentioned increase the reactivity of FAs, which can undergo intra- and intermolecular reactions such as formation of estolides: dimers or polymers formed by the esterification of FAs to the alcohol group of hydroxy FAs (http://www.cyberlipid.org).

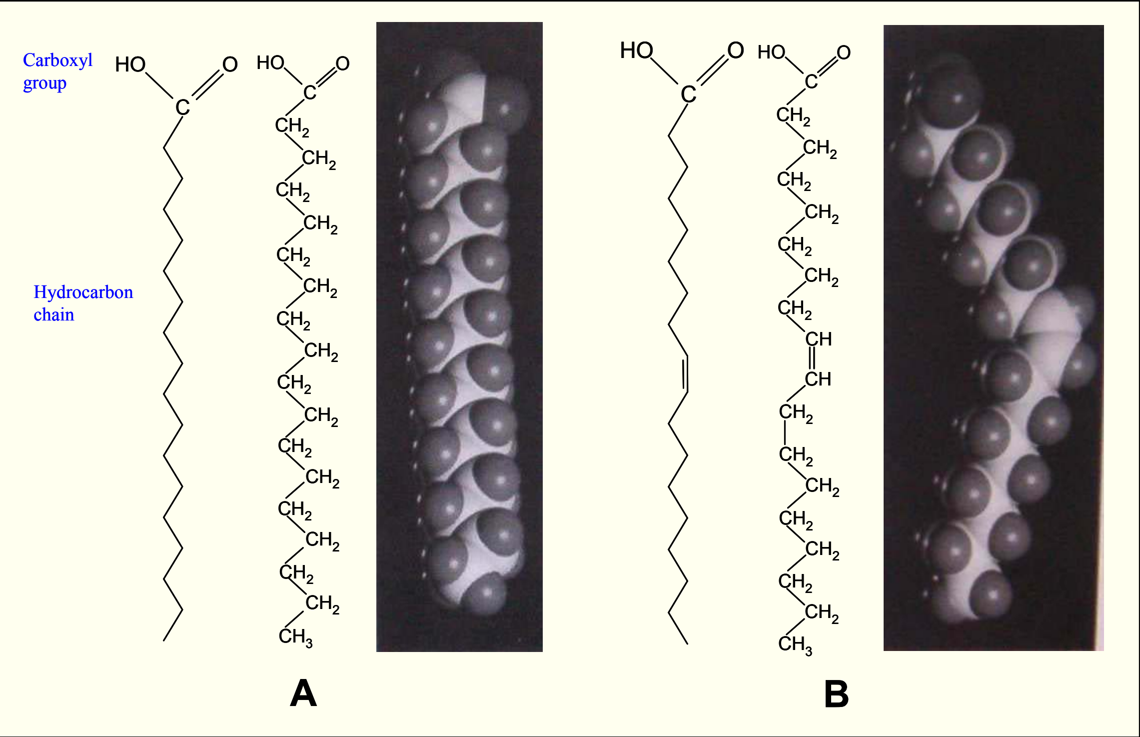


Figure I.4 Structure of a saturated (A and an unsaturated (B fatty acid.

Fatty acid structures are displayed in three different forms, from left to right: abbreviated structure, structural formula, and space-filling model. FAs are named either by a common name, by a systematic name: the name of the corresponding hydrocarbon ended in "-oic"; or by an abbreviation: X:YzΔa,b,… "X" being the number of carbon atoms, "Y" the number of double bonds, "z" the double bond configuration (c = *cis*, t = *trans*), and "a", "b", etc, where each double bond starts (counting from the carboxyl group). Thus, molecule A is named stearic acid, *n*-octadecanoic acid, or $C_{18:0}$, whereas molecule B is named oleic acid, *cis*-9-octadecenoic acid, or $C_{18:1c\Delta 9}$ (Mathews *et al.*, 2000).

FAs are weak acids with pKa values averaging about 4.5. Thus, they exist in the anionic form at physiological pH. They are amphipathic molecules with a polar or ionic head and a non-polar tail, and their solubility in water decreases when the tail length increases. The shortest FAs (C_2−C_4) are miscible with water in all proportions, whereas

those longer form micelles or are water insoluble (Mathews *et al*., 2000). However, water solubility of FAs increases in the presence of NaOH or KOH due to the formation Na^+ or K^+ salts of FAs, named soaps, which are very efficient in forming micelles. Soap formation takes place even when FAs are esterified (acylglycerols, sterol esters, etc). This reaction is named saponification, and it consists on an alkaline break of ester links and the subsequent release of FA salts and the corresponding alcohol. Therefore, lipids undergoing this reaction are named saponificable lipids (Boyer, 2000).

FAs can be found as free molecules, involved in metabolic processes of oxidation and synthesis, or as aromatic compounds. Moreover, metabolism of FAs is in the base of the biosynthesis of almost all existing lipids in living beings (Figure I.3). In fact, some FAs are direct precursors of compounds such as eicosanoids, and polyhydroxyalkanoates (Table I.1), whereas the majority of FAs are esterified in waxes, acylglycerols, phospholipids, glycolipids, ceramides, cyanolipids, etc (Boyer, 2000).

Table I.1 Lipids directly derived from fatty acids.

Hydrocarbons $CH_3(CH_2)_nCH_3$ **Fatty alcohols** $CH_3(CH_2)_nCH_2OH$ **Fatty aldehydes:** $CH_3(CH_2)_nCHO$	They are formed as products of FA cleavage during hydroperoxide decomposition processes or by specific metabolic routes (synthesis of isoprene, etc). They are found in free form (components of the cuticular lipids of plants, aromas, insect pheromones, etc), and integrated in other lipids.
Eicosanoids	They derive from araquidonic acid (20:4cΔ5,8,11,14). They are found in animals and have a hormone-like local effect. Involved in reproduction, inflammation, coagulation, fever, pain, etc.
• Prostanoids	Products of the ciclooxygenase pathway: prostaglandins, prostacyclins, thromboxanes, and their derivatives.
• Lipooxygenase products	Leukotrienes, lipoxins, and various peroxy or hydroxy FA-derivatives generated by the lipooxygenase.
• Isoprostanes and isofurans	Prostaglandin-like compounds generated by a free radical mechanism or by release from phospholipids.
Phytoprostanes	Structurally similar to isoprostanes, but they derive from linoleic acid (18:2cΔ9,12) by non-enzymatic oxidation. They are found in plants and have a phytohormone-like function.
Polyhydroxyalkanoates	Polyesters of 3-hydroxy FAs synthesized, mainly by prokaryotes, as carbon and energy storage materials.
Cutins and suberins	Polymers of hydroxy FAs or dicarboxylic acids, sometimes cross-linked, which protect plant outer surfaces.

FAs are synthesized by a multiprotein complex known as fatty acid synthase. The reaction starts from the terminal methyl end to the carboxylate end, and it consists in a condensation reaction of acetyl-CoA (or acyl-CoA in the following cycles) with acetyl units coming from malonyl-CoA. The resulting product, two-carbon longer each time, is then reduced and dehydrated to obtain a saturated FA (acyl-CoA), which can be further modified by elongation or unsaturation. FA degradation is also performed by a cyclic process named β-oxidation, although ω-oxidation and other processes are possible as well. β-oxidation consists in a oxidation, hydration and cleavage that eliminates the two carbon atoms of the carboxyl end each time. This process, and the complete oxidation of the acetyl-CoA units produced in each cycle, produces a high amount of energy, which makes of FAs better energy storage agents than other less reduced molecules such as carbohydrates and amino acids (Voet & Voet, 1995).

1.2.2 Acylglycerols

Acylglycerols (AGs) are also named glycerides or neutral fats, and they are glycerol esters of one, two or three FAs: mono- (MAGs), di- (DAGs), and triacylglycerols (TAGs), respectively. All FA combinations are possible, although the most frequently found are $C_{12–24}$ saturated or unsaturated FAs. DAGs and TAGs may have one type of FA (simple AGs), or a mixture of FAs (mixed AGs) (Boyer, 2000). Moreover, TAGs with acylated hydroxy FAs are known as estolide triglycerides (http://www.cyberlipid.org).

Besides these AGs, some parent lipids have one or two acyl chains substituted by terpene-derived alkyl chains (alkyl-diacylglycerols and alkyl-monoacyglycerols, respectively). Furthermore, AGs containing more than one glycerol (polyglyceryl esters) have been synthesized by alkaline-catalyzed random polymerization of glycerol followed by esterification with isolated FAs or TAGs. They are non-ionic surfactants with large applications in food and cosmetics industry (http://www.cyberlipid.org).

AGs can undergo the typical reactions of FAs such as hydrogenation, halogenation, etc, as well as other reactions such as chemical or enzymatic isomerization of MAGs and DAGs. Furthermore, they are hydrolyzed by alkaline

solutions (saponification) or by enzymes named lipases, which eliminate one FA each time. However, alkyl analogs are resistant to both hydrolysis. The composition and structure of AGs can be also modified by chemical or enzymatic processes of acyl exchange known as inter- and transesterification (Gunstone, 1999).

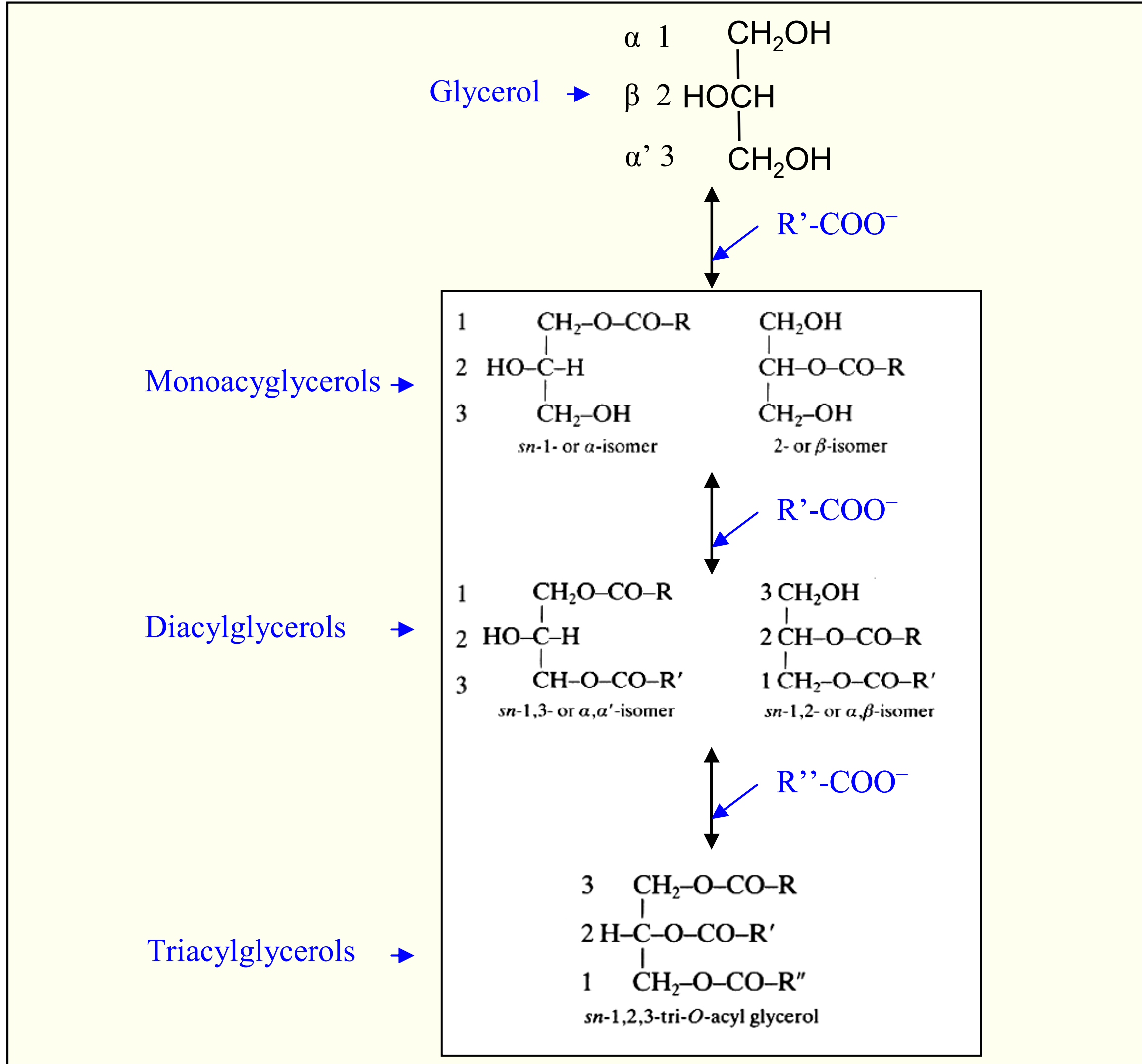


Figure I.5 Structure of glycerol and acylglycerols (AGs .

There is a precise nomenclature to stereospecifically number (*sn*) the carbon atoms of the glycerol molecule. Using the Fisher projection, if the secondary hydroxyl group is oriented to the left of carbon-2, the carbon atom above carbon-2 is designated carbon-1 and the other one carbon-3. The external carbons are also named "α", and the central one "β". R, R' and R" are acyl chains whose position in AGs is indicated by the prefix "*sn*" before the stem-name of the compound. Two isomeric forms exist for MAGs (*sn*-1 and *sn*-2) and DAGs (*sn*-1,2 and *sn*-1,3). Moreover, AGs display chiral properties when the substituents at the *sn-1* and *sn*-3 positions are different (http://www.cyberlipid.org).

MAGs are the most polar AGs and hence, they easily form micelles in water solutions and have detergent properties. On the contrary, TAGs are completely hydrophobic and do not form micelles in water very effectively, especially if they contain long-chain FAs thus, they form oily droplets. DAGs display an intermediate behaviour between MAGs and TAGs (Mathews *et al*., 2000).

Animal TAGs are named fats and they are more solid because they are rich in saturated long FAs that tend to pack closely together in regular semicrystalline structures. Plant TAGs, designated oils, are more liquid because they are rich in unsaturated FAs. The main function of TAGs is energy storage, although fats are also used for heat production and thermal insulation. In fact, TAGs provide six-fold the metabolic energy of an equal weight of hydrated glycogen because they are highly reduced and, being non-polar, are stored in an anhydrous form. In contrast with plants, where lipids are stored in seeds or fruits, in animals fats are found everywhere, although they are more abundant in the vacuoles of adipocytes (Boyer, 2000).

MAGs and DAGs are found in low amounts because they are intermediates in the metabolism of TAGs and phospholipids. However, some of them may have precise biological properties. For example, DAGs generated from membrane phospholipids play a fundamental role in cellular signalling and eicosanoid production (Boyer, 2000).

1.2.3 Waxes

Waxes are defined as the compounds formed by FAs esterified to the alcohol group of fatty alcohols or other lipid alcohols such as aminoalcohols, sterols, hydroxycarotenoids, or terpenols. Thus, they are saponificable, very hydrophobic compounds. Natural waxes are a mixture of waxes and other substances such as hydrocarbons, fatty alcohols, fatty aldehydes, FAs, terpenes, etc, that form a protective coating in plants and animals against dessication and parasites (Boyer, 2000).

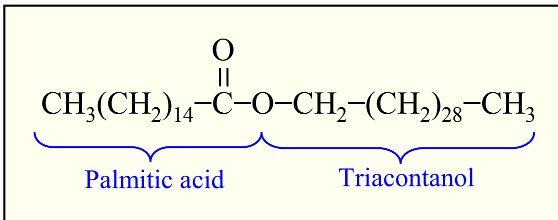


Figure I.6 Honeybee wax.

1.2.4 Cyanolipids

These compounds are FAs esterified to mono- or dihydroxynitrile moieties. Cyanolipids are mainly found in cyanobacteria and plants, and their hydrolysis usually produces HCN, which has a protective effect (http://www.cyberlipid.org).

1.2.5 Lipids containing amino–compounds

1.2.5.1 Aminoalcohols

They are long carbon chains (usually C_{18-20}) containing one or more alcohol groups and one amino group (normally branched) synthesized by condensation of an amino acid and an acyl-CoA. Aminoalcohols can have additional groups and be saturated or unsaturated, linear or branched. The most common ones are ethanolamine, choline, and sphingosine, which are largely found in complex form (http://www.cyberlipid.org).

1.2.5.2 Ceramides

Ceramides result from the condensation of an aminoalcohol and a FA through an amide bond (when the condensation is through an ester bond the result is a wax). The most common aminoalcohols are sphingosine and their derivatives, and the resulting ceramides are the simplest sphingolipids. Free sphingoceramides are found in some tissues or involved as messenger molecules, although their alcohol function is frequently linked to a glucide (glycosphingolipids), or is esterified by a phosphoric acid linked to a polar group (sphingomyelins) (Boyer, 2000; http://www.cyberlipid.org).

Another important group of ceramides are N-acylethanolamines (NAEs), ubiquitous trace constituents of animals, plants and bacteria, which are intracellular messengers tightly regulated, sometimes related to injuries (http://www.cyberlipid.org).

Figure I.7 Structure of a sphingoceramide.

1.2.5.3 Lipoamino acids and lipopeptides

Some microorganisms and animals contain in their membranes amphipathic lipids based on one (lipoamino acids) or more (lipopeptides) amino acids linked to a FA through an amide bond. A second FA can be linked to the amino acid through an ester bond. They may act as virulence factors, or have pharmacological activity (e.g. lipstatin is a lipase inhibitor used against obesity, see Figure I.26) (http://www.cyberlipid.org).

1.2.5.4 Other lipids formed by fatty acids linked to amino-compounds

Several compounds are made up by FAs linked to substances that contain amino groups. Among them, the commonest are acyl-CoAs (thioester bond), which are involved in the metabolism of FAs and other compounds (acetyl-CoA); acyl carnitines (ester bond), which are acyl transporters; and FAs linked to dopamine (amida bond), which are lipid mediators in mammalian nervous tissue (http://www.cyberlipid.org).

1.2.6 Terpenes

Terpenes are lipids constituted of a defined number of isoprene units (2-methyl 1,3-butanodiene; Figure I.8A). Isoprene units may be linked in a head to tail or in a head to head fashion, and the resulting compounds can be acyclic or cyclic, and saturated or

unsaturated. Many terpenes are hydrocarbons, although some of them, designated terpenoids, contain oxygen: alcohol (terpenols), aldehyde, or ketone groups. Terpenes are classified depending on the number of isoprene units incorporated into the basic molecular skeleton (Table I.2). Moreover, isoprenoid units are also found within the framework of several bioactive molecules such as certain phenols (quinones) and indole alkaloids (Table I.2). Terpenes are probably the most widespread group of natural products, and they are constituents of essential oils, resins, waxes, rubber, carotenoids, etc. They are also involved in the synthesis of steroids, vitamins, and chlorophyll, as well as in the acylation of proteins (Boyer, 2000; http://www.cyberlipid.org).

Figure I.8 Terpenes and related compounds.

A: Structure (left) and molecular formula (right) of the hydrocarbon isoprene. B: squalene, a triterpene precursor of steroids. C: β-carotene (provitamin A), a bicyclic carotenoid. D: Vitamin K1, a quinone with a diterpenic side chain.

Table I.2 Classification and biological functions of terpenes.

Terpenes	Isoprene units	Biological functions and examples
Monoterpenes	2	Constituents of essential oils, e.g. limonene.
Sesquiterpenes	3	Constituents of essential oils, insect hormones, and antibiotics, e.g. farnesol.
Diterpenes	4	Constituents of balsams, resins, plant hormones, chlorophyll, vitamins (E, K), and antibiotics, e.g. phytol.
Sesterpenes	5	Insect protective waxes, found also in fungi and diatomaceous algae (haslenes), e.g. ceroplastol.
Triterpenes	6	Essential oils, antibiotics, triterpenic sapogenins, and precursors of steroids, e.g. squalene.
Carotenoids	8	Pigments, antioxidants, provitamin A, precursors of xanthophylls and carotenoid-esters/sugars, e.g. β-carotene.
Rubber	>100	Rubber (insulation of injuries, water insulation, etc), e.g. *Hevea* rubber.
Others	Variable (n×)	Solanesol (9×): precursor of aromatic hydrocarbons, side chain for plastoquinone. Dolichols (16−19×; bacteria, yeast, and animals) and ficaprenols (10-13×; plants) are glycosyl carriers in the synthesis of polysaccharides.
Other molecules with isoprenoid units		
Lipid quinones	• Phylloquinone (vitamin K_1)	Naphthoquinone nucleous + a phytyl side chain: involved in photosynthesis in plants and in blood coagulation.
	• Menaquinones (vitamin K_2)	Naphthoquinone nucleous + a (4−13×)-isoprenoid side chain: several origins, involved in blood coagulation.
	• Ubiquinones (coenzyme)	Benzoquinone nucleus + a (6−10×)-isoprenoid side chain: electron transporters (all aerobic organisms), antioxidants.
	• Plastoquinones	Benzoquinone nucleus + a (6−9×)-isoprenoid side chain: electron transporters in chloroplasts (photosynthesis).
Vitamin alcohols	• Retinoids (vitamin A)	Monocyclic, 4 isoprenoid units + alcohol or aldehyde group (may be esterified with FAs): involved in vision, etc.
	• Vitamin E	Hydroxychroman ring + saturated (tocopherols) or unsaturated (tocotrienols) phytyl side chain: antioxidants.
Indole alkaloids		Indole-alkaloid nucleus + variable isoprenoid side chain: diverse biological and pharmacological activities.
Others		Several molecules such as chlorophyll, some alkyl glycerides, terpenol-waxes, etc.

1.2.7 Steroids

Steroids are modified triterpenes derived from squalene. Their nucleus is based on the saturated tetracyclic hydrocarbon 1,2-cyclopentanoperhydrophenanthrene or sterane (Figure I.9A), which can be modified by C-C bond scissions, ring expansions or contractions, dehydrogenation, and substitutions. They are divided into: sterols (including FA and sulphate sterol esters), brassinosteroids, bufadienolides, cardenolides, cucurbitacins, ecdysteroids, sapogenins, steroid alkaloids, withasteroids, bile acids, and vertebrate hormonal steroids. Steroids form complex lipids when they are linked to glucides or to acyl-glucides: (acylated) steryl glucosides, and many bufadienolides, cardenolides, cucurbitacins and sapogenins (Boyer, 2000; http://www.cyberlipid.org).

Steroids are present in animals, plants, fungi and some bacteria. They are important components of cell membranes, hormones, plant growth regulators, and vitamine D precursors (Boyer, 2000). Moreover, they are antimicrobial agents, insecticides, antiherbivores, poisons, detergents, etc, and some are used in pharmacology (http://www.cyberlipid.org).

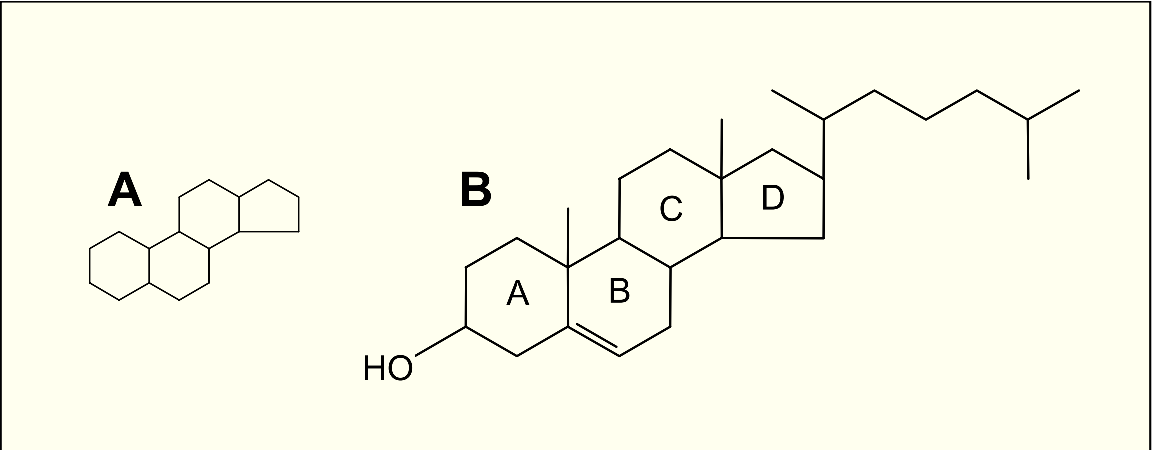


Figure I.9 Structure of sterane (A and cholesterol (B .

