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Microbial lipases as virulence factors

F. Stehr[a,*], M. Kretschmar[b], C. Kröger[a], B. Hube[a,c], W. Schäfer[a]

[a] *Institut für Allgemeine Botanik, AMP III, Universität Hamburg, Ohnhorststrasse 18, 22609 Hamburg, Germany*
[b] *Institut für Medizinische Mikrobiologie und Hygiene, Klinikum Mannheim, Postfach 10 00 23, 68135 Mannheim, Germany*
[c] *Robert Koch-Institut, NG4, Nordufer 20, 13353 Berlin, Germany*

Abstract

Up to now, lipases of microbial origin are known to be very useful in a palette of industrial applications. But it becomes more and more obvious that extracellular lipases also play a role during microbial infections. This review will focus on the virulent traits of these secreted enzymes from bacteria and fungi. Special emphasis will be laid on *Candida albicans* research. This human pathogenic fungus possesses a lipase gene family, which is expressed and differentially regulated under a variety of in vitro conditions. First results show that this isoenzyme family is also expressed during an experimental infection.

In addition, putative functions of extracellular lipases of pathogenic micro-organisms will be discussed.

Keywords: Lipases; Virulence factors; *Candida*; Human pathogen; Lipids

1. Introduction

Microbial lipases (EC 3.1.1.3) are widely used in biotechnological applications [1]. These enzymes are able to catalyse both the hydrolysis and synthesis of ester bonds of triacylglycerols (TAG) (Fig. 1). Due to their properties, e.g. being stable in organic solvents, lacking the need for cofactors and showing a high enantioselectivity, they are very valuable in the field of production of optically active compounds, esters, and lactones. Furthermore, they are added to detergents and used in the generation of food ingredients, e.g. to enrich polyunsaturated fatty acids from animal and plant origin, or for the flavour development of food.

Apart from these industrial applications there is increasing evidence that extracellular lipases are important microbial virulence factors. With regard to this aspect, research has been focused mainly on human pathogenic bacteria [2]. In contrast, lipases of pathogenic fungi as potential virulence factors have been widely neglected. In the following article, we will focus on bacterial and fungal lipases as virulence factors of these organisms.

2. Human pathogenic bacteria

The human skin is the natural environment of many different bacteria and a few fungi. Among these micro-organisms, some are opportunistic pathogens causing diseases when the natural defense of the host is weakened. One of these inhabitants is *Staphylococcus epidermidis*, which is described as a human cutaneous commensal living on the skin of its host. This bacterium is able to become an opportunistic pathogen. During the infection process, two secreted lipases might play a role in supporting growth and

Abbreviations: LIP, lipase gene; FFA, free fatty acid; TAG, triacylglycerol

* Corresponding author. Tel.: +49-40-42816-396; fax: +49-40-42816-513.
E-mail address: stehr@lipases.de (F. Stehr).

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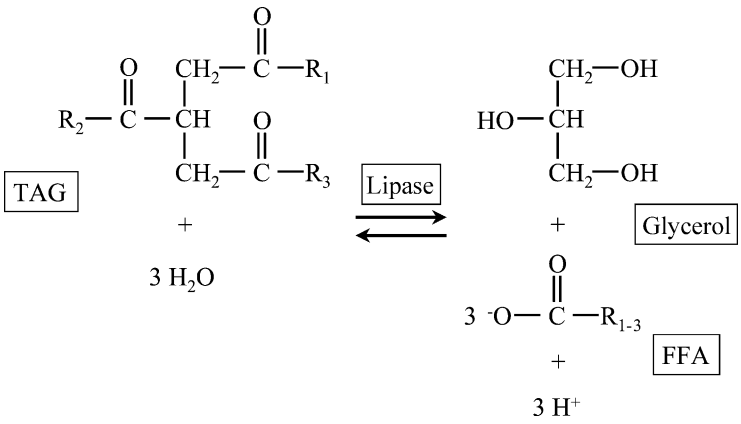


Fig. 1. Enzymatic reaction of a lipase catalysing hydrolysis and synthesis of a triacylglycerol. TAG: triacylglycerol, FFA: free fatty acids.

colonization through cleavage of sebum-derived triacylglycerols [3] and therefore might have an impact on the infection. Clinical isolates of *Staphylococcus aureus* that were isolated from deep infections produced higher amounts of lipase than those derived from superficial locations [4]. This indicates that lipase activity may be important for nutrition and/or dissemination of bacteria. Beyond that, lipases may play a direct role in virulence. It has been postulated that these particular enzymes may exert an influence on pathogenesis of *Staphylococcal* skin infection. In vitro studies have shown that purified lipase influenced functions of different human immune cells, such as the chemotaxis of neutrophils [5] and granulocytes [6]. In addition, granulocytes that were incubated with lipase showed a decreased phagocytotic killing of bacteria. A potential cause could be that the microbial enzyme damaged surface structures of the immune cells. The strongest hint that secreted lipases are involved in pathogenesis is the detection of anti-lipase IgG antibodies in patients suffering from *S. aureus* infections fortifying the virulent potential of extracellular lipases [7].