1.2.8 Phenolic lipids

Phenolic compounds are mainly present in plants, fungi, and bacteria. This heterogeneous group includes simple phenols and polyphenols as well as their derivatives, and can be classified into coumarins, quinones, and flavonoids, by far the largest group of phenolics. They have a wide range of biological effects: antimicrobials, antioxidants, toxics, enzyme inhibitors, etc. Among single-ring compounds, those considered as lipids are made up of a catechol, a resorcinol, or a hydroquinone nucleus alkylated by a variable non-isoprenoid carbon chain (http://www.cyberlipid.org).

Figure I.10 Ginkgolic acid, a catechol-derived lipid.

OH
COOH

1.2.9 Phospholipids

Phospholipids are complex lipids with a phosphoric acid attached to one (amino)alcohol linked itself to zero (simple phospholipids), one, or two fatty chains. They may include additional groups and chains. Phospholipids have a polar–ionic head, and usually two hydrophobic tails, thus tending to form lipid bilayers. In fact, they are the major structural constituents of all biological membranes, although they may be also involved in other functions such as signal transduction (Boyer, 2000). They are classified into two main groups: glycerophospholipids and sphingosyl phosphatides.

1.2.9.1 Glycerophospholipids

They derive from phosphatidic acid (1,2-diacyl-*sn*-glycerol-3-phosphate). In most glycerophospholipids the phosphate is in turn esterified to a polar head group: glycine, serine, choline, ethanolamine, inositol, glycerol, or phosphatidylglycerol. The polar head may include additional modifications. Glycerophospholipids can be hydrolyzed by alkaline solutions (saponification of the acyl chains), or by enzymes known as phospholipases (Figure I.11) (Boyer, 2000).

Figure I.11 Structure and degradation of glycerophospholipids.

"X" can be hydrogen (phosphatidic acid), glycine, serine, choline, ethanolamine, inositol, glycerol, or phosphatidylglycerol. Glycerophospholipids are degraded by phospholipases, which are designated with a letter according to the linkage they hydrolyze (Voet & Voet, 1995).

1.2.9.2 Sphingosyl phosphatides

These lipids derive from (sphingo)ceramides linked by its alcohol group to a phosphate group itself esterified to a polar head group: choline (in sphingomyelins), ethanolamine, glycerol; or to a glycoside moiety (in glycolipids) (Boyer, 2000).

1.2.10 Glycolipids

Glycolipids are complex lipids containing a glycosidic moiety. Glycolipids are major constituents of cell membranes in bacteria, plants and animals, where they regulate cell interactions with other cells or the environment (Boyer, 2000). According to their structure, glycolipids may be classified into the following groups: (1) glycosides of fatty acids, lipid alcohols and steroids, (2) glyceroglycolipids, (3) glycosphingolipids, (4) glycosides of lipoamino acids, and (5) lipopolysaccharides.

1.2.10.1 Glycosides of fatty acids, lipid alcohols and steroids

These compounds are made up of a glycosyl moiety (one or several units) linked to one or more FAs, fatty alcohols, or alkyl chains. Sterols and other steroids such as sapogenins (which form molecules named saponins as a result) can be also linked to glucides (http://www.cyberlipid.org).

1.2.10.2 Glycolipids based on glycerol (Glyceroglycolipids

These lipids consist of a mono-, di-, or oligosaccharide moiety linked glycosidically to the hydroxyl group of glycerol, which may be acylated (or alkylated) with one or two FAs. Furthermore, these glycolipids may contain additional groups and chains. They form compounds such as lipoteichoic acids of Gram-positive bacterial membranes, which consist of polymers of glycerol-1-phosphate linked to a (phosphatidyl)glycosyl diglyceride (http://www.cyberlipid.org).

1.2.10.3 Glycolipids based on ceramides

They are known as glycosphingolipids and they are based on a mono-, di-, or oligosaccharide moiety linked to the hydroxyl group of a ceramide backbone. The ceramide and the glycosyl group(s), which can be neutral (unsubstitued) or acidic (substituted with carboxyl, sulphate or phosphate group(s)), can have further modifications. The best known ones are cerebrosides (a ceramide linked to a hexose)

and gangliosides (a ceramide linked to an oligosaccharide containing sialic acid), both mainly found in membranes of brain and nerve cells (Boyer, 2000).

1.2.10.4 Glycosides of lipoamino acids

Two groups of complex lipoamino acids containing glycosyl moieties are known: (1) lipids having an amino acid with N-acyl and/or ester linkages, and (2) lipids having a glycerol and an amino acid with ether linkage (http://www.cyberlipid.org).

1.2.10.5 Lipopolysaccharides

These complex compounds are the endotoxic O-antigens found in outer membranes of Gram-negative bacteria. The lipid part (Lipid A), responsible for the toxic activity of these bacteria that results in septic shock, consists of a backbone of β-1,6-(1-phospho)glucosaminyl-(4-phospho)glucosamine. The 3-position of glucosamine II establishes a glycosidic linkage with a long-chain polysaccharide. The other hydroxyl and amine groups are substituted with normal or hydroxy FAs (Madigan *et al*., 2002).

1.2.11 Proteolipids

Proteolipids, also named fatty acylated proteins, are proteins that contain as a part of its primary structure one or more covalently associated acyl moieties. Proteolipids are divided into:

- **Myristoylated proteins**: myristic acid ($C_{14:0}$) is bound to the amino-terminal glycine residue of the protein (amide linkage).
- **Palmitoylated proteins**: palmitic acids ($C_{16:0}$), or other long FAs, are bound by thioester linkages to cystein residues of the protein.

Acylation is one of the most widespread modifications of cytosolic and membrane proteins in all living beings, and it is able to direct soluble proteins to membranes (http://www.cyberlipid.org).

2 LIPASES: GENERAL PROPERTIES

2.1 ORIGIN AND BIOLOGICAL FUNCTION OF LIPASES

The biological relevance and variability of lipids described above has led to the development of a great variety of lipid-degrading enzymes throughout all kingdoms of life. Among them, esterases, which belong to the group of enzymes that catalyze the cleavage of chemical linkages by the addition of a water molecule (hydrolases), are considered to be some of the most important biocatalysts due to their widespread biological functions and due to their biotechnological potential (Bornscheuer, 2002).

Esterases (EC 3.1.1.−; also named ester hydrolases) catalyze the hydrolysis of ester bonds of lipids and other organic compounds, although they are also able to hydrolyze non-ester bonds. In general, a particular esterase is specific for the alcohol or for the acid moiety, but not for both. Thus, they can be alcohol or carboxylic ester hydrolases (Fojan *et al*., 2000). Nowadays, esterases are divided into 78 enzymatic classes according to the specific bond, moiety and substrate they hydrolyze (http://www.expasy.org/cgi-bin/enzyme-search-ec). The carboxylic ester hydrolases which act on acylglycerols (AGs) to liberate fatty acids and glycerol are generically known as lipases (Gupta *et al*., 2004).

Lipases are widely distributed among bacteria, fungi, plants and animals (Bornscheuer, 2002), although they are more frequently found in microorganisms (Pandey *et al*., 1999). Their physiological functions are not yet clear for many of them, although they seem to be involved, in general, in the bioconversion of lipids (mainly TAGs) between different organisms or into the same organism (Pandey *et al*., 1999). Microbial lipases display wide substrate specificity, a property that seems to have evolved to ensure the access of these microorganisms to diverse carbon sources during plant cell wall degradation or during the recycling of lipid-containing nutrients (Gunstone, 1999; Bornscheuer, 2002). Moreover, some lipases are also involved in the turnover of membrane lipids and lipid-anchored proteins, in altering cell membrane

composition in order to change cell membrane functions or to adapt cell membrane to environmental changes, in cell signalling, in the controlled destruction of intracellular vacuoles, in cytolysis (Schmid & Verger, 1998; Titball, 1998), in detoxification of biocides and polluting agents (Khalameyzer *et al.*,1999; Margesin *et al.*, 2003), or in pathogenesis, acting as virulence factors (Jaeger *et al.*, 2004).

Triacylglycerol + 3 H_2O

Lipase (organic media) **Lipase** (aqueous media)

Glycerol + 3 Fatty acids

Figure I.12 Hydrolysis or synthesis of acylglycerols by lipases.

"Lipase" or "lipolytic enzyme" are generic terms that include two main groups of enzymes: (1) carboxylesterases, and (2) "true" lipases, which differ on their preference for short- or long-chain substrates, respectively. However, other enzymes which can hydrolyze AGs (cutinases) or similar lipids (phospholipases, etc) are also considered by some authors as lipolytic enzymes (Fojan *et al.*, 2000). Lipoprotein lipases (EC 3.1.1.34), enzymes that hydrolyze triglycerides of chylomicrons and very low density lipoproteins (VLDL), and closely related to the lipases of higher vertebrates

(http://www.expasy.org/cgi-bin/get-prosite-raw.pl?PDOC00110), can be considered as well as lipases, although they are usually regarded as a separate group.

2.1.1 Differences between "true" lipases and carboxylesterases

Several criteria (Table I.3) have been used to distinguish "true" lipases (TLs, EC 3.1.1.3), also known as triacylglycerol lipases, from carboxylesterases (CEs, EC 3.1.1.1), also called esterases although this name can bring to confusion with the whole group of esterases (EC 3.1.1.−). However, substrate specificity is the only criteria completely valid nowadays for this distinction due to the existence of several exceptions with respect to other criteria previously used (Jaeger *et al*., 1999; Fojan *et al*., 2000; Bornscheuer, 2002).

Table I.3 Differences between lipases and esterases.

Property	"True" lipases	Carboxylesterases
Preferred substrates	Triglycerides (long-chain) long-chain esters	Triglycerides (short-chain) simple esters
Scissile fatty acid binding site	Long	Short
Substrate hydrophobicity	High	High to low
Preferred substrate physical state	Insoluble and aggregated	More soluble
Interfacial activation and lid	Yes (with exceptions)	No
Solvent-exposed amino acids, and active site amino acids	More non-polar amino acids	Less non-polar amino acids
Optimum active-site electrostatic potential range (optimum pH)	Mostly 8−9, but (5−6 a few of them)	5.5−7
Substrate range	Broad	Broad to tight
Regio- and steroselectivity	High (usually)	High to low to zero
Organic solvent activity and stability	High	High to low

TLs display **substrate specificity** for long-chain AGs and other long-chain esters, whereas CEs act on short-chain AGs and simple esters (Bornscheuer, 2002). There is no strict definition of the terms long- and short-chain, but esters with an acyl chain length of ≥ 10 carbon atoms are considered TL substrates, with trioleoylglycerol (triolein) being the standard substrate. On the contrary, esters with an acyl chain length of < 10 carbon atoms are considered CE substrates, with tributyrylglycerol (tributyrin) being the standard substrate (Jaeger *et al*., 1999; Fojan *et al*., 2000). Nevertheless, it should be emphasized that most TLs are capable of hydrolyzing CEs substrates, and some CEs are active on TLs substrates (Jaeger *et al*., 1999; Fojan *et al*., 2000).

The mentioned differences in substrate specificity are reflected in the size and hydrophobicity of the **scissile acyl binding site** of the enzymes, which have to fit completely the acyl chain in both charge and, mainly, in size. A decreased size of the binding site would lead to steric conflicts with the substrate, whereas increasing its size would leave free space, which would lead to sub-optimal binding of substrates and thus, a decreased activity. For these reasons, CEs have a small acyl binding pocket which optimally fits the acyl moiety of their favourite substrates, whereas TLs have a long, hydrophobic scissile fatty acid binding site suitable to accommodate long acyl chains (Pleiss *et al*., 1998).

Furthermore, substrate specificity of these enzymes is directly correlated to the different preference that TLs and CEs show for the **hydrophobicity** and the **physical state** of their **substrates**. Thereby, TLs prefer highly hydrophobic substrates, which are water insoluble and tend to form aggregates (Fojan *et al*., 2000; Bornscheuer, 2002). Thus, the enzymatic reactions catalyzed by TLs occur at the lipid-water interface where the substrates usually form an equilibrium between monomeric, micellar, and emulsified states (Jaeger *et al*., 1999). Accordingly, TL activity is directly correlated to the substrate area, and not with the substrate concentration. On the contrary, CE activity is highest on more water soluble substrates, and it depends on substrate concentration (Fojan *et al*., 2000; Bornscheuer, 2002).

"**Interfacial activation**" is a unique feature which was used in the past years to assign an enzyme as a TL, and that is explained in more detail in General Introduction 2.2.4. Briefly, it consists in an activation of the enzyme by the presence of an interface,

that is, a sharp increase of TL activity is produced as soon as the substrate forms an emulsion. This phenomenon explains the preference of TLs for aggregated substrates, and it is usually correlated to the presence of a "**lid**" (see General Introduction 2.2.1) in the enzyme structure. The lid corresponds to a surface loop of the protein covering the active site of the enzyme and moving away in contact with the interface. However, these two criteria are not completely suitable to define TLs due to the existence of several TLs that do not show interfacial activation and/or do not have a lid loop (Verger, 1997; Jaeger *et al*., 1999).

TLs and CEs have a similar pI (isoelectric point) and amino acid residue composition. However, if solvent accessibility is taken into account, some differences among them are found with respect to the **amino acids most exposed to the solvent**. CEs show an expected decrease in non-polar residues with increasing solvent accessibility, a feature commonly observed in water soluble proteins. On the contrary, TLs display an enhanced content of non-polar residues around 50−80% solvent accessibility. These hydrophobic residues (usually short: valine, leucine and isoleucine) are found to cluster mainly in the protein hemisphere where the active site is located, and they could facilitate the lipase attachment to the hydrophobic substrate aggregate (Fojan *et al*., 2000). Moreover, TLs show a higher content on small non-polar **amino acids** than CEs in the **active site**, which enhances the interaction between the enzyme and its substrates when the lid is moved away (Fojan *et al*., 2000)

TLs and CEs differ also in their **electrostatic signature,** and this fact seems to be correlated, in general, with their **optimum pH** (8−9 and 5.5−7 respectively). In fact, these enzymes display optimum activity when the active site is slightly negatively charged, which occurs at pH 5.5−6.5 for CEs. On the contrary, TLs report an optimum active-site electrostatic potential range around pH 8−9 (Petersen *et al*., 2000).

In addition, most TLs display a broader **substrate range**, a higher **regio-** and **stereoselectivity**, and a higher **activity and stability in organic solvents** than do most CEs (Fojan *et al*., 2000; Bornscheuer, 2002).

2.1.2 Catalytic versatility of lipases

Lipases are very versatile enzymes that catalyze a large number of reactions (Table I.4). They catalyze the hydrolysis of AGs and a wide range of other esters (simple esters, phospholipids, acylglycosides, etc), including non-natural substrates. When they act in the presence of organic solvents (although sometimes with a small requirement for water), lipases are capable of performing the reverse reaction (ester synthesis), or the exchange of acyl groups (inter- and transesterification) among different AGs, alcohols, esters, glycosides and amines, or even among different chemical groups of the same compound (Schmidt-Dannert, 1999; Pandey *et al*., 1999; Ferrer *et al*., 2000; Bornscheuer, 2002; Gupta *et al*., 2004).

Table I.4 Reactions catalyzed by lipases.

Ester hydrolysis:

$R_1{-}COO{-}R_2 + H_2O \rightarrow R_1{-}OH + R_2{-}COOH$

$R_1{-}COO{-}R_2 + H_2O_2 \rightarrow R_1{-}OH + (Peroxy)R_2{-}COOH$

Ester synthesis:

$R_1{-}OH + R_2{-}COOH \rightarrow R_1{-}COO{-}R_2 + H_2O$

Intramolecular esterification:

$HO{-}R{-}COOH \rightarrow R{-}COO{-}R$ (Lactones)

Synthesis of estolides and other polymers:

$R_1{-}COOH + HO{-}R_2{-}COOH \rightarrow R_1{-}COO{-}R_2{-}COOH$

Interesterification:

$R_1{-}COO{-}R_2 + R_3{-}COO{-}R_4 \rightarrow R_1{-}COO{-}R_4 + R_3{-}COO{-}R_2$

Transesterification by acidolysis:

$R_1{-}COO{-}R_2 + R_3{-}COOH \rightarrow R_1{-}COO{-}R_3 + R_2{-}COOH$

Transesterification by alcoholysis:

$R_1{-}COO{-}R_2 + R_3{-}OH \rightarrow R_1{-}COO{-}R_3 + R_2{-}OH$

Transesterification by aminolysis:

$R_1{-}COO{-}R_2 + R_3{-}NH_2 \rightarrow R_1{-}CONH{-}R_3 + R_2{-}OH$

Lipases usually carry out all these reactions with a high chemo-, regio- or enantioselectivity, which can be different between the reactions of hydrolysis and the reactions of synthesis or acyl exchange (Gunstone, 1999). Thereby, lipases can be:

● **Substrate specific:** they display a different ratio of lipolysis when they act on different compounds such as TAGs, DAGs, MAGs, or other esters (Pandey *et al*., 1999).

● **Nonspecific or regiospecific:** nonspecific lipases act at random on TAGs, which results in their complete breakdown to FAs and glycerol. In contrast, most extracellular lipases are regiospecific, that is, they have a positional preference for the ester bonds they hydrolyze. Regiospecific lipases are usually 1,3-specific lipases which act only on primary ester bonds (i.e. ester bonds at atoms C_1 and C_3 of glycerol) thus hydrolyzing TAGs into FAs, 1,2(2,3)-DAGs and 2-MAGs. However, there are also some 2-specific lipases, which hydrolyze only secondary bonds (i.e. ester bonds at atom C_2 of glycerol) producing FAs and 1,3-DAGs (Gunstone, 1999).

● **Fatty acid-specific:** these lipases exhibit a pronounced preference for one FA or for a tight range of FAs (Gupta *et al*., 2004). They are capable of distinguish structural features of acyl chains such as the length, the number, position, or configuration of double bonds, or the presence of branched groups, as well as the nature of the acyl source: free acid, alkyl ester, glycerol ester, etc (Gunstone, 1999).

● **Enantio-/stereoselective:** these lipases are capable of discriminating between the enantiomers of a racemic mixture. The stereospecificity of a lipase depends largely on the structure of the substrate, on the interactions at the active site, and on the reaction conditions. Stereospecificity is one of the most important properties of lipases for their application in biotechnology (Muralidhar *et al*., 2002; Gupta *et al*., 2004).

2.2 STRUCTURE AND CATALYTIC MECHANISM OF LIPASES

2.2.1 The fold of lipases and the lid

The number of lipases that have been studied has grown tremendously over the last decade, some of them having been studied by X-ray crystallography and nuclear magnetic resonance (NMR) (Fojan *et al*., 2000). From these studies appeared that almost all lipases have a similar structural fold, although they display low sequence similarity (Jaeger *et al*., 1999). This structure is named α/β hydrolase fold (Figure I.13), and it is common for lipases and many other hydrolases (Ollis *et al*., 1992).

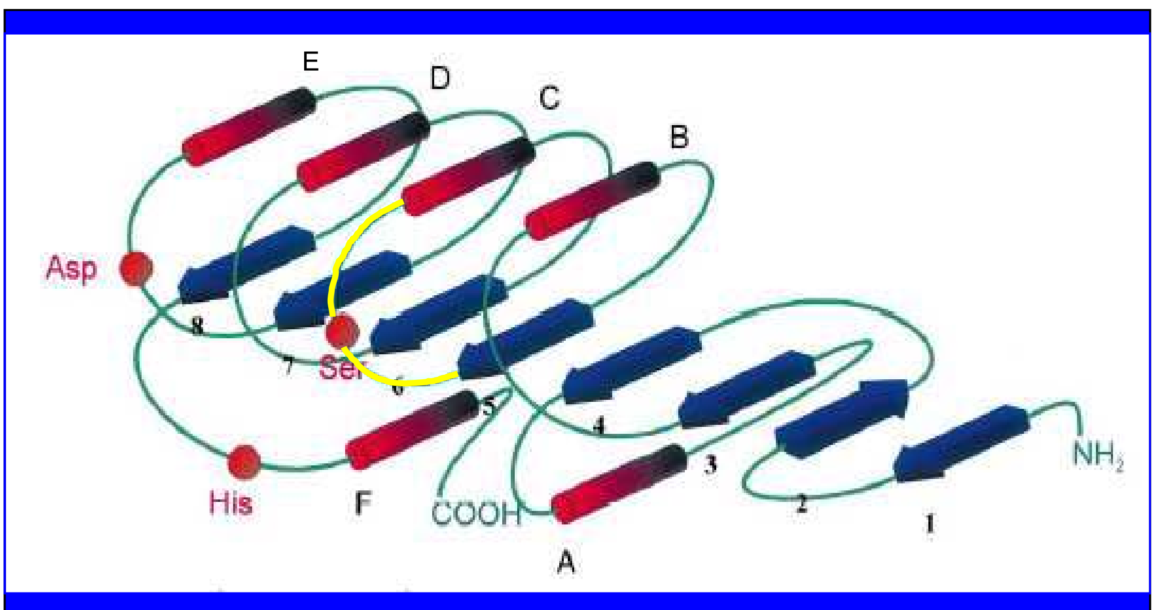


Figure I.13 Schematic representation of the canonical α/β hydrolase fold.

β Sheets (1−8) are shown as blue arrows, and α helices (A−F) as red columns. The relative positions of the amino acids of the catalytic triad are indicated as red circles, and the "nucleophile" elbow is in yellow (Bornscheuer, 2002).

The canonical α/β hydrolase fold (Figure I.13) consists of a central, mostly parallel β sheet of eight strands with the second strand antiparallel. The parallel strands β3 to β8 are connected by α helices, which pack on either side of the central β sheet. The β sheet has a left-handed superhelical twist such that the surface of the sheet covers

about half a cylinder and the first and last strands cross each other at an angle of ~90º. The curvature of the β sheet may differ significantly among the various enzymes, and also, the spatial positions of topologically equivalent α helices may vary considerably. Variations of the peptide chain at the C-terminal ends of strands in the C-terminal half of the β sheet form the binding subdomains of the α/β hydrolase fold proteins. They differ substantially in length and architecture, in agreement with the large substrate diversity of these enzymes (Jaeger *et al*., 1999). The lipase structures known so far obey the α/β hydrolase fold although they can display additional variations in the number or in the disposition of the α helix and β strands (Schrag & Cygler, 1997).

Furthermore, most TLs have a particular structure, designated as "lid", located at the protein surface, close to the active site. The lid consist of a single α helix, two helices, or a loop region, which is mainly hydrophobic on the side directed towards the active site, and hydrophilic on its external face. The lid displays a variable position depending on the physicochemical environment of the enzyme (Grochulski *et al*., 1994; Cygler & Schrag, 1997). Thereby, the lid covers the active site of the enzyme in the absence of an interface. On the contrary, the presence of an interface produces a structural rearrangement that displaces the lid, which is stabilized by hydrophilic interactions with the enzyme surface, allowing a free accession of the substrate and the solvent to the active site of the enzyme. However, exceptions such guinea pig pancreatic lipase do not have lid (Verger, 1997; Jaeger *et al*., 1999; Cygler & Schrag., 1999).

2.2.2 The active site and the catalytic residues of lipases

The active site of the α/β hydrolase fold enzymes has three catalytic residues: a nucleophilic residue (serine, cysteine, or aspartate), a catalytic acid residue (aspartate or glutamate), and a histidine residue, always placed in this order in the amino acid sequence (Ollis *et al*., 1992). Nevertheless, this order is different from that observed in any of the other proteins that also contain catalytic amino acid triads. In lipases the nucleophilic amino acid has so far always been found to be a serine residue, whereas the

catalytic acid can either be an aspartate or a glutamate residue (Jaeger *et al*., 1999; Bornscheuer, 2002). However, there are rare exceptions to this rule such as that of *Streptomyces scabies* esterase, which possesses a catalytic dyad (Ser and His) instead of a catalytic triad. In this case, the role of the acidic side chain is carried out by the backbone carbonyl of an amino acid located close to the His residue due to the existence of a modified α/β tertiary fold (Wei *et al*., 1995).

The nucleophilic serine residue is located in a highly conserved Gly-Xaa-Ser-Xaa-Gly pentapeptide (Ollis *et al*., 1992). This motif can be used as an indicator for a lipase classification, although there are some exceptions: (1) the first glycine is replaced by an alanine in lipases of the subfamilies I.4 and I.5 of bacterial lipases, (2) (carboxyl)esterases of the family II of bacterial lipases have their nucleophilic serine in an alternative Gly-Asp-Ser-(Leu) motif (Arpigny & Jaeger, 1999), and (3) the conserved pentapeptide has also been found in other proteins (Fojan *et al*., 2000).

The conserved Gly-Xaa-Ser-Xaa-Gly pentapeptide forms a sharp, γ-like turn between β5 of the canonical α/β hydrolase central β sheet and the following α helix. The main characteristic of the γ turn is the fact that the main chain of the nucleophilic serine forms energetically unfavourable Φ and φ torsion angles. β5, the γ turn and the following α helix have been called the "nucleophile elbow", and form the most conserved structural arrangement of the α/β hydrolase fold (Figure I.13). The nucleophile elbow positions the nucleophilic residue free of the active site surface and allows easy access by the catalytic histidine residue on one side and by the substrate on the other side. The sharp turn also optimally positions the nucleophilic residue at the N-terminal end of the following helix (helix C), thereby helping to stabilize the ionized form of the nucleophile and the tetrahedral intermediate produced during the catalytic process. The complete stabilization of this tetrahedral intermediate is achieved by the collaboration of the helix macrodipole of helix C (see Figure I.13) and the "oxyanion hole", which consists in a pocket with at least two main-chain NH groups that form hydrogen bonds with the negatively charged carbonyl oxygen atom of the tetrahedral intermediate (the "oxyanion"). One of the NH groups belongs to the residue just behind the nucleophilic serine; the other one comes from the residue at the end of strand β3 (Jaeger *et al*., 1999).

In the prototypic α/β hydrolase fold, the catalytic acid residue (Asp or Glu) occurs in a reverse turn after strand 7 of the central β sheet. However, the topological position of the acid seems to be variable, although its position in the active site is maintained in different lipases by several structural changes. The catalytic acid is hydrogen-bonded to the catalytic histidine, which is located in a loop with a variable length and configuration after β strand 8 (Ollis *et al*., 1992; Jaeger *et al*., 1999).

The active site of lipases contains also other structures that facilitate the catalytic process such as the "scissile fatty acid binding pocket", which is a variable pocket responsible for the accommodation of the acyl chain of the ester linkage that is going to be hydrolyzed. Additional binding pockets for the other acyl chains of substrates such as TAGs are also frequent, which contributes to maintain the substrate attached to the active site of the enzyme during catalysis (Jaeger *et al*., 1999).

2.2.3 The catalytic mechanism of lipases

Evidence of the mechanism of ester hydrolysis or synthesis has come from various studies, particularly crystallographic analyses of inhibitor–lipase complexes. It is constituted by the four steps represented in Figure I.14. During the first step, the substrate binds to the nucleophilic serine yielding a tetrahedral intermediate stabilized by the catalytic His and Asp residues, the helix C, and the oxyanion hole. Next, the alcohol is released and an acyl–enzyme complex is formed. Attack of a nucleophile (water in hydrolysis, alcohol or ester in trans- or interesterification) forms again a tetrahedral intermediate, which after resolution yields the final product (an acid or an ester) and free enzyme (Bornscheuer, 2002).

Figure I.14 Mechanism of hydrolysis.

[1] The hydrolysis starts with the lipid binding and the activation of nucleophilic serine residue by the neighbouring active histidine, to which a proton from the serine hydroxyl group is transferred. Proton transfer is facilitated by the presence of the catalytic acid, which precisely orients the imidazole ring of the histidine and partly neutralizes the charge. Activation is followed by an attack by the oxygen atom (O^-) of the serine hydroxyl group on the activated carbonyl carbon of the susceptible lipid ester bond (Jaeger *et al*., 1999).

[2] A transient tetrahedral intermediate is formed, which is characterized by a negative charge on the carbonyl oxygen atom of the scissile ester bond and four atoms bonded to the carbonyl carbon atom arranged as a tetrahedron. The intermediate is stabilized by the macrodipole of helix C (see Figure I.13), and by hydrogen bonds between the negatively charged carbonyl oxygen atom (the "oxyanion") and at least two main-chain NH groups (the "oxyanion hole"). Subsequently, the additional proton of histidine is donated to the ester oxygen of the susceptible bond, which is thus cleaved. At this stage, the acid component of the substrate is esterified to the nucleophilic serine (the "covalent intermediate"), whereas the alcohol component diffuses away (Jaeger *et al*., 1999).

[3] The next stage is the deacylation step, in which an incoming water molecule hydrolyzes the covalent intermediate ("acyl enzyme") and the acid component of the substrate is esterified to the enzyme's serine residue. The catalytic histidine activates the water molecule by drawing a proton from it. The resulting OH^- ion performs a nucleophilic attack on the carbonyl carbon atom of the acyl group covalently attached to the serine. Again, a transient negatively charged

tetrahedral intermediate is formed, which is stabilized by interactions with the oxyanion hole (Jaeger *et al*., 1999).

[4] The histidine residue donates its additional proton to the oxygen atom of the active serine residue, which breaks the ester bond between serine and the acyl component, and releases the acyl product. After diffusion of the acyl product, the enzyme is ready for another round of catalysis (Jaeger *et al*., 1999).

2.2.4 Interfacial activation

As mentioned in General Introduction 2.1.1, most “true” lipases (TLs) are characterized by a phenomenon designated interfacial activation (Figure I.15), which consists in a drastically increased activity when they act at the lipid-water interface of micellar or emulsified substrates (Sarda & Desnuelle, 1958). This increase in enzymatic activity is triggered by certain structural rearrangements of the lipase active-site region, as witnessed from the crystal structures of lipases complexed with small transition-state analogs (Jaeger *et al*., 1999), and it is facilitated by the particular amino acid composition of the enzymes themselves (Fojan *et al*., 2000). However, this phenomenon depends highly on the quality of the interface of the lipids used as substrates, as well as on the reaction conditions: ionic strength, presence of detergents or emulsifying agents, stirring, etc (Verger, 1997).

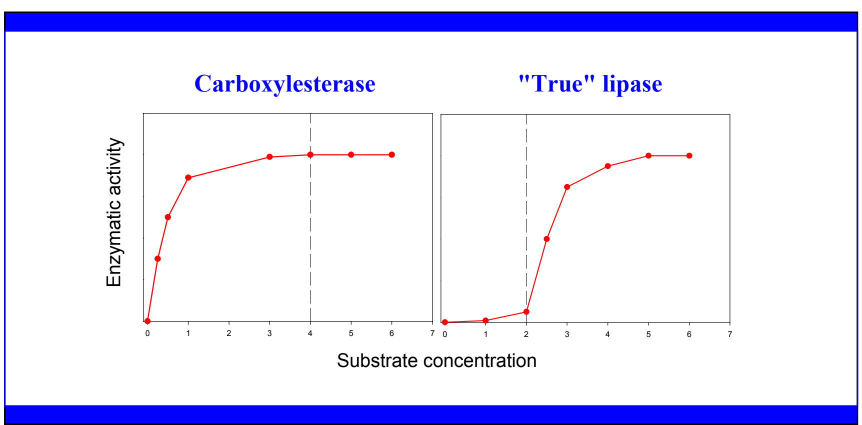


Figure I.15 Comparison of the enzymatic activity of carboxylesterases and “true” lipases.

Carboxylesterases show a typical Michaelis-Menten behaviour with an activity increase parallel to the increase of substrate concentration. They prefer short-chain substrates, which remain in monomeric state up to high concentrations (dashed line). On the contrary, “true” lipases display low activity until their substrate reaches a concentration high enough to form an emulsion or micelles (dashed line), then their activity increases drastically (interfacial activation).

TLs display an enhanced content of short, non-polar residues (usually valine, leucine and isoleucine) which cluster at the protein hemisphere where the active site is located. These residues could facilitate the lipase attachment to the hydrophobic substrate aggregate, which would then be followed by the structural rearrangements responsible for the opening of the lid (Fojan *et al*., 2000). Once opened the lid, the active site would be accessible to the substrate, which would explain the resulting increased activity (interfacial activation) of the enzyme. For this reason, lipases that do not have a lid do not show interfacial activation (Verger, 1997; Jaeger *et al*., 1999). However, there are some exceptions of lipases having lid but showing no interfacial activation (*Pseudomonas glumae* lipase and others), or having lid but showing interfacial activation only on some substrates (*Staphylococcus hycus* lipase) (Verger, 1997; Schmid & Verger, 1998).

The closed conformation of TLs (with the active site covered by the lid), predominates in aqueous solution, although persistence of a low level of activity in these solutions shows that open conformations are also present, at least transiently. However, the open conformation of TLs produced by lid opening predominates when the protein contacts the hydrophobic components of lipid-water interfaces. The open conformation makes the catalytic residues accessible to substrate and rearranges the oxyanion hole in a more suitable conformation; moreover, it also exposes the large hydrophobic surface surrounding the catalytic residues (Figure I.16; Cygler & Schrag, 1999). Because close contacts exist between the residues of the active-site region, the amino acids located two positions before and two positions behind the nucleophile are usually glycines, or occasionally other small residues such as alanine or valine (Jaeger *et al*., 1999). Moreover, the increased content of small non-polar residues in the active-site region of TLs in comparison with that of CEs and other enzymes enhances even more the interaction between active site of TLs and the substrate, thus increasing the enzyme activity when the lid is moved away (Fojan *et al*., 2000).

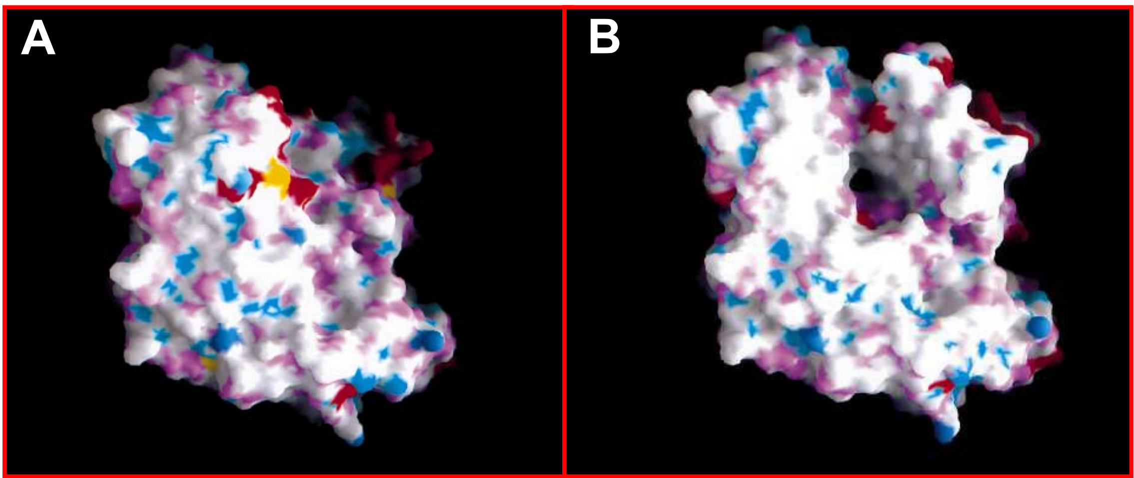


Figure I.16 Closed (A and open (B state of *Candida rugosa* lipase.

C. rugosa lipase covered (A) and non-covered (B) by the lid. Both states are displayed in the same orientation, showing the active site in the centre of the image. Colour scheme: charged oxygens, dark red; polar oxygens, magenta; charged nitrogens, dark blue; polar nitrogens, light blue; sulphurs, yellow; non-polar atoms, white. It should be emphasized the increased non-polar area around the active site (Cygler & Schrag, 1999).

The fact that evolution of TLs chose small non-polar residues to populate the region around the active site responds to several reasons. Small non-polar residues have a smaller entropic penalty for their solvation, they are flexible and can more easily intercalate into an interface than bulky amino acids, which tend to stack with membrane-like structures. This intercalation of small residues into hydrophobic interfaces disturbs them and facilitates the acquisition of triacylglycerol molecules into the active site. Furthermore, these residues are very unspecific, and no predefined attachment point on the hydrophobic surface has to be present (Fojan *et al*., 2000).

2.2.5 Substrate binding and selectivity of lipases

Extensive research has been carried out to identify the binding regions of the acyl and alcohol portions of the substrate in the various lipases, and to rationalize the observed substrate specificity and enantioselectivity (Jaeger *et al*., 1999).

A breakthrough came with the work of Lang & Dijkstra (1998) and Lang *et al*. (1998), who determined the X-ray structure in the open conformation of *Burkholderia cepacia* lipase in complex with a medium-chain TAG analog. They detected that the analog assumes the bent-tuning-fork conformation preferred by lipids at an interface (Pascher, 1996), and that the active site is divided into four binding pockets: an oxyanion hole and three pockets that accommodate the *sn*-1, *sn*-2, and *sn*-3 FA chains (Figure I.17). They also determined that Van der Waals interactions are the main forces that keep the radyl groups of the TAG analog in position, together with a hydrogen bond between the ester oxygen atom of the *sn*-2 chain and the catalytic histidine.

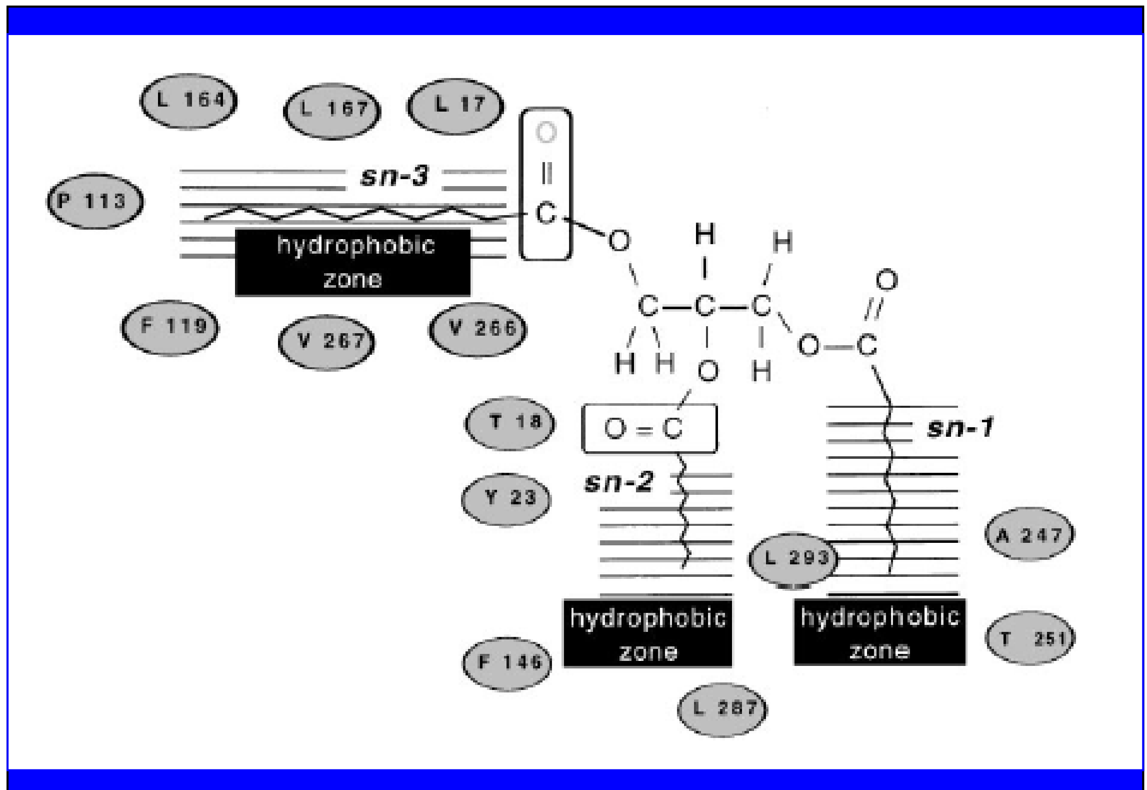


Figure I.17 Active site of *Burkholderia cepacia* lipase.

The binding pockets for the *sn*-1, *sn*-2, and *sn*-3 moieties of the lipid substrate, and the residues lining these binding pockets, are indicated. These pockets consists of a large hydrophobic groove in which the *sn*-3 acyl chain snugly fits, a mixed hydrophilic/hydrophobic cleft that accommodates the *sn*-2 moiety of the substrate and a smaller hydrophobic groove for the *sn*-1 chain (Lang *et al*, 1998).

Subsequently, studies focused on the geometry and properties of the scissile FA binding site revealed that lipases share a common catalytic machinery located at the bottom of a deep, elliptical binding pocket, where the ester substrates bind keeping their acid–alcohol axis parallel to the long axis of the pocket (Pleiss *et al*., 1988). The orientation of the carbonyl oxygen of the scissile FA is also the same in different lipases with respect to the binding pocket, and the relative position with respect to the nucleophile elbow and the Ser-His catalytic diad is also maintained (Cygler & Schrag; 1997). The short axis of the scissile FA binding pocket has nearly the same width for different lipases (4–4.5 Å), the appropriate size to fix the ester substrate. However, the length of the acyl binding site varies from 3.5 Å in esterases to 22 Å in the longest TLs, whose site is also more hydrophobic (Figure I.18; Pleiss *et al*., 1998).

Moreover, TLs were also subdivided into three sub-groups according to the geometry of their scissile FA binding site: (1) lipases with a hydrophobic, crevice-like binding site located near the protein surface (*Rhizomucor miehei* family); (2) lipases with a funnel-like binding site (*Burkholderia cepacia* family); and (3) lipases with a tunnel-like binding site (*Candida rugosa* family) (Figure I.18). These differences in the anatomy of the scissile FA binding site could explain the diverse substrate specificity found in TLs. For example, a lipase with a tunnel-like binding-site is more likely to accept substrates with long-chain FAs than bulky substrates. The opposite should apply to lipases with crevice- or funnel-like binding sites (Pleiss *et al*., 1998).

From these results, and with the support of mutants with changed FA chain-length specificity (Joerger & Haas, 1994; Klein *et al*., 1997), by Pleiss *et al*. (1998) identified also the residues involved in chain-length specificity, which led to protein engineering of lipases for changed chain-length specificity. These studies, and additional assays of changing the enantioselectivity of lipases by mutations in the residues lining the binding pockets of lipases (Hirose *et al*., 1995), allowed to demonstrate that the differences in shape, size and in the hydrophilicity/hydrophobicity of the various pockets, as well as the structure of the substrate and the reaction conditions, determine the specificities for the acyl moiety and the enantio- and regiopreferences of these enzymes (Pleiss *et al*., 1998; Jaeger *et al*., 1999; Muralidhar *et al*., 2002).

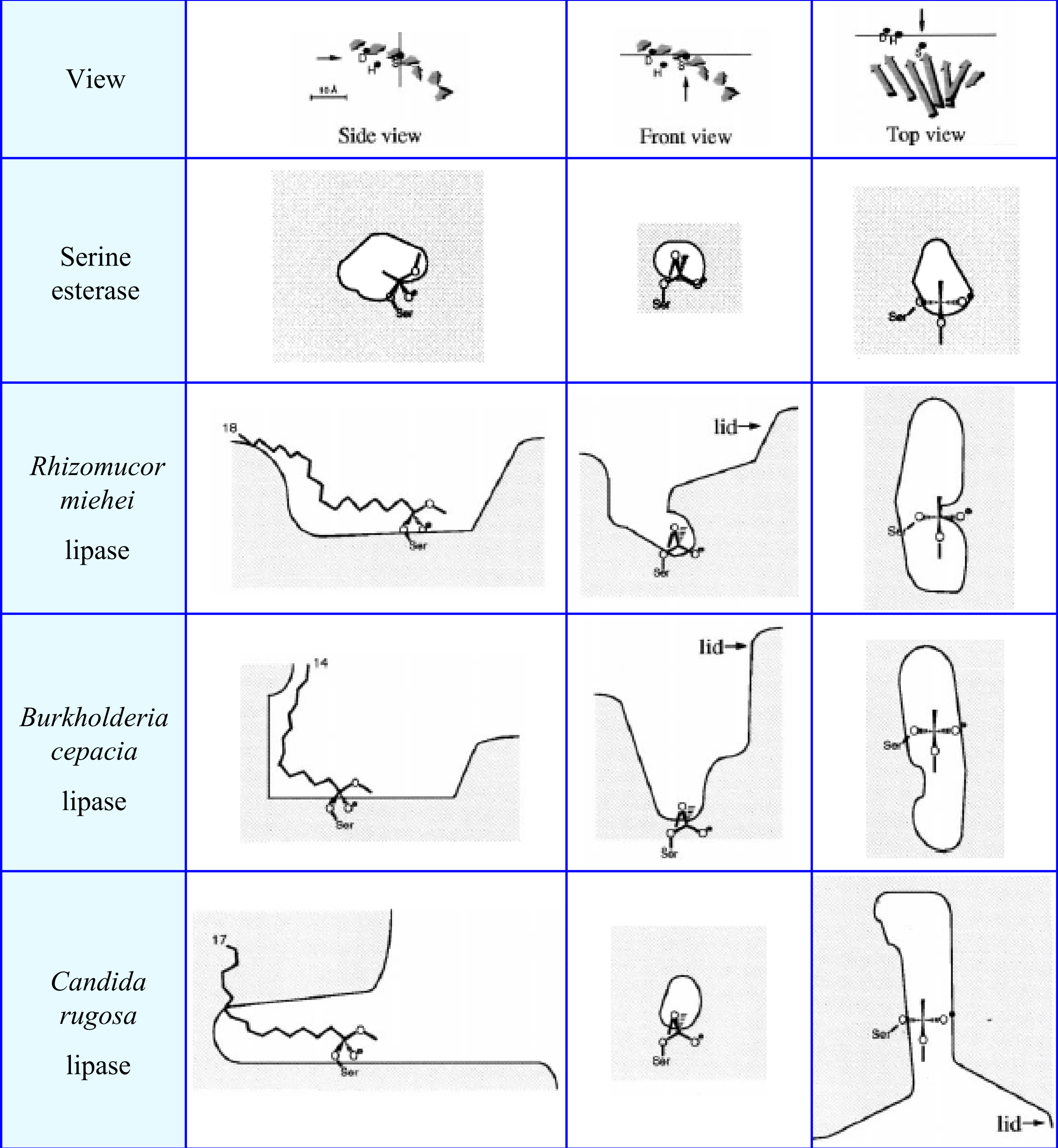


Figure I.18 Shape of the scissile fatty acid binding site of esterases and "true" lipases.

A number indicates the length of the longest FA which completely binds inside the binding pocket of TLs (Pleiss *et al*., 1998).

2.3 METHODS FOR LIPASE ACTIVITY DETERMINATION

Lipolytic activity is difficult to determine due to the fact that lipases are water soluble enzymes acting on water insoluble substrates. Therefore, additional factors, such as the substrate concentration at the interface or the use of different detergents, must be taken into account to interpret the activity and the enzyme kinetics obtained. For these reasons, a large number of methods for measuring the activity of lipases, or their inhibition, have been reported and reviewed (Beisson *et al*., 2000; Wahler & Reymond, 2001; Pencreac'h *et al*., 2002). These methods differ on the process used for substrate solubilization, on the activity marker employed, and on the detection system, which makes the comparison of the results obtained difficult (Beisson *et al*., 2000). Moreover, most of the methods used are not suitable for non-purified samples or for large number of assays because they are expensive, time-consuming, or limited to a few samples. Thus, there is an increasing utilization of chromogenic and fluorimetric assays, by far the simplest, the most reliable, and the easiest for large experiments (Whaler & Reymond, 2001). The most common methods for measuring the activity of lipases are briefly summarized below:

● **Plate assays**: they are non-quantitative methods. Lipase activity is usually detected by the appearance of degradation haloes on culture media supplemented with mechanical emulsions of the desired substrates: tributyrin, triolein, other AGs, olive oil, etc (Jaeger *et al*., 1999). The release of FAs from these substrates can be also detected by adding pH indicators, whose colour changes in response to the acidification produced by free FAs (Samad *et al*., 1989), or by adding fluorophores such as Rhodamine B, which forms complexes with FAs producing orange–pink fluorescence under UV light irradiation (Kouker & Jaeger, 1987). Another approach is the use of media supplemented with polysorbates of AGs (Tweens) and salts, in which released FAs are detected because they form insoluble soaps (Ionita *et al*., 1997).

● **Spectrophotometric methods (colorimetric assays :** these quantitative assays are widely used due the fact that they are fast and simple. Some of them are based on the detection of the FAs released by lipases acting on natural substrates. Detection can be direct or indirect (by the formation of FA soaps of Cu^{2+}, by additional enzymatic reactions, etc) (Beisson *et al*., 2000). For example, Pencreac'h *et al*. (2002) reported a direct assay based on the use of long-chain TAGs from *Aleurites fordii* seeds. These TAGs contain α-eleostearic acid ($C_{18:3c\Delta9,11,13}$), a chromophore released after hydrolysis that can be detected by UV spectrophotometry. On the other hand, it is also common the use of analogs of AGs such as *p*-nitrophenyl esters of FAs (*p*-NPs), which hydrolysis releases *p*-nitrophenol, a chromophore easily detected at 400−410 nm (Jaeger *et al*., 1999; Prim *et al*., 2000). However, care must be taken to interpret these results because certain short-chain *p*-NPs can be also hydrolyzed by other enzymes or by basic solutions (Beisson *et al*., 2000). This problem can be overcome by using the TAG derivative 1,2-O-dilauryl-rac-glycero-3-glutaric acid resorufin ester. The hydrolysis of this compound yields resorufin, which can be determined spectrophotometrically at 572 nm, or fluorimetrically at 583 nm (Jaeger *et al*., 1999).

● **(Spectro fluorimetric methods:** these qualitative and quantitative methods are fast, simple, and very sensitive (Beisson *et al*., 2000). They are usually based on the use of AGs analogs marked with fluorophores such as dansyl, resorufin, or 4-methylumbelliberone (MUF) groups. Lipolytic activity on these analogs releases the fluorophores, which can be detected fluorimetrically (Díaz *et al*., 1999; Beisson *et al*., 2000; Prim *et al*., 2000). Other approaches include the detection of FAs released from AGs by fluorimetric determination of Rhodamine B–FAs complexes (Beisson *et al*., 2000).

● **Chromatographic assays: C**hromatographic techniques can be used to detect or quantify FAs released from TAGs and other lipids. These techniques are very sensitive, although they are time-consuming. The most common ones are: silicic acid columns, thin-layer chromatography, gas-liquid chromatography of FA

methyl esters, and high-performance liquid chromatography (HPLC) (Beisson *et al.*, 2000). The latest is usually performed by detecting β-naphtol or *p*-nitrophenol released as a result of lipolytic activity on their corresponding FA-ester derivatives (Maurich *et al.*, 1991a; Maurich *et al.*, 1991b; Grippa *et al.*, 1999). Chromatographic assays are also used for detecting the reaction products of lipases in reactions of synthesis or acyl exchange, as well as for assaying the enantioselectivity of these enzymes (Jaeger *et al.*, 1999).

● **Titrimetric methods:** they are quantitative assays widely used as reference methods for the determination of the specificity and the kinetic parameters of lipases, although they have low sensibility. Lipase activity is measured on a mechanically stirred emulsion of natural or synthetic TAGs by a pH-stat equipment, which neutralizes the FAs released by adding titrated NaOH in order to maintain the pH at a constant end point value (Beisson *et al.*, 2000).

● **Radiometric methods:** these quantitative assays are very sensitive, although they require special substrates and installations. They detect the FAs released by lipases from AGs with radioactively-marked acyl chains (Beisson *et al.*, 2000).

● **Tensiometric methods:** they are quantitative assays very suitable to determine the kinetic parameters of TLs, although they require expensive equipment and experienced personnel. They are based in the monolayer technique, in which a lipid film is spread at the air/water interface. Lipase-catalyzed hydrolysis of the lipid monolayer results in changes of the surface pressure, which can be readjusted automatically by a computer-controlled barostat (Jaeger *et al.*, 1999).

● **Other methods:** Conductimetric, turbidimetric and infra red assays are also used to detect the hydrolysis or synthesis catalyzed by lipases. Moreover, atomic force microscopy, NMR, and crystallography techniques are employed to analyze the interactions between lipases and their substrates or inhibitors (Beisson *et al.*, 2000).

2.4 THE LIPASE OF *Candida rugosa* AS A MODEL ENZYME

Candida rugosa, formerly *C. cylindracea*, is a non-sporogenic, pseudofilamentous, unicellular yeast. *C. rugosa* is considered as GRAS (generally regarded as safe) microorganism (Benjamin & Pandey, 1998), although some *C. rugosa* strains refractory to antifungal therapy are involved in veterinary mycology and in emerging pathogenesis on immunocompromised patients (Colombo *et al*., 2003).

C. rugosa secretes a mixture of closely related lipase isoenzymes (CRLs) encoded by different genes of a "lipase minigene family" (Lotti *et al*., 1994). Up to the present, the genes coding for seven of such lipases (LIP1 to LIP7) have been identified and sequenced. The isoforms fully characterized (LIP1 to LIP5) show 85−90% amino acid similarity among themselves, being LIP1 the major constituent of CRL. All five are proteins of 534 amino acids with a molecular weight of approx. 60 kDa. Furthermore, they have a similar pI (3.9−4.0), optimum temperature (30−40 ºC), thermostability (up to 50 ºC), optimum pH (6.5−7.5), pH stability (8.0−8.5), and glycosilation degree (5%) (Schmidt-Dannert, 1999; Cygler & Schrag, 1999). Chromatographic resolution of the various isoforms obtained from natural sources has been difficult and only three of them have been purified and crystallized: LIP1 (Grochulski *et al*., 1993; Grochulski *et al*., 1994), LIP2 (Mancheño *et al*., 2003) and LIP3 (Ghosh *et al*., 1995).

CRL (Figure I.19) displays the general features of lipases: α/β hydrolase fold, a Ser-Glu-His catalytic triad, and the typical Gly-Xaa-Ser-Xaa-Gly pentapeptide. However, owing to its large size, CRL contains more strands in the central β sheet, and more α helix than in the canonical α/β hydrolase fold (Cygler & Schrag, 1997). Furthermore, CRL contains a lid formed by one loop encompassing residues 65 to 93 (LIP1, Grochulski *et al*., 1994). This loop contains two α helix, and its topological position is between strands β1 and β2, N-terminal to the nucleophile. The lid makes the active site inaccessible to the solvent in the closed conformation. In the open conformation, the active site is accessible and lies at the bottom of a large depression created by the lid movement. This conformation of the lid, which then forms one of the walls of the substrate binding site, is stabilized by interactions with the carbohydrates and residues of the protein surface (Cygler & Schrag, 1997).

Figure I.19 Ribbon diagram of the three-dimensional structure of *Candida rugosa* lipase.

The central mixed β-sheet is light blue and a smaller N-terminal β-sheet is dark blue. Helices which pack against the central β-sheet are dark green. The closed conformation of the lid is yellow and the open conformation is red. The residues forming the catalytic triad (S: Ser^{209}, D: Glu^{341} and H: His^{449}) are shown in red (Cygler & Schrag, 1999).

Despite the presence of a lid structure, some CRL isoenzymes (e.g. LIP3 but not LIP1) do not show interfacial activation in the presence of substrate interfaces or detergent micelles due to they are also active in aqueous solution. This fact seems to be related to the existence of both monomers and dimers of these isoenzymes. The closed conformation of monomers predominates in aqueous solution, whereas the open conformation is present only transiently in the absence of an interface. On the contrary, dimers form an equilibrium between the open and the closed state because both conformations are stable in aqueous solutions. Thus, there is also enzymatic activity in the absence of an interface (Turner *et al*., 2001; Pernas *et al*., 2001).

CRL displays a more complex substrate-binding site than other lipases, in which the scissile acyl chains lie on the surface of the protein. In CRL, the polypeptide chain folds over this site forming a deep tunnel penetrating towards the centre of the molecule

that has no solvent molecules (Grochulski *et al*., 1994; Ghosh *et al*., 1995; Pleiss *et al*., 1998). The tunnel has an unusual "L" shape, it is 25 Å long (it can fit up to C_{18} FAs), and it has a diameter of 4 Å (Cygler & Schrag, 1999). A phenylalanine-rich area near the tunnel entrance contains the catalytic Ser^{209}, whereas the tunnel walls are lined with hydrophobic residues, and the tunnel bottom is rich in aliphatic residues (Cygler & Schrag, 1997; Mancheño *et al*., 2003). The size of the tunnel indicates that it can accommodate only one acyl chain. The positions of the other acyl chains of the substrate would be located in hydrophobic patches under the tunnel entrance, on the protein surface. It seems that the TAG molecule must adopt the tuning fork conformation to be accommodated by this lipase, although it is unclear how the TAG enters, or how the acid products leave the tunnel (Cygler & Schrag, 1997 and 1999).

CRL catalyzes a broad range of reactions (hydrolysis, synthesis, trans- and interesterification) with a wide range of specificities (substrate, positional, FA, and stereopreference), mainly due to the occurrence of the different isoforms. In general, CRL is capable of hydrolyzing FAs on all positions of glycerol, and it displays preference for aggregated substrates. Moreover, CRL shows preference for short-chain FAs, although CRL can also distinguish among FAs of similar chain-length (e.g. palmitic and oleic acids are liberated before than stearic acid) (Benjamin & Pandey, 1998). Nevertheless, the diverse isoenzymes (LIP1 and LIP3 are the best known) can be very different with respect to their enzymatic properties (Rúa *et al*., 1993; Rúa & Ballesteros, 1994; Plou *et al*., 1997; Diczfalusy *et al*., 1997).

CRL is commercially available from NOVOZYMES (Denmark), which has led to an extensive study of the properties of this enzyme, probably the best known lipase with respect to its structure and function. In fact, many of the lipase features mentioned in the previous pages, such as the folding, the molecular basis of the interfacial activation, or the anatomy of the active site, have been determined using this lipase as a model (Benjamin & Pandey, 1998). CRL has been also used as a model in several lipase inhibition assays (Grippa *et al*., 1999; Gatto *et al*., 2002). All this knowledge, as well as its catalytic versatility have made of this lipase the most widely used in biotechnology (Benjamin & Pandey, 1998).

3 BACTERIAL LIPASES

Bacterial lipases are gaining an increasing interest due to their potential as biotechnological catalysts, as well as by their role as virulence factors in some pathogenic bacteria (Rosenau & Jaeger, 2000).

3.1 PHYSIOLOGY AND PROPERTIES OF BACTERIAL LIPASES

Understanding the mechanisms of gene expression, folding, and secretion of bacterial lipases, as well as their enzymatic properties, is essential for improving the biotechnological application of these enzymes, and for the treatment of lipase-related diseases (Rosenau & Jaeger, 2000)

3.1.1 Regulation of gene expression

Until recently, a significant number of prokaryotic lipase genes have been cloned, but the molecular mechanisms regulating their expression remain largely unknown because these mechanisms have been only studied in some lipases produced by pathogenic microorganisms, or produced by biotechnologically-interesting strains during fermentation processes. From these studies, it has been concluded that lipase gene expression is a highly regulated system, mainly for extracellular lipases. A cell density-dependent regulation seems to be the most generalized regulatory mechanism, although additional activators or repressors of transcription responding to a variety of environmental signals exist as well (Jaeger *et al.*, 1999; Gupta *et al.*, 2004).

General regulation of lipases forms part of a global regulatory network that coordinately controls the transcription of: (1) genes involved in the use of additional carbon sources, (2) genes encoding extracellular proteins, or (3) genes involved in pathogenesis. This sensing system can be mediated through regulators of one or more

components, and by alternative sigma factors of RNA polymerase. Lipase transcription is usually activated when the microorganism enters the stationary growth phase, although some lipases show a basal constitutive synthesis. Moreover, different regulatory mechanisms seem to coexist in microorganisms producing several lipases. In general, some of these lipases are activated or repressed in response to environmental signals, whereas the others are expressed constitutively. Figure I.20 summarizes the current status of knowledge about the regulatory network controlling expression of the lipase operon *lipA/H* of *Pseudomonas aeruginosa*, one of the most studied bacterial lipases with respect to its regulation (Jaeger *et al*., 1999).

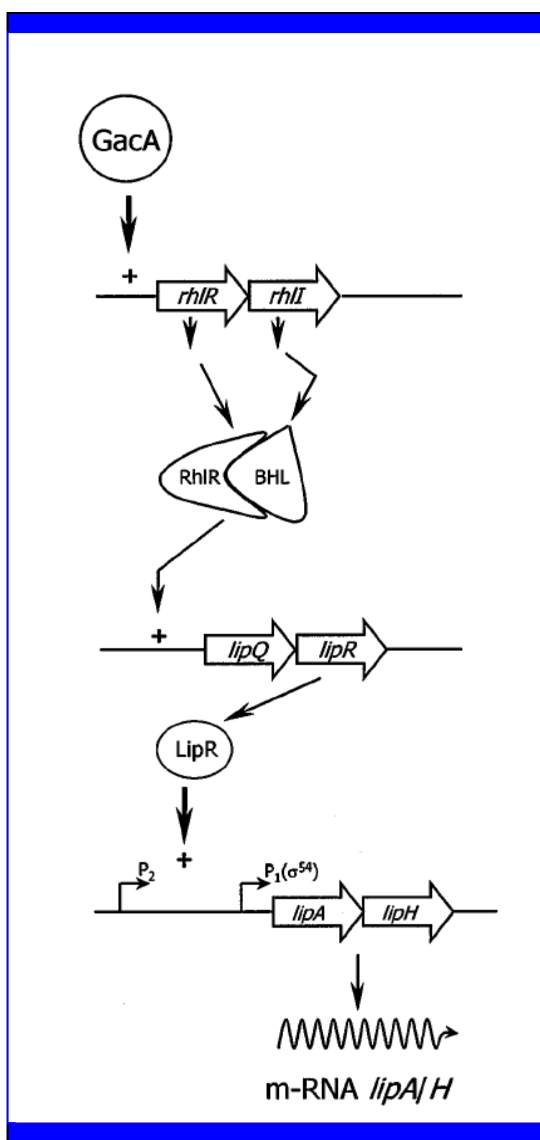


Figure I.20 Model for the regulatory network controlling expression of the lipase operon *lipA/H* in *Ps.* aeru*ginosa*.

The extracellular lipase of *Ps. aeruginosa* is secreted when the bacteria enters the stationary growth phase. Lipase gene expression seems to be the result of a cell density-dependent regulation involving the *rhlR/I* system (also named *vsmR/I*), which includes the autoinducer *N*-butyryl-homoserine lactone (BHL). The *rhlR/I* system is one of the quorum sensing systems identified in *Ps. aeruginosa*, and it controls the expression of several extracellular enzymes, as well as the synthesis of the transcriptional activator LipR. LipR, which could be part of a two-component system involving the putative protein Lip , is the cognate transcriptional activator required by the alternative sigma factor σ^{54} for the transcription of the lipase operon *lipA/H* from promotor P1. A second promotor (P2) located ~300 base pairs upstream of P1 exists, although its regulation is still unknown. Further assays have led to the identification of the additional global regulator gene *gacA*, which probably acts as part of another two-component system responsible for the activation of the *rhlR/I* system (Jaeger *et al*., 1999).

The general regulatory system of lipase gene expression is modulated by a variety of environmental signals by means of additional activators or repressors of transcription. The major factor controlling the expression of lipase activity is carbon. Lipase expression is induced by the presence of lipid sources such as olive oil, TAGs, alkanes, polysorbates, bile salts, and FAs, although the latter ones can also act as repressors. Furthermore, lipase production is significantly influenced by other carbon sources such as sugars, polysaccharides, amino acids, and other complex compounds. In addition, lipase synthesis is influenced by other nutritional and physicochemical factors such as temperature, pH, type of nitrogen source (organic or inorganic), inorganic salts, agitation, and oxygen concentration (Gupta *et al.*, 2004).

3.1.2 Folding of bacterial lipases

Correct protein folding is essential for the activity of biocatalysts, and for their translocation through bacterial membranes, in the case of extracellular lipases (most of bacterial TLs). Many lipases are directly folded by themselves in the cell cytoplasm or during secretion. However, some lipases require chaperones which help them in overcoming a kinetic barrier along their folding pathway. In addition, chaperones may also function as temporary competitive inhibitors of their cognate enzymes, thus avoiding an undesirable activity of the enzyme on cell lipids (Jaeger *et al.*, 1999; Jaeger & Eggert, 2002). These chaperones can be divided into:

1. Intramolecular chaperones: lipases produced by the Gram-positive bacteria of the genus *Staphylococcus* are synthesized as pre-proenzymes. They have an N-terminal pro-region of about 260 amino acids acting as a folding catalyst, which is cleaved in the extracellular medium by a specific protease. The pro-region facilitates the translocation of these lipases through the cytoplasmic membrane, but also protects them from proteolytic attack (Götz *et al.*, 1998).

2. Intermolecular chaperones: specific lipase intermolecular foldases (Lif proteins) have been identified in *Pseudomonas* spp. and other Gram-negative bacteria. These foldase proteins are required for the correct folding of lipases in the bacterial periplasm. Therefore, Lif coding-genes are usually located in an operon with their

cognate lipase genes. The interaction of a Lif and a lipase is almost always specific with respect to both proteins. Lif proteins contain a hydrophobic N-terminal domain that keeps them anchored to the inner membrane. Lif anchorage prevents their secretion, thus rendering them reusable (Jaeger *et al*., 1999). Correct folding of foldase-dependent lipases is necessary for their subsequent translocation through the outer membrane. However, these lipases usually require additional accessory proteins involved in disulfide bond formation (Dsb proteins). These disulfide bonds seem to be necessary for the final secretion step of these enzymes as well as for the enzyme stability against proteolysis and denaturing agents (Jaeger *et al*., 1999). On the contrary, lipases are generally not sulphydryl proteins, thus in most of them neither free –SH nor S–S bridges are important for their catalytic activity (Gupta *et al*., 2004).

3.1.3 Secretion of bacterial lipases

Many lipases from Gram-positive and Gram-negative bacteria are secreted enzymes. At present, three major secretion pathways have been identified for bacterial lipases (Jaeger & Eggert, 2002; see Figure I.21):

1. The Sec–Xcp system: many lipases from Gram-positive and Gram-negative bacteria possess an N-terminal signal sequence. This leader peptide mediates their secretion through the plasmatic membrane by means of the Sec translocase, a protein complex formed by soluble and membrane-embedded multisubunits that secretes the prelipase and cuts off the signal peptide. The Sec machinery has been found, among others, in *Escherichia coli* and *Bacillus* spp. (Fekkes & Driessen, 1999; Jaeger *et al*., 1999). In Gram-negatives, a second step is necessary to achieve complete secretion. The exported lipase, which is folded in the periplasm by means of Lif and Dsb proteins, is transported through the outer membrane by the secreton (Xcp machinery), a complex of 14 different proteins that form the type II, or general secretion pathway (Pugsley, 1993; Jaeger *et al*., 1999).

2. The Tat system: a second translocation mechanism has been described to operate in both, Gram-positive and Gram-negative bacteria. It was named Tat pathway

because proteins using this pathway contain a unique Twin arginine translocation motif in their signal sequence (Jaeger & Eggert, 2002).

3. The ABC system: some Gram-negative secreted lipases lack a typical N-terminal signal sequence, although it has been found that some of them may have a C-terminal motif involved in their mechanism of folding and secretion (Eggert & Jaeger, 2002). These lipases are secreted by the type I secretion pathway, also known as the ABC exporter. This pathway consists of three different proteins: (1) an inner membrane protein containing an ATP-binding cassette (ABC protein) and responsible for the substrate specificity to the system, (2) a membrane fusion protein (MFP) which can be associated with both the inner and the outer membrane, and (3) an outer membrane protein (OMP) (Jaeger *et al*., 1999).

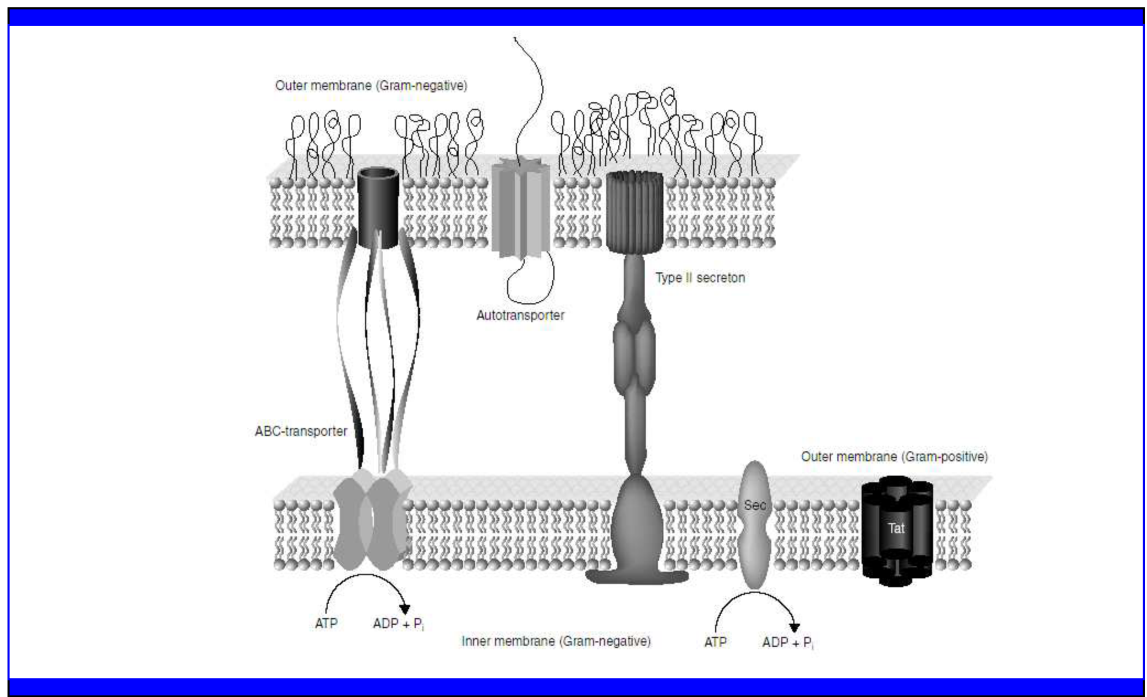


Figure I.21 Major pathways used by bacteria to secrete lipases (Eggert & Jaeger, 2002 .

In addition, some esterases of the GDSL family (Arpigny & Jaeger, 1999) contain an additional C-terminal domain that includes about one-third of their entire sequence. This domain shows high similarity to the autotransporting domains of a

newly identified family of virulence factors which can be translocated through the outer membrane of Gram-negative bacteria by themselves (Loveless & Saier, 1997; Henderson *et al.*, 1998). In these proteins the C-terminal domain is presumably folded into approximately 12 amphipathic β-sheets that would form an aqueous pore in the outer membrane. This pore would allow the transit of the catalytic N-terminal domain, which would be subsequently released into the extracellular medium by a specific proteolytic process (Arpigny & Jaeger, 1999).

3.1.4 Enzymatic properties of bacterial lipases

The features of bacterial lipases have been studied mainly due to the industrial or clinical interest of these enzymes. They are very diverse enzymes with a wide range of enzymatic properties, although some conclusions can be obtained by comparing them. These conclusions, mainly focused on TLs, are briefly summarized below.

Generally, bacterial lipases have neutral or alkaline pH optima although there are some exceptions (Bornscheuer, 2002). They are active over a broad pH range (pH 4–12), mainly in the case of lipases from the genus *Bacillus* (pH 3–12). Bacterial lipases generally have temperature optima in the range of 30–60°C, and they show thermal stability up to 60ºC, although lower and higher (mainly for thermophilic *Bacillus* lipases) ranges have been reported. Furthermore, the thermostability of lipases may be enhanced by the addition of stabilizers such as ethylene glycol or glycerol. In addition, lipases are generally stable in organic solvents, such as ethanol or acetone, with few exceptions of stimulation or inhibition (Gupta *el al.*, 2004).

Cofactors are usually not required for lipase activity, but divalent cations such as calcium often stimulate enzyme activity. This has been suggested to be due to the formation of calcium salts of long-chain FAs, which reduce the interference of FAs at the interface, or their inhibition on the enzyme. In contrast, some lipases are inhibited by the presence of calcium ions. Furthermore, heavy metals like Fe^{3+}, Co^{2+}, Ni^{2+}, Hg^{2+} and Sn^{2+} inhibit drastically lipase activity, whereas Zn^{2+} and Mg^{2+} are slight inhibitors (Patkar & Björkling, 1994).

3.2 CLASSIFICATION OF BACTERIAL LIPASES

Classification of enzymes can be done either based on their substrate specificity or by sequence alignments. The former requires that all enzymes have to be assayed with the same or similar substrates and reaction conditions to compare them. This fact is very difficult as there are wide differences among the substrates and methods used in the different laboratories. On the contrary, the increasing availability of sequence information in public databases allows the comparison of amino acid sequences, which can provide a clearer picture about the similarity and evolutionary relationships between lipases. Therefore, classifications based on sequence similarity comparisons are the most used nowadays, although they have some limitations. In fact, sequence similarity cannot be often related to the enzymatic properties of a defined lipase such as substrate specificity, stereoselectivity, pH and temperature optima, etc. Furthermore, some lipases show a considerable sequence similarity to other non-lipolytic enzymes, which makes it difficult the interpretation of the similarity results obtained for these lipases (Bornscheuer, 2002).

Arpigny & Jaeger (1999) elaborated the most accepted classification of bacterial lipases. They grouped bacterial lipases into eight families based on the conserved sequence motifs and biological properties of 53 lipases and esterases. Subsequently, several extensions of the original classification scheme such as the enlargement of family I from 6 to 7 subfamilies have been proposed attending to novel research results (Nthangeni *et al*., 2001; Jaeger & Eggert, 2002). An extended version of the original classification is shown in Table I.5, and a brief explanation of the properties of the eight lipase families is included in the following pages.

Table I.5 Families of lipolytic enzymes (Arpigny & Jaeger, 1999; Jaeger & Eggert, 2002 .

Abbreviations: Sf, subfamily; OM; outer membrane; PHA, polyhydroxyalkanoate.

Family	Sf	Strain	Accession number	Similarity (%		Properties
				Family	Sf	
I	**1**	*Pseudomonas aeruginosa* (LipA)	D50587	100		"True" lipase
		Pseudomonas fluorescens C9	AF031226	95		
		Vibrio cholerae	X16945	57		
		Pseudomonas aeruginosa (LipC)	U75975	51		
		Acinetobacter calcoaceticus	X80800	43		
		Pseudomonas fragi	X14033	40		
		Pseudomonas wisconsinensis	U88907	39		
		Proteus vulgaris	U33845	38		
	2	*Burkholderia glumae*	X70354	35	100	
		Chromobacterium viscosum	05489	35	100	
		Burkholderia cepacia	M58494	33	78	
		Pseudomonas luteola	AF050153	33	77	
	3	*Pseudomonas fluorescens* SIKW1	D11455	14	100	
		Serratia marcescens	D13253	15	51	
	4	*Bacillus subtilis* (LipA)	M74010	16	100	Secreted carboxylesterase
		Bacillus pumilus	A34992	13	80	
		Bacillus licheniformis	U35855	13	80	
		Bacillus subtilis (LipB)	C69652	17	74	
	5	*Geobacillus stearothermophilus* L1	U78785	15	100	"True" lipase
		Geobacillus stearothermophilus P1	AF237623	15	94	
		Geobacillus thermocatenulatus	X95309	14	94	
		Geobacillus thermoleovorans	AF134840	14	92	
	6	*Staphylococcus aureus*	M12715	14	100	
		Staphylococcus haemolyticus	AF096928	15	45	
		Staphylococcus epidermidis	AF090142	13	44	
		Staphylococcus hyicus	X02844	15	36	Phospholipase
		Staphylococcus xylosus	AF208229	14	36	"True" lipase
		Staphylococcus warneri	AF208033	12	36	
	7	*Propionibacterium acnes*	X99255	14	100	
		Streptomyces cinnamoneus	U80063	14	50	

Family	Sf	Strain	Accession number	Similarity (% Family	Sf	Properties
II		*Aeromonas hydrophila*	P10480	100		Secreted acyltransferase
(GDSL		*Streptomyces scabies*	M57297	36		Secreted esterase
		Pseudomonas aeruginosa	AF005091	35		OM-bound esterase
		Salmonella typhimurium	AF047014	28		OM-bound esterase
		Photorhabdus luminescens	X66379	28		Secreted esterase
III		*Streptomyces exfoliatus*	M86351	100		Extracellular lipase
		Streptomyces albus	U03114	82		Extracellular lipase
		Moraxella sp.	X53053	33		Extracellular esterase 1
IV		*Alicyclobacillus acidocaldarius*	X62835	100		Esterase
(HSL		*Pseudomonas* sp. B11-1	AF034088	54		Lipase
		Archaeoglobus fulgidus	AE000985	48		Carboxylesterase
		Alcaligenes eutrophus	L368176	40		Putative lipase
		Escherichia coli	AE000153	36		Carboxylesterase
		Moraxella sp.	X53868	25		Extracellular esterase 2
V		*Pseudomonas oleovorans*	M58445	100		PHA-depolymerase
		Haemophilus influenzae	U32704	41		Putative esterase
		Psychrobacter immobilis	X67712	34		Extracellular esterase
		Moraxella sp.	X53869	34		Extracellular esterase 3
		Sulfolobus acidocaldarius	AF071233	32		Esterase
		Acetobacter pasteurianus	AB013096	20		Esterase
VI		*Synechocystis* sp.	D90904	100		Carboxylesterase
		Spirulina platensis	S70419	50		
		Pseudomonas fluorescens	S79600	24		
		Rickettsia prowazekii	Y11778	20		
		Chlamydia trachomatis	AE001287	16		
VII		*Arthrobacter oxydans*	01470	100		Carbamate hydrolase
		Bacillus subtilis	P37967	48		*p*-Nitrobenzyl esterase
		Streptomyces coelicolor	CAA22794	45		Putative carboxilesterase
VIII		*Arthrobacter globiformis*	AAA99492	100		Stereoselective esterase
		Streptomyces chrysomallus	CAA78842	43		Cell-bound esterase
		Pseudomonas fluorescens SIK	AAC60471	40		Esterase III

3.2.1 Family I

This family includes lipases from both Gram-positive and Gram-negative bacteria, most of them “true” lipases. Subfamilies I.1, I.2 and I.3 include “true” lipases with a molecular weight of 30−32 kDa from *Pseudomonas* spp. and other Gram-negatives. Lipases from subfamilies I.1 and I.2 have a Sec signal that mediates their secretion through the type II secretion pathway (Sec–Xcp system). Stabilization of their active site requires two aspartic residues involved in a Ca^{2+}-binding site, and their folding and secretion requires Lif and Dsb proteins. Subfamily I.3 enzymes have no N-terminal signal peptide and no cysteine residues. They are secreted by the type I pathway (ABC transport system) (Arpigny & Jaeger, 1999).

Subfamilies I.4 and I.5 include the secreted lipases of the genera *Bacillus* and *Geobacillus*. These lipases have in common that an alanine residue replaces the first glycine in the conserved pentapeptide that contains the catalytic serine (Ala-Xaa-Ser-Xaa-Gly). Lipases from the mesophilic *Bacillus* species are grouped in subfamily I.4. They are the smallest lipases known (~20 kDa) and share very little sequence similarity with the other *Bacillus* lipases. Lipases from the generally thermo- and alkalophilic *Geobacillus* species have a molecular mass of ~45 kDa, and display maximal activity approximately at pH 9.0 and 65 °C (Arpigny & Jaeger, 1999; Jaeger & Eggert, 2002).

Subfamily I.6 groups Staphylococcal lipases, which are large enzymes (~75 kDa) secreted as precursors. The propeptide (about 260 residues), an intramolecular chaperone which facilitates the folding and secretion of the enzyme, is cleaved at the extracellular medium by a specific protease, yielding a mature protein of ~400 residues. Interestingly, the lipase from *S. hyicus* also displays a remarkable phospholipase activity, which is unique among “true” lipases (Arpigny & Jaeger, 1999).

Subfamily I.7 includes the lipases from *Propionibacterium acnes* (339 residues) and from *Streptomyces cinnamoneus* (275 residues), which show significant similarity to each other. Their central region (50−150 residues) shows ~50% similarity to lipases of subfamily I.2, and to the small lipases from *B. subtilis* belonging to subfamily I.4 (Arpigny & Jaeger, 1999).

3.2.2 Families II−VIII

The enzymes grouped into family II (GDSL family) do not exhibit the conventional pentapeptide Gly-Xaa-Ser-Xaa-Gly. They instead display a Gly-Asp-Ser-(Leu) [GDS(L)] motif containing the active-site serine, which lies much closer to the N-terminus than in other lipolytic enzymes. A very interesting member of this family is the esterase from *Streptomyces scabies*, previously mentioned in General Introduction 2.2.2 because of having, in contrast to most lipases, a Ser-His catalytic dyad and a particular tertiary fold (Arpigny & Jaeger, 1999).

Family III includes extracellular lipases from a psicrophilic *Moraxella* sp. and from several *Streptomyces* species. Enzymes of family IV, also named HSL (hormone-sensitive lipase) family, display amino acid sequence blocks similar to those of mammalian hormone-sensitive lipases, which indicates that HSLs could derive from a catalytic domain homologous to bacterial lipases. These sequence blocks were thought to be related with cold adaptation although this family includes also mesophilic and thermophilic enzymes (Arpigny & Jaeger, 1999; Bornscheuer, 2002).

Enzymes grouped into family V originate also from psicrophilic and mesophilic bacteria. They share significant amino acid sequence similarity (20−25%) to various bacterial non-lipolytic enzymes which also possess the typical α/β-hydrolase fold and a catalytic triad, such as epoxide hydrolases, dehalogenases and haloperoxidases. Enzymes of family VI are among the smallest carboxylesterases known (molecular mass of 23−26 kDa). They display ~40% sequence similarity to eukaryotic lysophospholipases (Arpigny & Jaeger, 1999).

Family VII includes large esterases (50–65 kDa) with significant amino acid sequence similarity to eukaryotic acetylcholine esterases and intestine/liver carboxylesterases. Family VIII is formed by three enzymes of ~380 residues that show a striking similarity to several class C β-lactamases. Two of them have a Gly-Xaa-Ser-Ala-Gly motif strangely not followed by a histidine, and located near the C-terminus of the protein. Site directed mutagenesis assays seem to indicate that the catalytic serine is not located in this pentapeptide, but laying in a Ser-Xaa-Xaa-Lys motif contained at the enzyme's N-terminal region (Arpigny & Jaeger, 1999; Bornscheuer, 2002).

3.3 BACTERIAL LIPASES IN BIOTECHNOLOGY

3.3.1 Relevance of microbial enzymes in biotechnology

Microorganisms or their enzymes are used in a wide range of biotechnological activities such as synthesis of antibiotics, vitamins, or biopolymers; decontamination of soils; food, paper or textile industry; etc. Among these activities, the improvement of industrial processes with microbial enzymes is one of the most important fields of research because enzyme-catalyzed reactions are highly efficient and selective, are less polluting, and usually require mild conditions and less energy, which leads to the lowering of costs (Cherry & Fidantsef, 2003). Thus, there is an increasing interest for isolating new enzymes and new enzyme-producing strains for their use in industrial conversions. Among these enzymes, lipases, esterases, cellulases, xylanases, pectinases, amylases and proteases are some of the most important (Cherry & Fidantsef, 2003; Gupta *et al*., 2004).

Cellulases (endoglucanases, exoglucanases and β-glucosidases) are a group of enzymes that catalyze the hydrolysis of glucosidic bonds in cellulose, the most abundant polysaccharide in vegetable biomass. They are currently used for removal of stained fibres in paper recycling, in textile fibre treatment, for alcohol and biofuel production, etc (Bhat, 2000).

(Endo)xylanases produce the hydrolysis of the main polyxylose backbone from xylans, which are the second most abundant vegetable polysaccharides. Some of the main industrial processes improved by xylanases are: removal of xylan and lignin from paper pulp, biofuel and dissolvent production, wine clearing and juice extraction (Beg *et al*., 2001; Viikari *et al*., 2001).

Pectinases are a complex group of enzymes responsible for the degradation of pectin polysaccharides of plant cell walls such as polygalacturonic acid (PGA). These enzymes are used in the clarification, extraction or concentration of juices and other beverages, in tea or coffee fermentation, in vegetable biomass liquefaction, for textile fibre production, etc (Kashyap *et al*., 2001).

3.3.2 Properties of lipases useful in biotechnology

Bacterial (and fungal) lipases represent the most versatile and widely used class of enzymes in biotechnological applications and organic chemistry. This is reflected by more than 1000 original articles and reviews on lipases and lipase applications that appear each year, and by a the fact that the use of lipases in biotechnology was a business of more than 1.5 billion U.S. dollars in year 2000 (Schmidt-Dannert, 1999; Jaeger & Eggert, 2002; Gupta *et al*., 2004). The reasons for the success of lipases in biotechnology are the following:

1. **Versatility**: lipases are very versatile biocatalysts which can carry out many different reactions of hydrolysis and, in organic solvents, synthesis and acyl exchange, using a wide range of natural and non-natural compounds (Bornscheuer, 2002; Gupta *et al*., 2004).

2. **Specificity and selectivity:** some of them show high substrate specificity, or high chemo-, regio- and stereoselectivity (Gunstone, 1999; Jaeger & Eggert, 2002).

3. **Absence of subproducts:** most lipases do not perform lateral reactions (Jaeger & Eggert, 2002).

4. **Stability:** lipases are active and stable in organic solvents, and in a wide range of pH and temperatures (Schmidt-Dannert, 1999; Gupta *et al*., 2004).

5. **Knowledge:** their structure and function is well known, and they can be modified to adapt them to novel uses (Schmidt-Dannert, 1999; Jaeger & Eggert, 2002).

6. **Availability:** lipases, mainly those which are secreted, are available in large amounts by fermentation processes of natural or recombinant strains (Schmidt-Dannert, 1999; Gupta *et al*., 2004).

7. **No cofactors:** most lipases do not require cofactors (Gupta *et al*., 2004).

8. **Low-cost and green:** processes involving the use of lipases have a lower cost and are less polluting, because these enzymes act under mild conditions and with low energy and equipment requirements (Gunstone, 1999; Pandey *et al*., 1999; Jaeger & Eggert, 2002).

3.3.3 Biotechnological applications of lipases

The most common industrial applications of lipases are summarized below:

1. Food industry: lipases are used *in situ*, and sometimes together with other enzymes, during the elaboration of bread, cheese, and other foods to improve their shelf-life and their reological properties, or to produce aromas or emulgents. Moreover, they are used *ex situ* to produce flavours, and to modify the structure or composition of AGs by inter- or transesterification, in order to obtain AGs with an increased nutritional value, or suitable for parenteral feeding (Gunstone, 1999; Reetz, 2002).

2. Organic chemistry: organic chemistry is the most important application of lipases after food industry. They are used to produce specific products that can not be produced chemically, or whose elaboration by classical chemical means is difficult or expensive. For example, they are used in pharmaceutical and agrochemical industries to the modification or synthesis of antibiotics, anti-inflammatory compounds, pesticides, etc, and to the production of enantiopure compounds or the resolution of racemic mixtures (Gunstone, 1999; Pandey *et al*., 1999; Reetz, 2002).

3. Detergency and cleaning: an important application of lipases resistant to high temperatures, proteolysis, and denaturation by surfactants, is their use in the composition of laundry detergents (together with proteases) to improve the removal of lipid stains. They are also used in the synthesis of surfactants for soaps, shampoos and dairy products (Schmidt & Verger, 1998; Pandey *et al*., 1999).

4. Paper industry: lipolytic enzymes are used to remove the “pitch”, the lipid fraction of wood that interferes during the elaboration of paper pulp. They also help in the removal of lipid stains during paper recycling and to avoid the formation of sticky materials (Pandey *et al*., 1999; Gutiérrez *et al*., 2001).

5. **Management of waste and toxic compounds:** lipases can be used in the management of waste produced during fat or food processing, and to the treatment of lipid–contaminated waters and sludge. Moreover, they are useful in the treatment of biofilm deposits, oil-contaminated soils, and poisonous gases (Pandey *et al*., 1999).

6. **Other applications:** lipases can synergistically collaborate with cellulases, pectinases, and proteases in the elaboration and bleaching of cotton fibres (Li & Hardin, 1997). Lipases are also used as components of biosensors, in biodiesel production, in leather processing, in hard-surface cleaning, in single-cell protein production, and in the synthesis of polymers, biodegradable plastics, lubricants or cosmetics (Schmidt & Verger, 1998; Schmidt-Dannert, 1999; Pandey *et al*., 1999; Reetz, 2002).

3.3.4 Optimization of lipase application in biotechnology

The increasing contribution of lipases in the field of biotechnology has led to high efforts aimed to improve their use (Jaeger & Eggert, 2002). These efforts have been focused into four different areas:

3.3.4.1 Optimization of the reaction conditions

Improving the reaction conditions catalyzed by lipases has been achieved by: (1) using new solvents, acyl donors, surfactants, salts, and other additives, or by changing their proportions, (2) continuous removal of the final product to avoid reaching the reaction equilibrium, and (3) immobilization of the enzyme to recycle it (Reetz, 2002; Jaeger & Eggert, 2002; Bornscheuer, 2002).

3.3.4.2 Optimization of lipase production and purification

Production of large amounts of lipases, occasionally purified, is essential for their large-scale application. Therefore, optimization of the lipase production has been focused on: (1) improving fermentation conditions such as carbon or nitrogen source supply, temperature, pH, or aeration, (2) using inductors for lipase expression and synthesis, (3) cloning and overexpresion of the lipase of interest in a host capable of producing and, if possible, secrete large amounts of enzyme, which sometimes requires the modification of the wild lipase-coding gene to add/change its signal peptide or certain codons to adapt them to those more suitable for the host, and (4) improving the purification process (Schmidt-Dannert, 1999; Sánchez *et al*., 2002; Gupta *et al*., 2004).

3.3.4.3 Lipases with new catalytic properties or an increased stability

New biotechnological applications require usually lipases with new catalytic properties such as different substrate specificity, enantioselectivity, etc, or with an increased stability (Gupta *et al*., 2004). This can be solved by two different ways:

1. **Modification of the existing enzymes**: this can be achieved by protein engineering (rational mutation), which requires knowledge about the structure and mechanism of action of the enzyme (Reetz, 2002); or by direct evolution, a process based on successive cycles of random mutagenesis (error-prone PCR, or random recombination of DNA fragments by "DNA shuffling") and selection of the best variants of the modified enzymes. Therefore, this method requires having the nucleotide sequence of the gene, a suitable expression system, and a high-throughput screening method (Jaeger & Eggert, 2002).

2. **Isolation of novel lipases:** a promising approach to obtain new lipases consists on using sequence similarity searches to find new lipase-coding genes among the whole genome of sequenced microorganisms (Ro *et al*., 2004). However, the most common approach is the screening of microorganisms previously isolated from natural sources. Among them, soils are the main source of enzyme-producing strains because they are nutrient-rich environments where there is a high

proliferation of microorganisms. The microbial diversity of each soil depends on nutrient availability and several physicochemical properties related to the climate and type of soil. In general, many soil microorganisms are highly active in nutrient recycling, mainly in the degradation of lipids and vegetal polysaccharides (Atlas & Bartha, 2001). Nevertheless, some strains can not be isolated and cultured, which has led to complementary processes such as the metagenome approach. This method consists on the direct cloning and/or sequencing of DNA isolated from environmental sources, and it has revealed to be very useful for obtaining new lipase or other enzyme-coding genes (Gupta *et al*., 2004).

3.3.5 Relevance of Bacillales in biotechnology

Bacillus-related species are facultative anaerobic or aerobic, Gram-positive, endospore former, rod-shaped bacteria commonly found in soil, water sources or associated with plants (Priest, 1993). The original *Bacillus* genus, as is defined in Bergey's Manual of Systematic Bacteriology (Claus & Berkeley, 1986), includes a wide and heterogenous group of species, and therefore, it has been divided into the genera: *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Aneurinibacillus*, *Alicyclobacillus*, *Halobacillus*, *Gracilibacillus*, *Geobacillus, Salibacillus* and *Virgibacillus* (Nazina *et al*., 2001; Stackebrandt & Swiderski, 2002).

Bacillus and related genera are one of the most important sources of industrial enzymes because: (1) most of them are considered GRAS microorganisms, (2) they produce and secrete large amounts of homologous or heterologous enzymes including lipases, xylanases, cellulases and pectinases, etc, (3) their genetics, biochemistry and physiology are well characterized in some of them, and (4) they are easy to handle (Ferrari, 1993).

For these reasons, there is an increasing interest in isolating new lipases or new lipase-producing strains from these genera, which has led to the isolation of several lipolytic enzymes showing high biotechnological potential (Gupta *et al*., 2004). Many efforts have been performed on *B. subtilis*, the best known Gram-positive bacterium. Its genome (Kunst *et al*., 1997), proteome (Hirose *et al*., 2000) and secretome (Tjalsma *et*

al., 2000; Antelman *et al*., 2001) have been determined, allowing the discovery, location, and function designation of several previously unknown genes in this strain.

In *Bacillus subtilis*, several intracellular and extracellular esterases have been found, including the non-secreted carboxylesterase PnbA, an enzyme suitable for biotechnological processes such as synthesis of antibiotics (Zock *et al*., 1994), and classified into family VII of bacterial lipases (Arpigny & Jaeger, 1999).

Moreover, two secreted carboxylesterases from *B. subtilis* (LipA and LipB), classified into subfamily I.4 of bacterial lipases (Arpigny & Jaeger, 1999), have been also cloned and characterized (Dartois *et al*., 1992; Eggert *et al*., 2000). LipA was the first of these enzymes in being characterized, which revealed that LipA was a 19-kDa (mature) enzyme with activity on triolein. For these reason, it was considered as a “true lipase”, although further assays showed that the enzyme displayed a marked preference towards short–medium triacylglycerols and *p*-nitrophenyl esters, the typical substrates of carboxylesterases (Kennedy & Lennarz, 1979; Dartois *et al*., 1992; Lesuisse *et al*., 1993; Eggert *et al*., 2000). The molecular and biochemical features of LipA have led to the overexpression of this enzyme in several hosts (Dartois *et al*., 1992; Sánchez *et al*., 2002), as well as to its application in several biotechnological applications such as the production of β-blocker compounds (Dröge *et al*., 2000).

LipB carboxylesterase, showing 73% identity to LipA at the protein level, showed similar substrate preference than LipA, although it not hydrolyzed at all triolein, (Yamamoto *et al*., 1996; Kuntz *et al*., 1997; Eggert *et al*., 2000). However, further studies revealed that LipA and LipB differ in regulation of gene expression, in their secretion pathway, in some biochemical properties, in the residues located at the protein surface (Tjalsma *et al*., 2000; Eggert *et al*., 2001 and 2003).

Analysis of other lipolytic strains and species from *Bacillus* and related genera has led to the isolation of additional biotechnologically-interesting lipases, including lipases belonging to subfamily I.4 from other mesophilic *Bacillus* species such as *B. pumilus* (Moeller *et al*., 1991; Kim *et al*., 2002) and *B. licheniformis* (Nthangeni *et al*., 2001). Moreover, several lipases grouped into subfamily I.5 have been obtained from thermophilic *Bacillus* (now named *Geobacillus*): *Gb. thermocatenulatus* (Schmidt-Dannert *et al*., 1996), *Gb. thermoleovorans* (Lee *et al*., 1999) and *Gb.*

stearothermophilus (Kim *et al*., 1998). Two cell-bound type B esterases belonging to family VII, and similar to *B. subtilis* PnbA, have also been described: *Paenibacillus* sp. BP-23 EstA (Prim *et al*., 2000), and *Bacillus* sp. BP-7 EstA1 (Prim *et al*., 2001).

3.4 BACTERIAL LIPASES INVOLVED IN VIRULENCE

Many lipolytic bacteria and fungi are involved in infectious diseases (Table I.6), although lipases seem to play a significant role only in some of them: *Staphylococcus* spp., *Pseudomonas* spp. (Jaeger *et al*., 1994), *Propionibacterium acnes* (Higaki, 2003) and *Helicobacter pylori* (Smoot, 1997). During the last decades, both *Propionibacterium acnes* and *Helicobacter pylori* have generated a high interest because of their social and clinical implications. For these reasons, they are treated with more detail in the following pages.

Table I.6 Main lipolytic microorganisms involved in pathogenesis.

Microorganisms	Diseases	References
Acinetobacter spp.	Lung/urinary infections, endocarditis	Breuil & Kushner, 1975; Simons *et al*., 1998a
Acrhomobacter lacticum	Urinary infections	Fryer *et al*., 1967
Actinobacillus actinomycetemcomitans	Plaque	Dogan *et al*., 1999
Aspergillus niger	Otitis, nasal sinus and lung infections	Tombs & Blake, 1982
Bacillus subtilis	Opportunistic infections	Kouker & Jaeger, 1987; Thomson *et al*., 1999
Burkholderia cepacia	Cystic fibrosis	McKenney *et al*., 1995
Candida albicans	Candidiasis, opportunistic diseases	Stehr *et al*., 2004
Candida rugosa	Candidiasis, opportunistic diseases	Lotti *et al*., 1998; Colombo *et al*., 2003

Microorganisms	Diseases	References
Escherichia coli	Intestinal and urinary (opportunistic) infections	Nantel & Proux, 1973; Linder *et al*., 1988
Dermatophytes *(Microsporum canis, Gypseum* spp., *Epidermophyton floccosum, Trichophyton* spp.)	Cutaneous mycosis, warts	Nobre & Viegas, 1972
Fusobacterium necrophorum	Plaque	Amoako *et al*., 1993
Gardnerella vaginalis	Vaginitis	Piot *et al*., 1984
Giardia lambdia	Intestinal infections	Zenian & Gillin, 1987
Helicobacter pylori	Gastritis, peptic ulcer, stomach cancer, endocarditis, etc	Slomiany *et al*., 1989a and 1989b; Smoot, 1997
Pityrosporum orbiculare	Vesicular pitiriasis, seborreic dermatitis	Ran *et al*., 1993; Plotkin *et al*., 1996
Prevotella spp.	Periodontal diseases	Maeda *et al*., 1998
Propionibacterium acnes	Acne	Gribbon *et al*., 1993; Miskin *et al*., 1997; Higaki, 2003
Propionibacterium granulosum	Acne	Puhvel *et al*., 1975; Greenman *et al*., 1983
Proteus vulgaris	Cystitis	Kim *et al*., 1996; Simons *et al*., 1998a
Pseudomonas aeruginosa	Cystic fibrosis, queratitis, urinary infections, etc	Konig *et al*., 1996; Thomson *et al*., 1999
Serratia marcescens	Periodontitis	Kouker & Jaeger, 1987
Spirochaetes	Periodontitis	Fiehn, 1986
Staphylococcus epidermidis	Blepharitis, acne, etc	Jaeger *et al*., 1994; Simons *et al*., 1998a
Staphylococcus hyicus	Foruncolosis, meningitis, etc	Simons *et al*., 1998b
Staphylococcus aureus	Blepharitis, systemic infections	Rollof *et al*., 1988; Jaeger *et al*., 1994
Streptococcus mutans	Caries	Chen *et al*., 1989
Vibrio cholerae	Cholera	Fiore *et al*., 1997
Yarrowia lipolytica	Enterocolitis, conjuntivitis	Fickers *et al*., 2005
Yersinia enterocolitica	Enterocolitis	Aulisio *et al*., 1983

3.4.1 *Propionibacterium acnes* lipase

Propionibacterium acnes (formerly *Corynebacterium acnes/parvum*) (Figure I.22A) is a Gram-positive, pleomorphic, rod-shaped, non-spore former, non-motile, obligate anaerobe (some strains are aerotolerant), non-toxigenic, indole and/or nitrate-positive, and catalase-producer bacterium, which produces propionic acid, acetic acid, and CO_2 as major products of fermentation of lactate, glucose, and other sugars (Toyoda & Morohashi, 2001; http://web.umr.edu/~microbio/BIO221_1998/P_acnes.html). *P. acnes* strains have been divided into two serotypes and five biotypes, being biotype III (included in serotype 1) the most common one (Higaki *et al*., 2004).

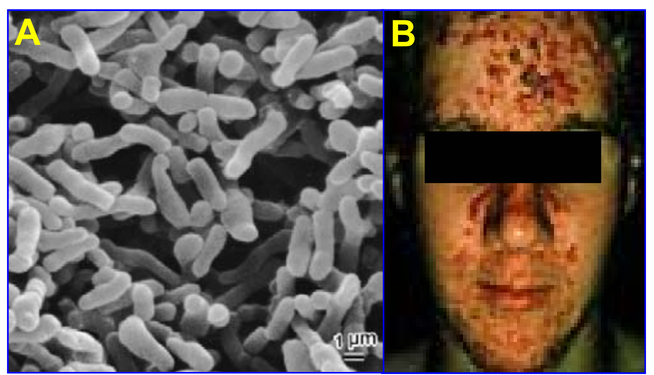


Figure I.22 *P. acnes* and acne.

A: Scanning electron microphotograph of *P. acnes* (Toyoda & Morohashi., 2001). B: a patient with severe acne (http://www.aad.org/pamphlets/acnepamp.html).

P. acnes is a usual inhabitant of human skin. This bacterium resides within sebaceous follicles (special pilosebaceous units located on the face, chest, and back), usually as a harmless commensal even though it is involved in acne formation. Moreover, *P. acnes* is associated with other diseases such as endocarditis, infections in the central nervous system, corneal ulcers, and SAPHO syndrome (of synovitis, acne, pustulosis, hyperostosis, and osteitis), all of them thought to be initiated by its colonization of the keratinized surface of the skin (Eady & Ingham, 1994; Jappe, 2003).

Acne vulgaris is the most common disease associated to *P. acnes*. This disease affects 80% of total population at least once during life, and it is the main cause of dermatologic consults. Acne develops chiefly (95%) in patients in age brackets of 10−30 years, although it can be present in some patients up to 50 years, or during menstruation, drug treatments, or stress. Many patients undergo spontaneous and

complete resolution of their lesions, whereas others have continuous acne or long-term consequences such as disfigurative scarring and keloids (Figure I.22B). Moreover, acne can lead to psychological disorders such as depression and suicide, therefore, an extensive research on this disease has been done during the last decades (Jappe, 2003).

Acne is an inflammatory chronic disease of the sebaceous follicles consisting of a follicular rash that starts as an open (black point) or closed (white point) comedo. Further, inflammation and breakage of the comedo leads to the formation of red papules, pustules and, in the most severe cases, to the formation of nodules, cysts, and fistulas that can produce atrophic or hypertrophic scarring. According to the number and severity of lesions, acne is divided into mild, mild-moderate, moderate and severe (Brown & Shalita, 1998; Toyoda & Morohashi, 2001).

The aetiology of acne is multifactorial (see Figure I.23), and its pathogenesis is still not fully understood. Overproduction of sebum, ductal hypercornification, multiplication of *P. acnes* (and sometimes other microorganisms such as *P. granulosum*, and *Staphylococcus epidermidis*), and inflammation (as a result of the previous ones) are the main causes of the disease development. Other factors such as changes in hormonal production, stress, psychosomatic factors, environmental conditions, use of cosmetics, use of corticoids and other drugs, or excess of cleaning, contribute to the pathogenesis of acne, which seems to be also related with an autosomal–dominant inherited familiar trait of variable expression (Brown & Shalita, 1998; Jappe, 2003).

Each one of these pathogenic factors is a potential target for acne therapy, although the main targets are: reduction of sebum production, reduction of hypercornification, inhibition of *P. acnes* growth (and reduction of the synthesis and/or activity of *P. acnes* enzymes and pro-inflammatory antigens), and reduction of inflammation. Mild acne is treated using topic compounds such as: (1) retinoids (tretionin, tazarotene, adapalene), which are sebostatic, keratolytic, and anti-inflammatory drugs, or (2) benzoyl peroxide or azelaic acid, which are keratolytic and anti-*P. acnes*. These compounds have a limited effectiveness, thus mild-moderate acne requires the combination of the previous treatments with topic antibiotics such as tetracyclines (tetracycline, clindamycine, minocycline, etc) or macrolides (erythromycin

or roxithromycin). Topic antibiotics are replaced by oral ones in moderate acne. Topic and systemic antibiotics are effective against *P. acnes* growth and its effects, and they are anti-inflammatory. However, they have a slow effect, and are associated to the appearance of resistances. Severe acne usually requires the use of oral isotreonin (13-*cis* retinoic acid), a very effective compound with sebostatic, keratolytic, anti-*P. acnes*, and anti-inflammatory activities, but associated to many secondary effects and forbidden in some countries like Japan (Haider & Shaw, 2004). Alternative therapies such as hormonal treatments or Kampo formulations have been also developed. Kampo formulations, officially authorized in 1976 in Japan, contain several traditional herbal drugs, and they are used in treating acne, alone or in conjunction with western therapy, due to their anti-inflammatory, antioxidant, and anti-*P. acnes* properties (Higaki, 2003).

The role of *P. acnes* in acne and other *P. acnes*–associated diseases seems to be related to the production of numerous enzymatic activities involved in the degradation of host molecules, including lipase, protease, hyaluronidase, and acid phosphatase activities. Moreover, *P. acnes* produces surface-associated and secreted immunogenic and chemotactic factors which seem to be involved in triggering inflammation in acne and other *P. acnes*–associated diseases (Toyoda & Morohashi, 2001; Jappe, 2003). Complete sequencing of *P. acnes* genome has revealed the existence of additional putative enzymes and immunogenic factors (neuraminidases, endoglycoceramidases, pore-forming factors, heat shock proteins, and other antigens) which could have also a pathogenic role (Brüeggemann *et al*., 2004).

Among these virulence factors, *P. acnes* lipase (GehA, glycerol-ester hydrolase A) has been recognized as one of the most important factors in the pathogenesis of acne (Higaki, 2003), although several investigations disapproved in part this statement. Weeks *et al*. (1977) reported a 40% reduction of free FAs in the skin of acne patients, but not a decrease in the number of *P. acnes* or acne lesions using GehA inhibitors (halopyridyl phosphorus compounds). However, Akamatsu & Horio (2000) reported that roxithromycin produced a decrease of free FAs in the sebum of acne patients, which was strongly correlated to a decrease in the number of *P. acnes* cells and inflammatory papules, confirming the earlier reports of Marples *et al*. (1971) about a decline in the number of *P. acnes* cells as a result of a previous reduction of free FAs in the follicle.

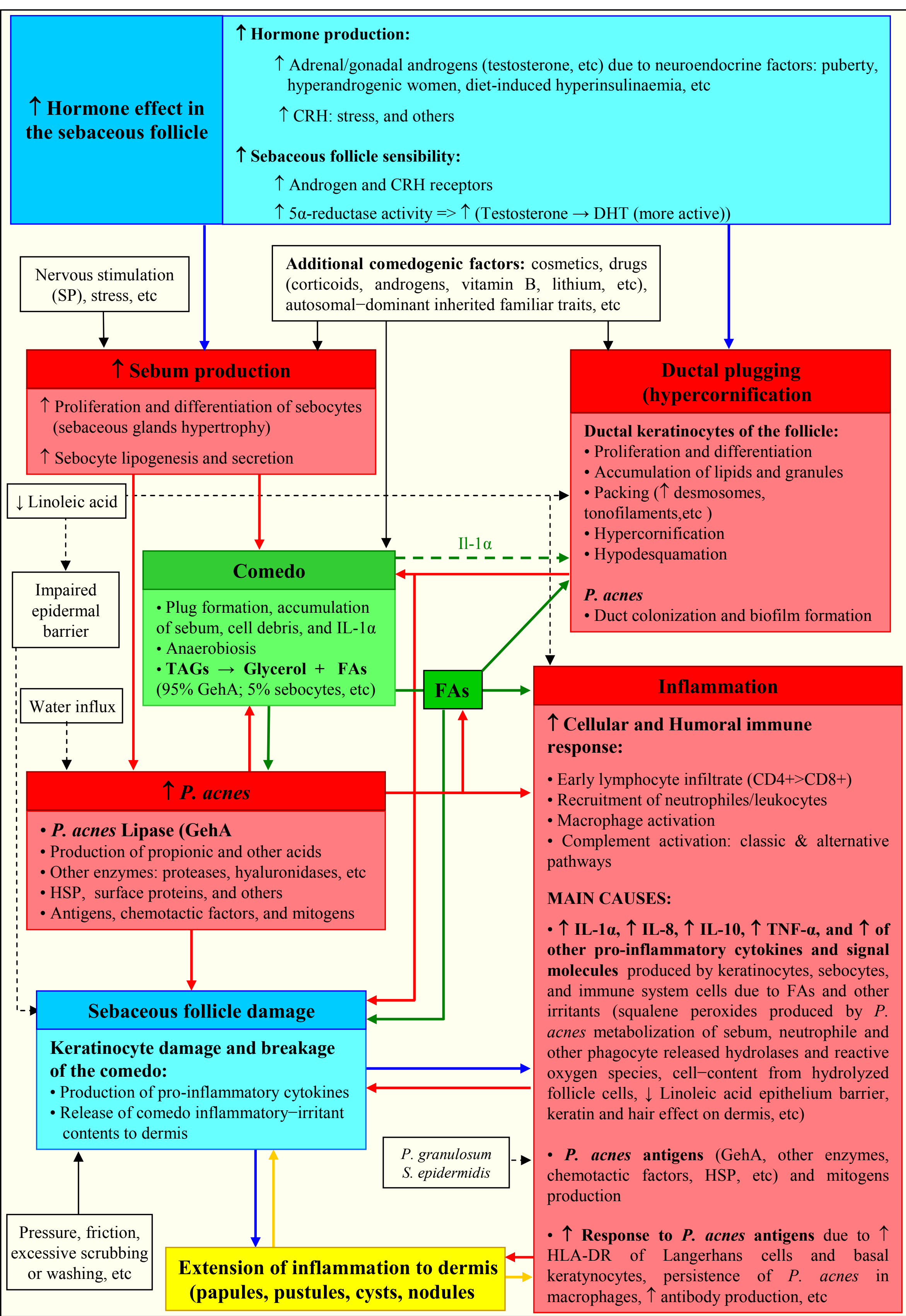


Figure I.23 Synopsis of main factors involved in the pathogenesis of acne.

Colours: main factors involved in the pathogenesis of acne are in red, whereas other important factors are in blue. Continuous lines: facts; dashed lines: hypothesis. Abbreviations: CRH, corticotrophin releasing hormone; HLA, human leucocyte antigen; HSPs, heat shock proteins; IL, interleukin; GehA, *P. acnes* lipase; TNF-α, tumor necrosis factor alpha; SP, Substance P (neuropeptide).

[1] Acne starts when an increased effect on the sebaceous follicle of androgens, other hormones, and other factors such as nervous stimulation, cosmetics, etc, produces an increase of sebum production, and a phenomenon named hypercornification that consists on an increased proliferation, differentiation, hyperkeratinization, lipid accumulation, and a reduced desquamation of the keratinocytes of the follicular ducts. Hypercornification can be also increased by additional factors such as IL-1α or decreased linoleic acid, and leads to plugging of pilosebaceous ducts (comedo formation) and to the subsequent accumulation of sebum and keratinous debris, which distends the follicle (Toyoda & Morohashi, 2001; Jappe, 2003).

[2] Sebum accumulation and anaerobiosis, are two factors favouring the multiplication of *P. acnes* and other bacteria in comedones. *P. acnes* produces a lipase (**GehA**) responsible for the 95% of the hydrolysis of sebum TAGs to glycerol and FAs (the other 5% seems to be related to sebocyte metabolism and other factors) (Jappe, 2003).

On the one hand, FAs trigger the inflammatory response in the comedo and the surrounding area because they are highly inflammatory and chemotactic (Strauss & Pochi, 1965; Jappe, 2003). Moreover, FAs are irritating for the sebaceous follicle cells, which release pro-inflammatory cytokines as a result (Toyoda & Morohashi, 2001; Jappe, 2003). In addition, FAs seem to be related with an increased adhesion and package between keratinocytes (Toyoda & Morohashi, 2001), as well as to an increased adhesion between *P. acnes* cells and between *P. acnes* cells and follicle cells, which favours *P. acnes* colonization and biofilm formation in the follicle and the follicle ducts (Gribbon *et al*., 1993; Burkhart & Burkhart, 2003). *P. acnes* biofilm consists in a community of bacteria that encase themselves within an extracellular polysaccharide which they secrete after adherence to a surface. This gylcocalyx polymer acts as a protective exoskeleton and serves as a physical barrier, limiting the effectiveness of antiacne drugs (Burkhart & Burkhart, 2003). On the other hand, glycerol is a nutrient source for *P. acnes*, which proliferates and produces propionic acid and other products that contribute to inflammation.

Furthermore, GehA itself is a strong chemotactic and pro-inflammatory antigen (Lee *et al*., 1982). GehA, and other antigens and mitogens produced by *P. acnes* such as secreted enzymes, HSPs, surface-proteins, and other chemotactic factors, are responsible for increasing the cellular and humoral inflammatory responses (Brown & Shalita, 1998; Jappe, 2003).

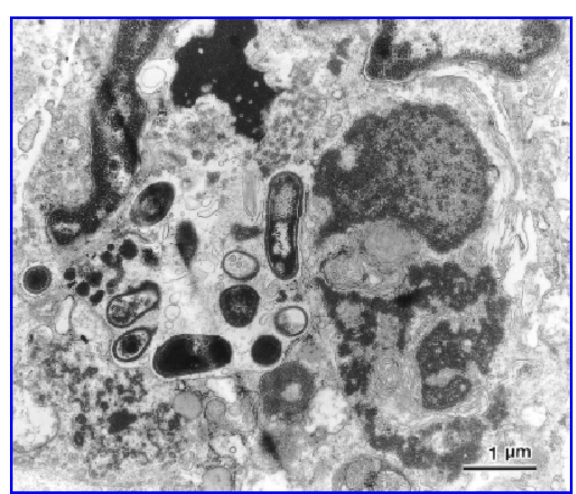
[3] Inflammation, cell damage resulting from inflammation, FAs and other direct or indirect products resulting from *P. acnes* activity, manipulation, etc, as well as accumulation of sebum, keratinocytes and *P. acnes* cells, can finally cause comedo rupture. Release of comedo contents to the dermis extends the inflammation, and leads to the formation of more severe lesions (papules, pustules, cysts, or nodules).

Nowadays, it is clear that GehA plays a very important role in acne development (see Figure I.23). Additional investigations reporting the inhibition of GehA synthesis and/or activity by many antiacne compounds such as antibiotics (tetracyclines and others), Kampo formulations, etc, have increased the certainty of a major role of this

enzyme in acne, and have led to the consideration of GehA as a very interesting therapeutic target (Higaki, 2003). In fact, this enzyme is the main responsible for the hydrolysis of sebum TAGs to glycerol and FAs (Jappe, 2003). Glycerol is a nutrient source for *P. acnes*, whereas released FAs are highly inflammatory, chemotactic, and irritating for the sebaceous follicle cells (Strauss & Pochi, 1965; Toyoda & Morohashi, 2001; Jappe, 2003). Moreover, FAs favour ductal hypercornification because they are related to an increased adhesion and package between keratinocytes (Toyoda & Morohashi, 2001), between *P. acnes* cells, and between *P. acnes* cells and follicle cells, which also favours *P. acnes* colonization and biofilm formation (Gribbon *et al*., 1993; Burkhart & Burkhart, 2003). Furthermore, GehA itself is a strong chemotactic and pro-inflammatory antigen (Lee *et al*., 1982; see Figure I.24).

Figure I.24 Interaction between *P. acnes* and immune cells.

Transmission electron microphotograph showing numerous *P. acnes* cells inside the phagosomes of an infiltrating macrophage attracted to an inflammatory lesion due to *P. acnes* lipase (GehA) and other chemotactic factors (Toyoda & Morohashi, 1999).



Therefore, GehA has generated a high interest as a pharmacological target. First GehA preparations were obtained by Hassing (1971), Fulton *et al*. (1974), and Pablo *et al*. (1974). These authors reported that this enzyme was a serine lipase of approximately 54 KDa and a pI of 3.8. Enzyme preparations were stable in a pH range of 4−8, and showed an optimum pH of 7.5−9 (at 23 ºC). Moreover, the enzyme displayed non-linear enzyme kinetics, acted equally on positions α and β of glycerol (non-regiospecific), and was active on a wide range of substrates: TAGs (activity against emulsions of tributyrin > triolein > tricaprylin > trilaurin > tristearin), DAGs, MAGs, *p*-nitrophenyl acetate, etc.

Later, Ingham *et al*. (1981) confirmed the non-regiospecificity of GehA using more purified (4800-fold) GehA preparations. These lipase preparations had a MW of 47.8 KDa, an optimum pH of 6.8, were more stable in the pH range of 5−6, and were completely inactivated after 30 minutes at 60 ºC. These GehA preparations hydrolyzed trilaurin, triolein, trimyristin and tripalmitin at decreasing rates and did not exhibit phospholipase or other enzymatic activities. Finally, Miskin *et al*. (1997) cloned and overexpressed in *E. coli gehA*, the gene encoding GehA. This gene was present in *P. acnes* genome as a single copy. The nucleotide sequence of *gehA* was determined, and shown to contain a single ORF of 1017 kb encoding a protein of 339 amino acids with a predicted molecular mass of 36 kDa. The protein sequence displayed 37.8% identity over a 74-residue overlap region of *B. subtilis* LipA, and contained the hypothetic catalytic serine located in the consensus pentapeptide of lipases. A 33 kDa polypeptide, probably corresponding to GehA after cleavage of the putative 26 amino acid residue signal peptide, was detected from recombinant *E. coli* preparations. The recombinant enzyme formed insoluble aggregates, although active lipase was produced when the overexpressing strain was incubated at a reduced temperature and in the presence of sucrose. Purification near to homogeneity from *P. acnes* culture supernatant fluids confirmed the production of a 33 kDa lipase in this bacterium. Thus, the MWs determined by other authors were probably due to by the presence of higher-MW contaminant proteins in their GehA preparations.

3.4.2 Lipolytic activity of *Helicobacter pylori*

Helicobacter pylori (formerly named *Campylobacter pylori/pyloridis*) is a Gram-negative, spiral-shaped, flagellated (unipolar or bipolar), microaerophilic rod (Figure I.25A). However, it may have coccoid or slightly spiral shape when it grows in vitro or under unfavourable conditions. This bacterium, mainly found in humans and other animals (primates, cats, etc), lives in the antral and fundic segments of the stomach (between the mucus layer and the surface of epithelial cells), and in the duodenum. The mode of transmission of *H. pylori* infection is not well recognized,

although it seems to be related to faecal–oral transmission, direct transmission, or to nosocomial infections (Nagorni, 2000; http://www.helicobacterspain.com).

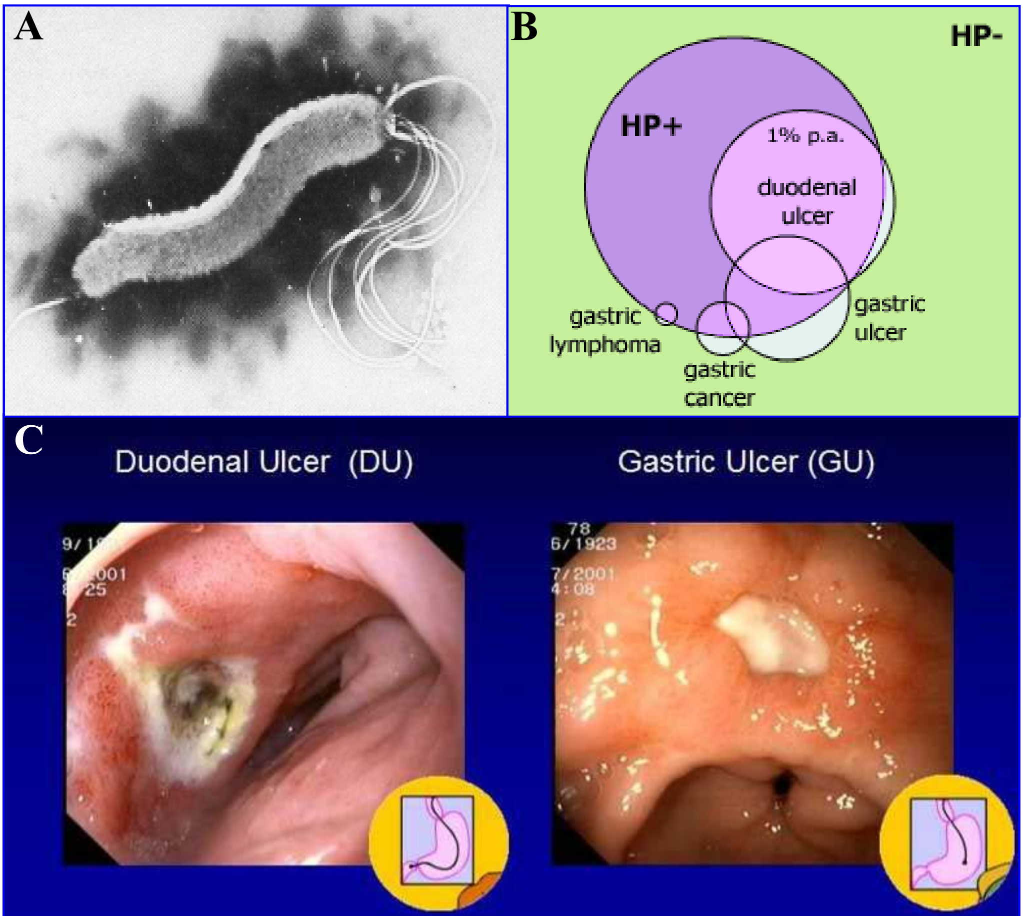


Figure I.25 *Helicobacter pylori* and peptic ulcer.

A: Transmission photomicrograph of *H. pylori* (http://www.helicobacterspain.com). B: Gastric diseases and their relationship with *H. pylori* prevalence. *H. pylori* is present (HP+) in all patients with chronic active antral gastritis, in 90-95% of those with duodenal ulcer, and in 70% of patients with gastric ulcer, whereas the other 30% of gastric ulcers (HP−) are related to aspirin and other non-steroidal anti-inflammatory drugs. Gastric adenocarcinomas (50−70%) and ~90% of gastric lymphomas (MALT -mucosa associated lymphoid tissue- cancers) are also associated with *H. pylori*, although in the final stage *H. pylori* can no longer be detected on biopsy (immunologic studies show evidence of past infection) (http://www.helico.com). C: Gastric (right) and duodenal (left) peptic ulcers are seen as a white-brownish alteration of the lining mucosa (http://www.helicobacterspain.com).

H. pylori has generated a great interest since it was isolated by Warren and Marshall in 1983 (Nagorni, 2000) from gastric biopsy, because it causes one of the most

extended infections worldwide. *H. pylori* colonizes about 60% of the world's population, although a higher prevalence is associated with low socio-economic status, overcrowding, and higher age, being as high as 90% in adults from developing countries. Most infected individuals develop asymptomatic gastritis, but for approximately 1 in 10 people, *H. pylori* infection is associated with the development of chronic gastritis (the underlying condition which causes ulcers and possibly stomach cancer), peptic ulcers (sores on the lining of the stomach or the duodenum; Figure I.25C), or systemic, cardiovascular, respiratory, neurological, autoimmune, and growth disorders, among other diseases (Figure I.25B). Furthermore, *H. pylori* has been recognized as a member of group 1 carcinogens (IARC, 1994) strongly associated with gastric carcinoma and primary low-grade (B cell) gastric lymphoma. *H. pylori* populations in humans are highly diverse (genotypic variation between strains, and variations in populations within an individual host), and this diversity is extremely important in relation to the clinical outcome of infection. Therefore, an extensive research is being carried out to understand the pathogenic mechanisms of this bacterium, and to its eradication (Nagorni, 2000; http://www.helicobacterspain.com).

Numerous diagnostic tests have been developed (histology, culture, urease breath test, PCR, serology, etc) to detect this pathogen. Maastricht guidelines strongly recommended eradication therapy for unequivocally diagnosed *H. pylori* positive patients with ulcer, low grade MALT gastric lymphoma, gastritis with severe macro- or microscopic changes, and after resection of early gastric cancer. The most accepted therapeutic approach recommends an inhibitor of acid secretion from parietal cells (proton pump inhibitors: omeprazole, pantoprazol, etc; or H_2 receptor antagonists: ranitidine, famotidine, etc) plus two antibiotics (clarithromycin or metronidazole in combination with amoxycillin), or an alternate combination of ranitidine bismuth citrate with clarithromycin or amoxycillin in combination with metronidazole or tetracycline (Perna *et al*., 2003). However, recent studies have shown disappointing results with these regimens, mainly due to the rapid emergency of bacterial resistance to the antibiotics used. uadruple regimens, which comprise a proton-pump inhibitor combined with bismuth, tetracycline and metronidazole is often suggested as optimal second-line therapy, although even the best therapies fail in 5-10% of infected patients. Moreover, apart from resistances, some of these compounds can produce allergic

reactions and other secondary effects, or are not suitable during pregnancy (Nagorni, 2000; http://www.helico.com). Thus, research on alternative therapies is in progress, including the use of antiulcer compounds obtained from herbal drugs, which usually have a wide range of beneficial activities such as anti-*H. pylori*, anti-inflammatory, and antioxidant effects, inhibition of acid secretion, etc (Borrelli & Izzo, 2000). Vaccination is also an interesting approach that is being studied (http://www.helico.com).

Surface gastric epithelial cell injury is a characteristic feature of gastritis, although there are different opinions on whether most of epithelial damage occurs as a direct result of *H. pylori*-induced cell injury or due to the autoimmune cell damage resulting from the abundant inflammatory response appearing after *H. pylori* infection (Smoot, 1997). In vitro studies have shown that *H. pylori*, by direct contact and by the elaboration of enzymes and cytotoxins (see Figure I.26), can directly injure gastric cells and may be directly responsible for the majority of gastric cell damage found in infected persons. It is thought that cytotoxins (VacA, CagA, IceA, etc) are the main responsible for cell lysis, directly and through the inflammatory response they produce, whereas enzymes (urease, protease, lipase, phospholipase, etc) would be mainly involved in favouring bacterial colonization of the epithelium, and in the degradation of the proteins and lipids of the tissue matrix and the mucin-lipid barrier that protects the epithelium from the detrimental agents of gastric juice: gastric acid, pepsin, and other proteases (Smoot, 1997; Tsang & Lam, 1999).

The existence of *H. pylori*-produced lipase and phospholipase activities towards the lipids of the gastric mucosa was first reported by Slomiany *et al*. (1989a and 1989b), who also reported their inhibition by colloidal bismuth subcitrate and sofalcone, two antiulcer agents. Further studies revealed the inhibition of these enzymatic activities by sucralfate, ranitidine bismuth citrate and other antiulcer agents (Piotrowski *et al*., 1991 and 1994; Slomiany *et al*., 1992a and 1992b; Ottlecz *et al*., 1999), suggesting the importance of these enzymes in ulcer development. Nowadays, it is thought that lipase and phospholipase activities produced by *H. pylori* are involved in hydrolyzing the lipids of gastric mucus, thus reducing its thickness and hydrophobicity. Weakening of mucus barrier properties favours *H. pylori* colonization of the host cell surface, and makes the epithelium more accessible to gastric acid and pepsin. Moreover, lipase and, mainly, phospholipase activities seem to be related to the disruption of the apical

membrane of epithelial cells, to haemolysis and to the generation of lysophospholipids and other cytotoxic and pro-inflammatory lipids (eicosanoids, etc) detrimental for the mucous gel and the epithelium integrity (Figure I.26; Smoot, 1997; Tsang & Lam, 1999; Berstad *et al*., 2002).

Nevertheless, knowledge about these enzymes is still low despite the knowledge of the genome sequence of *H. pylori* strains 26695 (Tomb *et al*., 1997) and J99 (Alm *et al*., 1999). The presence of carboxylesterase activity has been confirmed employing NMR spectroscopy (Mendz *et al*., 1993), although the number and biochemical properties of the lipase(s) produced by this bacterium remain unknown. *H. pylori* possesses also several different phospholipase activities (A_1, A_2, C) (Ottlecz *et al*., 1993), although phospholipase A_1 activity is almost unknown, and phospholipase C has been only partially purified and preliminarily characterized (Weitkamp *et al*., 1993). Phospholipase A_2 is the only enzyme acting on lipids whose gene (*pldA*) has been identified, cloned and expressed (Dorrell *et al*., 1999). Experiments with isogenic mutants revealed that this enzyme was responsible for more than 90% of the phospholipase A_2 activity, and for 50% of the haemolytic activity of the bacterium. *pldA* was not essential for adhesion, although *pldA* mutants failed to colonize the gastric mucosa probably because of an inability to break down and penetrate through the mucous layer, which would impair a stable colonization (Dorrell *et al*., 1999; Xerry & Owen, 2001). Furthermore, phase variations in *pldA* gene are involved in the adaptation of *H. pylori* to the acidic environment of the stomach lining by changing bacterial surface-lipid composition, which leads to an increased adherence to epithelial cells, and to a higher release of urease, VacA, and other factors involved in colonization, persistence, and epithelium damage (Tannaes *et al*., 2001).

On the other hand, *H. pylori* can indirectly induce the release of phospholipases A_2 from the cytosol of the epithelial cells from the stomach, from Paneth cells of the intestinal mucosa, and from polymorphonuclear leucocytes. Current evidences suggest that these enzymes would be the main responsible for the mucus and epithelium damage, and for the increased production of eicosanoids and other pro-inflammatory and cellular signalling factors associated to phospholipase A_2 activity (Nardone, 2001; Pomorski *et al*., 2001; Huhtinen *et al*., 2002).

Helicobacter pylori (pathogenic mechanisms

OTHER FACTORS

Motility and spiral shape:

● Its spiral shape favours *H. pylori* penetration through gastric mucus layer by a corkscrew movement.

● 4−6 polar flagella give *H. pylori* a high motility, allowing a high penetration through mucus layer, and avoiding bacterial removal by the local nonspecific host-defence mechanisms (peristalsis, ciliar activity and turnover of epithelial cells and the mucous layer) (http://www.helicobacterspain.com).

Adhesins:

● *H. pylori* adhesins specifically recognize receptors in gastric mucosa favouring bacterial colonization and preventing bacterial removal by nonspecific host-defence mechanisms.

● *H. pylori* adhesion to gastric epithelial cells alters their cytoskeleton, which is associated with formation of adherence pedestals, induction of tyrosine phosphorylation, apoptosis, etc.

● Adherence allows an efficient transfer of bacterial toxins to gastric epithelial cells, which could secrete IL-8 as a result (Smoot, 1997; Nagorni, 2000).

● However, other mechanisms such as constitutively shed outer membrane vesicles could play a role in promoting gastritis independently form *H. pylori* adhesion (Salim *et al*., 2003).

Defence from host immune system and others:

● The immune system responds to an *H. pylori* infection by sending white cells, killer T cells and other fighting agents. However, they cannot reach *H. pylori* because they cannot easily get through the stomach lining, they are thus accumulated and the immune response grows. Extra nutrients sent to reinforce the white cells feed *H. pylori* (http://www.helico.com).

● The LPS could inhibit mucin glycosilation. Moreover, the LPS of 89% of *H. pylori* isolates contains regions that are exact analogues of Lewis blood group antigens (Lewis X, Y, A, B and Sialyl-Lewis X) (Tsang & Lam, 1999; Nagorni, 2000).

● Moreover, *H. pylori* may release antibacterial peptides and stimulate immune response to prevent invasion of the stomach by other competing bacteria (Walsh, 1999).

ENZYMES

Urease:

● Conversion of urea into bicarbonate and ammonia (also produced by amidases and arginases), strong bases that protect *H. pylori* by neutralizing gastric acid.

● Ammonia is also an important nitrogen source for the synthesis of *H. pylori* proteins necessary for bacterial adhesion. Moreover, amonia is cytotoxic, impedes gastric cell cycle progression, and induces inflammation (Bury-Mone *et al*., 2003).

Protease:

● Degradation of the gastric mucous layer that protects the epithelium from acid and pepsin by disintegration of the mucin polymeric structure (Smoot, 1997).

Lipases and phospho-lipases :

● Weakening of gastric mucous layer by lipid degradation and loss of mucosal surface hydrophobicity, which favours bacterial colonization and ulceration.

● Disruption of the apical membrane of epithelial cells and haemolysis.

● Generation of lysophospho-lipids and other pro-inflammatory and cytotoxic lipids detrimental to mucus and epithelial cells (Smoot, 1997; Tsang & Lam, 1999).

● Bacterial colonization and adaptation to acid environments (Tannaes *et al*., 2001).

Other enzymes:

Catalase, oxidase, alcohol dehydrogenase, hyaluronidase, sphingomyelinase and others: host damage, bacterial metabolism and persistence, etc (http://www.helicobacterspain.com).

CYTOTOXINS

Several genes, some of them showing genotipic variation, could play a role in the pathogenicity of *H. pylori*:

Vacuolating cytotoxin gene (*vacA* :

● VacA is a toxin which causes vacuolation of epithelial cells after *H. pylori* attachment.

● VacA is activated by gastric acid to form a membrane pore which causes cells to become leaky, especially to urea.

Cytotoxin associated gene (*cagA* :

● *cagA* is one of about 30 genes in *cag* pathogenicity island. These genes form a secretion system which injects CagA protein into cells, where it causes disorganized cellular structure due to "growth factor like" effects.

● CagA could be also related to activation of VacA.

● *cagA*-positive strains are also associated to faster and successful *H. pylori* colonization of stomach, increased expression of Lewis antigens, epithelial cell proliferation and damage, induction of IL-8 secretion from gastric epithelial cells, increased inflammation and alteration of gastric acid secretion, etc (Nagorni, 2000).

Other genes:

● *iceA*, *bab*, *nap*, *mcp-1*, and other genes are involved in: favouring *H. pylori* colonization of the gastric mucosa, inhibition of gastric mucin synthesis and secretion, alteration of epithelial cells proliferation and apoptosis, stimulation of inflammation, etc (van Doorn *et al*., 1998; http://www.helico.com).

Helicobacter pylori (effects

INFLAMMATION

● *H. pylori* induces humoral (blood antibodies and complement activation) and cellular (neutrophiles, monocytes, T cells, B cells and plasma cells) immune responses, which produces a chronic-active inflammation.

● Cellular changes, epithelium damage and *H. pylori* products are associated with an increased production of pro-inflammatory and chemotactic factors (IL-1b, IL-6, IL-8, PGE_2, PAF, TNF-α, etc), with an increase in the number of IFN-γ producing cells; and with an increased death of polymorphonuclear leucocytes, which spill their destructive compounds (superoxide radicals, proteases, phospholipases A_2 and other hydrolases) on stomach lining cells.

● These responses do little to confer protective immunity against *H. pylori*, but they accumulate inducing numerous changes in the gastric epithelium and promoting additional inflammation and epithelial cell damage (including apoptosis). Therefore, it may not be *H. pylori* itself which causes gastritis or peptic ulcer, but the inflammatory response to *H. pylori.*

● Environmental factors (hygiene, stress, etc), the host (predisposition, age, blood group, etc) and the bacterium features (virulence, pathogenicity, and genetic characteristics) are important to the infection expression (Nagorni, 2000).

CELL/EPITHELIUM DAMAGE

● Cytokines produced due to cell damage and inflammation (including TNF-α, IFN-γ, IL-1b, etc) can increase the production and secretion of gastric epithelium molecules that increase inflammation: phospholipases, lysozymes, metalloproteinases (endopeptidases involved in extracellular matrix remodelling, cell proliferation and inflammatory processes), IL-8, class II MHC molecules and the polymeric immunoglobulin receptor, among others (Gooz *et al.*, 2003; http://www.helicobacterspain.com).

● Inflammation and *H. pylori* products induce severe cell damage, apoptosis and acid hypersecretion (by a trophic effect on parietal cells and by gastric somatostatin reduction), which increases the risk of peptic ulcer and leads to gastric atrophy or epithelial metaplasia (aberrant cell renovation and epithelium repair) (Nagorni, 2000).

CHRONIC GASTRITIS AND PEPTIC ULCER

● The host immune response fails to eliminate *H. pylori* in the majority of individuals. Accumulation of chronic inflammatory cells in the infected mucosa, combined with epithelium damage gives way to active chronic gastritis with antral predominance.

● A greater degree of antral inflammation combined with hyperacidity predisposes to duodenal and pre-pyloric damage. Increased acid extends mucosal damage producing gastric metaplasia. Metaplasia is a protective response to acid, but counterproductive since the infection is able to better colonize the damaged mucosa, which leads to more mucosal damage. Exacerbation of this process can lead to gastric or duodenal peptic ulcers (Nagorni, 2000).

CANCER

● Production of toxics, impairment of mismatch repair, cell cycle alteration atrophic gastritis and/or gastric metaplasia induced by *H. pylori* can lead to diffuse gastric cancer in genetically predisposed persons. In others persons increasing atrophy in the corpus renders the parietal cells less sensitive to gastrin, producing a hypochlorhydria related to intestinal-type gastric cancer (Nagorni, 2000).

● Lymphoid follicular gastritis combined with genetic factors can lead to the development of gastric lymphoma in some *H. pylori*-infected subjects (http://www.helicobacterspain.com).

Figure I.26 Main factors involved in *Helicobacter pylori*-induced pathogenesis.

Previous page: *H. pylori* mechanisms involved in pathogenesis; this page: effects of *H. pylori* in the host. Abbreviations: IL, interleukin; IFN-γ, interferon-gamma; MHC II, major histocompatibility complex II; LPS, lipolysaccharide, PAF, platelet-activating factor, PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor alpha.

4 LIPASE INHIBITORS

Lipase inhibitors have been one of the most useful tools in the characterization of the structure, catalytic properties, substrate specificity and selectivity of lipases. In this sense, inhibition studies on lipases contribute to better understand their mechanism of action in order to design novel substrate specificities, and new regio- or enantioselective lipases for increasing and improving the biotechnological applications of these enzymes (Simons *et al*., 1999).

Furthermore, lipase inhibitors have a high pharmacological interest because they could help in the therapy of diseases in which lipases play an important role such as obesity or infective diseases produced by lipolytic microorganisms (Schmid & Verger, 1998; Grippa *et al*., 1999; Gupta *et al*., 2004). In fact, several compounds useful in the therapy of these diseases have demonstrated a high antilipase activity:

1. **Tetracycline** and other antiacne drugs inhibited *P. acnes* lipase (Higaki, 2003).
2. **Sucralfate** and other antiulcer drugs inhibited *H. pylori* lipolytic activity (Slomiany *et al*., 1992b and 1994).
3. **Orlistat** (Xenical), the tetrahydro-derivative of lipstatin (Figure I.27), is currently used for the treatment of severe obesity because it is a very potent inhibitor of gastric and pancreatic lipases, the key enzymes for fat absorption during digestion (Eisenreich *et al*., 2003).

Figure I.27 Structure of lipstatin.

Lipstatin, isolated from *Streptomyces toxytricini* by Weibel *et al*. (1987), contains a β-lactone ring, two aliphatic chains, and an N-formyl-leucine moiety. The β-lactone ring is probably the responsible for the irreversible inhibition of human pancreatic lipase, by forming a covalent adduct with the catalytic serine of the enzyme through transesterification.

4.1 CLASSIFICATION OF LIPASE INHIBITORS

In general, inhibitors of enzymes are classified as reversible or irreversible. Reversible inhibitors are further divided into competitive, non-competitive, uncompetitive and mixed according to their effect on the kinetic parameters of enzymes that fulfil Michaelis-Menten kinetics. However, most TLs do not display a Michaelis-Menten behaviour, thus reversible and irreversible lipase inhibitors are classified as specific or non-specific, depending on if they act directly on the active site of the enzyme or not (Patkar & Björkling, 1994; Voet & Voet, 1995).

4.1.1 Non-specific reversible inhibitors

Compounds that inhibit lipase activity in a reversible way by changing the conformation of the enzyme or the properties of the substrate-water interface are defined as non-specific reversible inhibitors. Surfactants, bile salts, proteins and metal ions belong to this group of inhibitors, and usually produce non-competitive inhibition kinetics. Surfactants (anionic, non-ionic and cationic) and bile salts usually produce a weak activation of lipase activity at concentrations below their CMC by favouring the formation of the substrate-water interface. However, they inhibit lipase activity at concentrations above their CMC by a direct effect on the enzyme (conformational changes, denaturation, etc) or by disturbing the access of the enzyme to the interface and/or the substrate. Inhibition by proteins is produced by their binding to the enzyme or to the interface, which disturbs the lipase–substrate interaction. Metal ions such as calcium, iron and mercury destabilize the enzyme, although the mechanism of action is only known for some of them (e.g. mercury binds to the thiol groups of the enzymes forming stable complexes). On the contrary, some metal ions (including calcium in some cases) activate the enzyme due to the fact that they act as scavengers of free FAs, which minimises product inhibition from released FAs (Patkar & Björkling, 1994; Gupta *et al*., 2004).

4.1.2 Specific reversible inhibitors

Specific reversible inhibitors cause a loss of lipolytic activity by a reversible direct interaction with the active site of the enzyme. The most common ones are: (1) boronic acid derivatives, which form reversible but long-lived transition-state analogs with the active site serine of lipases (Lolis & Petsko, 1990), and (2) TAG analogs including monoalkyl glycerols, alkyl-diacylglycerols and glycerol triethers. Boronic acid derivatives inhibition depends on the reaction conditions. Thereby, changes in the substrate, pH, etc can produce changes in the inhibitory kinetics of these compounds, from non-competitive to mixed or competitive kinetics. TAG analogs are competitive inhibitors, although their affinity for the enzyme is low, compared with that of the substrate (Patkar & Björkling, 1994; Gupta *et al*., 2004).

4.1.3 Non-specific irreversible inhibitors

Non-specific irreversible inhibitors react with amino acids not belonging to the active site but important in maintaining the conformation of the enzyme. Thus, these inhibitors produce modifications in the protein conformation, causing loss of enzymatic activity. The most common ones are cysteine- and tryptophan-modifying agents such as 2-mercaptoethanol or *N*-bromosuccinimide (NBS), respectively (Gupta *et al*., 2004).

4.1.4 Specific irreversible inhibitors

Specific irreversible lipase inhibitors react with the amino acids located at the active site of lipases inhibiting the catalytic activity of the enzyme. Most of them are serine inhibitors, due to the fact that lipases are serine hydrolases, although lipases in which free –SH groups or S–S bridges are important components of the active site are also specifically inhibited by cysteine-modifying agents. The most common serine inhibitors are: phosphorous-containing compounds (*p*-nitrophenyl phosphates or phosphonates, etc), phenylmethylsulfonyl fluoride (PMSF), carbamates, and lactones (estearastin, lipstatin, tetrahydrolipstatin, etc) (Patkar & Björkling, 1994).

4.2 LIPASE INHIBITION BY NATURAL SUBSTANCES

Lipase inhibition by the compounds previously mentioned such as PMSF or heavy metals is interesting for structure-mechanism assays, or by their effect on biotechnological processes. However, these compounds are not suitable for pharmacological applications in the therapy of disease-related lipases due to their elevated toxicity and side effects, or due to their lack of activity on some lipases (Gupta *et al*., 2004). Therefore, there is an increasing interest in finding new lipase inhibitors of natural origin and with low toxicity such as fatty acids and plant secondary metabolites.

The general properties of fatty acids are explained in more detail in General Introduction 1.2.1. Apart from being products of lipolytic activity, FAs are well-known competitive inhibitors of the hydrolysis and synthesis reactions catalyzed by lipases (Bengtsson & Olivecrona, 1980; Markweg-Hanke *et al*., 1995; Hari Krishna & Karanth, 2001). Moreover, FAs are known to be involved in: (1) activation or inhibition of lipase production (Gupta *et al*., 2004, see General Introduction 3.1.1), (2) in inhibition of the expression of several bacterial virulence factors (Ruzin & Novick, 2000), and (3) in inhibition of the growth of several microorganisms, including lipolytic bacteria such as *H. pylori* (Sun *et al*., 2003) and *P. acnes* (Higaki, 2003).

Plant secondary metabolites present in herbal drugs and food have shown to be very useful in the prevention and treatment of many diseases (Singh *et al*., 2003). Among these metabolites, saponins, flavonoids and alkaloids are a promising source of lipase inhibitors since they are present at high concentrations in plant extracts capable of inhibiting porcine pancreatic lipase activity (Shimura *et al*., 1992). Furthermore, these compounds are also present in several plant extracts that have been used in traditional medicine for the treatment of diseases in which lipases could play an important role such as gastric ulcer (Borrelli & Izzo, 2000) or acne (Higaki, 2003). Recent studies have also demonstrated that saponins like platycodin D (Han *et al*., 2002), flavonoids like quercetin (Gatto *et al*., 2002), and alkaloids like berberine and sanguinarine (Grippa *et al*., 1999) are good lipase inhibitors. However, further studies are needed to elucidate the effect of other purified plant metabolites on lipolytic enzymes in order to select the most suitable ones for therapeutic or preventive pharmacological treatments.

4.2.1 Saponins

Saponins are natural compounds containing a steroid or triterpenoid sapogenin nucleus linked to one, two or three linear or branched glycosyl side chains (Figure I.28). In some cases, saponins are esterified with additional moieties. They are so-called because of being excellent foaming agents due to their amphiphilic nature and surface-active properties. Saponins are found in a great variety of plants and a few marine animals, in which they act as antimicrobial or antifungal agents, as well as toxic compound against cold-blood animals such as insects or fishes. Most of these effects are produced by the interaction of saponins with membrane sterols, which causes cell damage by disrupting the function and stability of cell membrane, and by inducing apoptosis (Milgate & Roberts, 1995; Singh *et al*., 2003).

Figure I.28 Structures of digitonin (A and glycyrrhizic acid (B .

Digitonin (A) is a triterpenoid saponin, and glycyrrhizic acid (B) is a steroidal saponin.

Saponins, and saponin-containing plant extracts and feed in which saponins are the main active compounds, have generated a great interest because of being antimicrobial, anti-inflammatory, hypocholesterolemic, antitumoral, antidiabetic and vaccine adjuvant agents, as well as due to the low toxicity they produce (Milgate & Roberts, 1995; Singh *et al*., 2003). Nevertheless, saponins produce haemolysis when high amounts are injected in the blood stream, although they are used in low concentrations due to displaying anti-inflammatory, anti-oedematous, and venotonic

properties, as well as low adverse effects (Sirtori, 2001). Moreover, saponin-derivatives are active against human pathogenic *Candida* species (Bader *et al*., 2000).

Saponins usually remain unabsorbed in the gastrointestinal tract, where they stimulate the intestinal microbiota, and where they act as scavengers of cholesterol, bile salts, pathogens, and impacted rubber-like materials. However, the intestinal microflora can also hydrolyze them and release the sapogenin nucleus, which can then be absorbed. Sapogenins produce similar but less intense effects than saponins, and are finally metabolized in the liver and excreted into the bile (Milgate & Roberts, 1995; Singh *et al*., 2003).

Furthermore, saponins and herbal drugs rich in saponins are active on several lipases and lipolytic microorganisms, as well as on several diseases in which lipases could play an important role. In fact, saponin-containing formulations are known inhibitors, *in vitro*, of porcine pancreatic lipase (Shimura *et al*., 1992) and *P. acnes* lipase (Higaki *et al*., 2000). Pancreatic lipase inhibition by purified saponins such as platycodin D and dioscin has been also reported recently (Han *et al*., 2002; Kwon *et al*., 2003). However, it must be taken into account that saponins from *Medicago sativa* caused activation of pancreatic lipase (Sroka *et al*., 1997). Thus, further studies are necessary to elucidate the exact effect of purified saponins on lipolytic enzymes.

In addition to their effect on *P. acnes* lipase, saponin-containing Kampo formulations contribute to the treatment of acne because of they display antibacterial effect on *P. acnes*, as well as anti-inflammatory activity (Higaki, 2003). Moreover, saponins and several plants containing high amounts of saponins have shown to possess anti-ulcer and gastro-protective activities (Borrelli & Izzo, 2000). Several mechanisms have been proposed to explain these effects: (1) activation of protective factors in the mucosa (Matsuda *et al*., 1998), (2) inhibition of gastric acid and pepsinogen secretion (Marhuenda *et al*., 1993), (3) improvement of blood flow (Marhuenda *et al*., 1994), (4) inhibition of *H. pylori* growth (Kim *et al*., 2000), (5) inhibition of *H. pylori* N-acetyl transferase (Chung, 1998), (6) stimulation of gastric mucus production by enhancing the rate of incorporation of sugars into the glycoproteins of the gastric mucosa, (7) promotion of mucosal cell proliferation and inhibition of mucosal cell exfoliation, (8) inhibition of prostaglandin degradation and increase of PGE_2 release, (9) reduction of

thromboxane B2 formation, and (10) regulation of DNA and protein synthesis rates in gastric mucosa (Borrelli & Izzo, 2000). However, the effect of most saponins on *P. acnes* and *H. pylori* growth and lipolytic activity is still unknown.

4.2.2 Flavonoids

Flavonoids (or bioflavonoids) are a group of about 4000 naturally occurring phenolic compounds. They are ubiquitous in all vascular plants, where they act as pigments, growing factors, defensive substances, etc. Structurally, flavonoids are benzo-γ-pyrone derivatives that can be grouped according to the presence of different substituents on the rings and to the degree of benzo-γ-pyrone ring saturation (Figure I.29). Flavonoids *per se* are formed by a benzene ring condensed with a six member ring which possesses a phenyl ring at the 2 position, whereas compounds with the phenyl ring at the position 3 are properly termed isoflavonoids. In addition, the six member ring can be either γ-pyrone (flavones and flavonols) or its dihydro-derivative (flavanones and flavanols). Flavonoids can be also glycosilated or methylated, and they can form dimers (pycogenols) (Di Carlo *et al*., 1999).

Figure I.29 General structure of a flavonoid.

Flavonoids are important constituents of human diet (~1 g per day) and of several herbal drugs. However, most of them are poorly absorbed due to the fact that they are usually bound to sugars as β-glycosides. Only colon microorganisms can break these bonds and allow their absorption, although they also degrade most of the resulting aglycone flavonoids. Once absorbed, flavonoids have high therapeutic potential due to

their low toxicity, and because they are capable of modulating the activity of many enzymes and cell systems producing, among others, antimicrobial, antioxidant, hypotriglyceridemic, cardioprotective, anti-inflammatory, antihepatotoxic, and antitumoral effects. Finally, they are metabolized in the liver, kidney and the intestinal wall (Di Carlo *et al*., 1999; Singh *et al*., 2003).

Among their wide range of effects, flavonoids are present in herbal drugs capable of inhibiting gastric lipase (Shimura *et al*., 1992), or used against lipase-related diseases such as acne (Higaki, 2003) and ulcer (Borrelli & Izzo, 2000). However, the role of purified flavonoids in lipase inhibition has been poorly studied, and only some of them such as hesperidin or neohesperidin (Kawaguchi *et al*., 1997), and quercetin (Gatto *et al*., 2002), are demonstrated lipase inhibitors.

In fact, flavonoids are important components of Kampo formulations, which are active against acne because they have antioxidant and anti-inflammatory effects, and because they inhibit *P. acnes* growth and *P. acnes* lipase activity. Flavonoids seem to contribute to these effects, mainly with respect to inhibition of *P. acnes* growth, that secondary leads to a decrease in *P. acnes* lipase activity (Higaki *et al*., 1993; Higaki, 2003). However, their direct effect on *P. acnes* lipase is still unknown.

Furthermore, flavonoids such as quercetin and kaempferol (Izzo *et al*., 1994), and several herbal extracts containing flavonoids have antiulcer and gastroprotective activities (Borrelli & Izzo, 2000). Several mechanisms have been proposed to explain the gastroprotective effect of flavonoids: increase of mucosal prostaglandin content (PGE_2) (Alcaraz & Hoult, 1985), decrease of histamine secretion from mast cells by inhibition of histidine decarboxylase (Bronner & Landry, 1985), inhibition of *H. pylori* growth (Beil *et al*., 1995), free radical scavenging (Baumann *et al*., 1980; Salvayre *et al*., 1982), and inhibition of platelet activating factor formation (Izzo *et al*., 1994). However, their effect on *H. pylori* lipolytic activity as an additional antiulcer mechanism remains unstudied.

4.2.3 Alkaloids

Alkaloids are very diverse natural substances that contain a basic nitrogen, usually as a part of a cyclic system. In general, synthetic compounds related or derived from alkaloids are also considered as members of this group. These heterogeneous compounds, more than 10000, are usually produced by higher plants, although they are also found in lower plants, fungi, animals and microorganisms. Their biological function in these organisms remains unclear for most of them. Some alkaloids seem to be lateral products, whereas others probably act as antimicrobial agents, toxic-protective compounds, storing and transporting compounds, antioxidants, and proton acceptors (Evans, 1996).

The large majority of alkaloids are produced from amino acids, and only a few of them derive from purines, pyrimidines, steroids and triterpenes. They are classified according to their ring structure into heterocyclic and non-heterocyclic alkaloids. Heterocyclic alkaloids (typical alkaloids) are further divided into the following groups (Figure I.30): (1) pyrrole and pyrrolydine, (2) pyrrolizidine, (3) pyridine and piperidine, (4) tropane, (5) quinoline, (6) isoquinoline, (7) aporphine, (8) norlupinane, (9) indole or benzopyrrole, (10) indolizidine, (11) imidazole or glyoxaline, (12) purine, (13) steroidal and steroid glycosides, and (14) terpenoid (Evans, 1996).

Alkaloids are active on many enzymes and biological systems producing a wide range of positive and negative effects such as stimulation or depression of the central nervous system, nerve endings and muscles, modification of blood pressure, and diuretic, anti-inflammatory, antimicrobial, mutagenic, or hepatotoxic effects (Evans, 1996). Moreover, alkaloids have an important effect on some lipases. Berberine, cheleritrine, chelidonine, and sanguinarine, but nor palmatine, are known inhibitors of CRL (Grippa *et al*., 1999). Other studies have demonstrated that reserpine increased LPL activity of heart tissue (Heinroth & Forster, 1980) and inhibited epididymal hormone-sensitive lipase but not that of other tissues (Matsuara, 1970). Moreover, physostigmine, a known inhibitor of microbial and other esterases (Steigleder & Rottscher, 1959), inhibited also lipases such as bile-salt stimulated lipase (Ellis & Hamosh, 1992) or pancreatic lipase (Hall, 1961), but not lipolytic liver extracts (Katz, 1957).

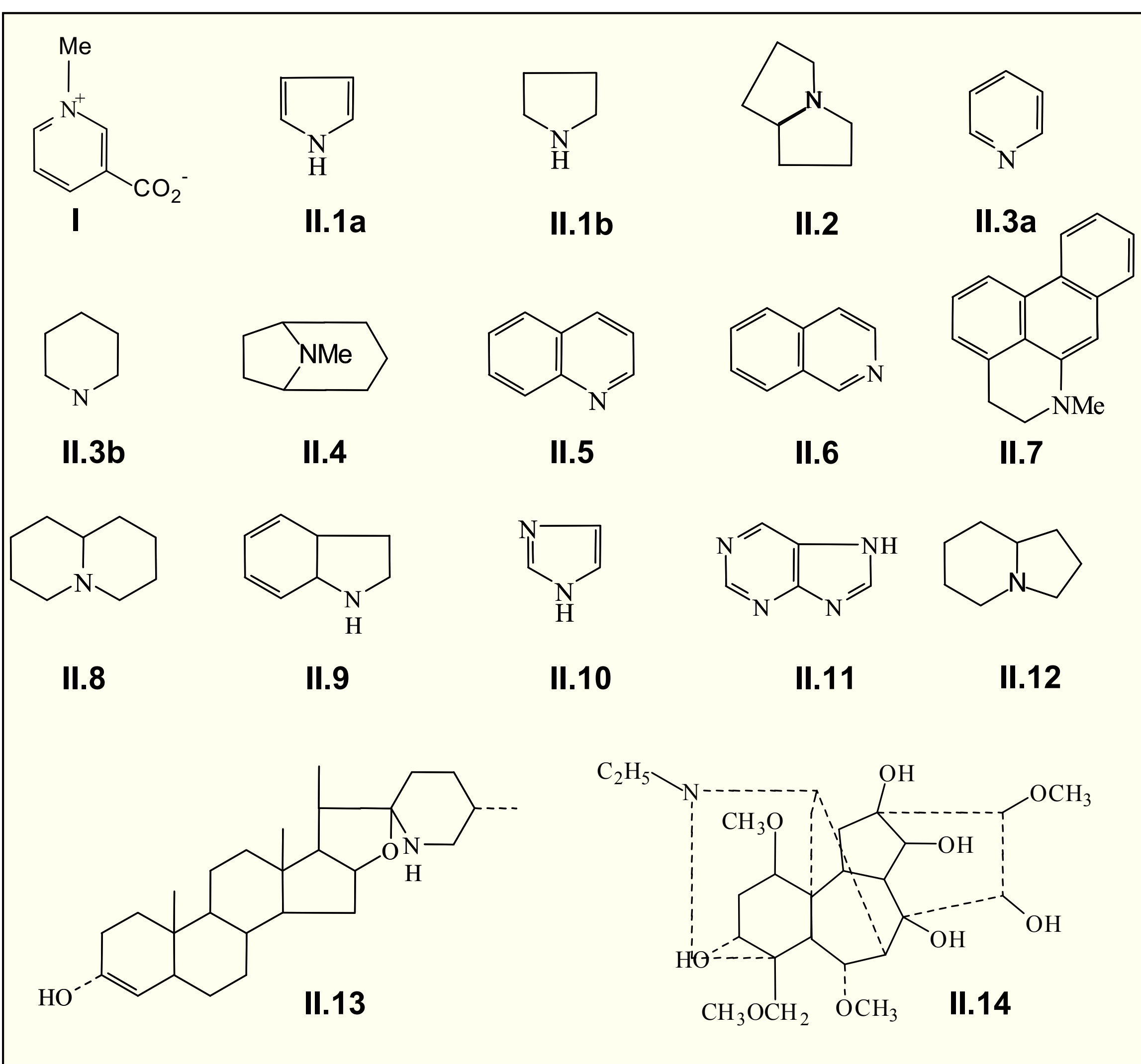


Figure I.30 Skeletal structures of alkaloids found in medicinal plants.

I. Complete structure of trigonelline, a non-heterocyclic alkaloid. II. Skeletal structure of heterocyclic alkaloids: (1a) pyrrole, (1b) pyrrolydine, (2) pyrrolizidine, (3a) pyridine, (3b) piperidine, (4) tropane, (5) quinoline, (6) isoquinoline, (7) aporphine, (8) norlupinane, (9) indole or benzopyrrole, (10) indolizidine, (11) imidazole or glyoxaline, (12) purine, (13) complete structure of solasodine, a steroidal alkaloid, (14) complete structure of aconine, a triterpenoid alkaloid.

Alkaloids are also effective against several lipase-related diseases. In fact, erythromycin, a non-heterocyclic alkaloid, is widely used in the treatment of acne due to its effectiveness. This compound did not inhibit *P. acnes* lipase directly (Puhvel & Reisner, 1972), although it has demonstrated to inhibit *P. acnes* growth and to have anti-inflammatory activity at sub-MIC concentrations through inhibition of lipase production by *P. acnes*, as well as through inhibition of leucocyte chemotaxis (Webster

et al., 1981; Tan, 2004). Moreover, the alkaloids obtained from Kampo formulations and from *Coptidis japonica* rhizoma had an antiacne effect by inhibiting *P. acnes* growth, which secondary reduced *P. acnes* lipase activity (Higaki *et al*., 1996). Other alkaloids such as berberine and jatorrhizine displayed also antimicrobial effect on clinical strains of *P. acnes* and *Candida* species (Slobodnikova *et al*., 2004). On the contrary, the effect of alkaloids with respect to *H. pylori* and ulcer is variable. Methanolic extracts containing trigonelline displayed antiulcer properties (Zia *et al*., 2001), whereas reserpine and rescinnamine were ulcerogenic (La Barre, 1960), in part by activating the releasing of lisosomal β-glucuronidase from gastric mucose (Pfeiffer *et al*., 1980). Therefore, alkaloids display a high potential as antilipase drugs, although their diverse structure and effects makes it necessary to study each one separately.

5 GENERAL OBJECTIVES

This PhD Thesis is focused according to the general objectives of our research group, which consist on the isolation, characterization, and overproduction of microbial enzymes with novel biotechnological potential. As explained before (General Introduction 3.3.1), microbial enzymes are used in a wide range of biotechnological activities because they are highly efficient and selective, as well as less polluting. In this sense, previous research in our group has allowed the isolation of microorganisms from natural sources showing high activity on lipids and polysaccharides (Blanco & Pastor, 1993; López *et al*., 1998). From these hydrolytic microorganisms, several lipases, cellulases, xylanases, and pectinases have been isolated, characterized and, in some cases, overproduced and evaluated on industrial processes (Blanco *et al*., 1995, 1998, and 1999; Prim *et al*., 2000, 2001, and 2003; Pastor *et al*., 2001; Soriano *et al*., 2000; Gallardo *et al*., 2003 and 2004; Sánchez *et al*., 2002 and 2003).

Since 2001, our research group is also interested in the isolation, characterization and inhibition of microbial lipases that could play an important pathogenic role in diseases such as acne and peptic ulcer. This interest is the result of a recent collaboration with the research group of Dr. Luciano Saso from the Università degli studi di Roma "La Sapienza", working on lipase inhibition by natural substances with pharmacological potential (Grippa *et al*., 1999; Gatto *et al*., 2002).

Therefore, the general objective of the present work was the isolation, cloning, characterization and inhibition of microbial lipases with interest in biotechnology and infectious diseases. The specific objectives are summarized in the next pages, although they are explained in more detail in the corresponding chapters:

Chapter 1

- To analyze the lipolytic system of *Bacillus megaterium* CECT370, and to isolate, clone, and characterize the lipase(s) of this microorganism.
- To analyze the lipolytic system of *Bacillus* sp. BP-6, and to isolate, clone, and characterize the lipase(s) of this strain.
- To isolate, clone, and characterize the lipase(s) of the previously described strain *Bacillus* sp. BP-7.

Chapter 2

- To isolate the lipid- and polysaccharide-degrading microorganisms from three soil samples obtained from a subtropical forest of Puerto Iguazú (Argentina), and to determine the lipolytic activity of the most active strains.
- To analyze the biochemical and physiological properties of the lipolytic isolates *Bacillus* sp. CR-179 and *Rhodococcus* sp. CR-53.

Chapter 3

- To develop a fast, simple, and reliable colorimetric assay suitable for high-throughput evaluation of lipolytic activity and lipase inhibition.
- To analyze the effect of unsaturated fatty acids on *Candida rugosa* lipase and five *Bacillus*-related lipolytic enzymes: *Paenibacillus* sp. BP-23 EstA, *Bacillus* sp. BP-7 EstA1, *Bacillus subtilis* LipA, *Bacillus megaterium* LipA, and *Bacillus* sp. BP-6 LipA.

Chapter 4

- To evaluate the inhibitory effect of several saponins, flavonoids and alkaloids on the model lipase of *Candida rugosa*.

Chapter 5

- To clone and characterize the lipase GehA of *Propionibacterium acnes* P-37.
- To isolate, clone, and characterize the lipase(s) of *Helicobacter pylori* 26695.
- To evaluate the effect on *Propionibacterium acnes* GehA and *Helicobacter pylori* HP0739 (EstV) of the most potent lipase inhibitors found in Chapter 4.