In addition to *Staphylococcus*, other bacterial species appear to employ lipases in the pathogenic repertoire. Another human skin inhabitant is *Propionibacterium acnes*, the causative agent of acne vulgaris from which everyone has to suffer during puberty. This bacterium produces a number of extracellular enzymes including a lipase, which has been implicated in the microbial colonization of human skin. Due to lipolytic activity, released free fatty acids (FFA) might assist bacterial adhesion and hence support the colonization of sebaceous follicles, the natural environment of *P. acnes* [8]. In more severe cases of bacterial infections certain bacteria can cause invasive and even systemic infections. For example, *Pseudomonas aeruginosa* is able to infect immuno-compromised patients. Furthermore, *P. aeruginosa* colonization of the respiratory tract is correlated with an increase in lung destruction of patients suffering from cystic fibrosis. This pathogen possesses a complex extracellular lipolytic system with at least two phospholipases C (PLC), one outer membrane-bound esterase (a member of the recently described GDSL family of lipolytic enzymes, [9]) and one secreted lipase [10]. Jaeger and co-workers discovered that the combined action of lipase and PLC led to an increase in the immune reactive substances 12-hydroxyeicosatetraenoic acid from human platelets and leukotrien B_4 from neutrophils [11]. These reactions might lead to disordered immune responses, which could initiate tissue damage and stimulate inflammatory processes. It was also shown that both enzymes had a synergistic effect on the cleavage of dipalmitoylphosphatidylcholine, the major lung surfactant lipid [12]. During infection, both enzymes are present, as patients suffering from cystic fibrosis have IgG antibodies against both lipase and PLC.

Based on these results it is evident that infections are multifactorial events and different compounds synthesized and secreted by the bacterial cells may

equally contribute to the colonization and persistence of the pathogenic micro-organism. It becomes more and more obvious that secreted lipases play a part in the group of bacterial virulence factors.

3. Pathogenic fungi

Another group of important pathogenic micro-organisms are fungi. There are many different virulence factors in discussion that might help fungi to colonize and infect humans, but the role of secreted lipases still needs to be clarified. It is of great importance to discover new virulence factors, because they could represent promising targets for novel antifungal drugs. The need for successful therapies is growing, as the number of life-threatening mycoses caused by opportunistic fungal pathogens is rising. One of the biggest problems of fungal infections in humans is the fact that fungi are eukaryotes and thus, antifungal drugs often have strong side effects for the patient. Yeast species are the predominant group of fungi that infect humans. These species benefit from host dysfunctions like the disruption of protective barriers to establish disease.

Even though *Malassezia* species belong to the opportunistic pathogens, they are also regarded as normal human commensal flora. So far, seven different *Malassezia* species have been discovered [13]. They are located at openings of sebaceous glands on the human skin. Predisposing factors like corticosteroid treatment and immunodeficiency can cause the switch from a commensal to a pathogenic state, which often results in skin diseases and sometimes even systemic infections. Among other diseases, this fungus is associated with tinea versicolor, seborrhoeic dermatitis and dandruff. Six out of seven *Malassezia* species are lipophilic requiring exogenous lipids, preferentially long chain fatty acids, for growth. Ran et al. [14] investigated the lipase activity of *Malassezia furfur* and discovered that the pH optimum lies at an acidic pH similar to the skin surface pH. The addition of sodium taurocholate, a lipase activator, enhanced cell growth in a dose dependent manner and induced the morphological transition of the yeast to the hyphal state, which is associated with disease [15]. This suggests that the lipolytic activity plays a role in cell growth and hyphal formation to support colonization. Next to nutrient acquisition another putative role of extracellular lipases is the enhancement of adhesion. Adhesion itself is often regarded as the initial stage of the infection process. For the black yeast *Hortaea werneckii*, the causative agent of tinea nigra, it has been shown that the cell surface of this fungus is very hydrophobic, enabling *H. werneckii* to adhere to the host skin. This is supported from studies by Göttlich et al. [16], which found that the lipolytic activity of the fungus increases hydrophobic interactions by liberation of FFA.

In addition to dermatophytes, opportunistic fungi such as *Candida* species are known to secrete extracellular lipases. *Candida* spp. are the fourth-leading cause of nosocomial infections in the US [17], which supports the view that *Candida* infections are a very important health issue. Among the different medically important yeasts, *Candida albicans* is the most commonly found *Candida* species in clinical samples. It is part of the human commensal flora living on human skin or mucosal surfaces. There are estimations that at least 50% of all individuals are colonized by this *Candida* species. However, in situations that cause a decrease in host defense, the fungus can become invasive and pathogenic. For example, indwelling catheters can serve as external sources of candidosis. In these cases, the bloodstream of the patient may get persistently inoculated with the yeast. The prolonged use of catheters is also associated with another pathogenic *Candida* species, *C. parapsilosis*. So far, proteolytic and lipolytic activities of this yeast have been detected [18,19]. Interestingly, the lipolytic enzyme of *C. parapsilosis* is called a lipase-acyltransferase, as it favours the synthesis of esters [20]. It is unknown to what extent this activity supports the growth of the fungus in lipid-rich environments, but the illumination of the apparent involvement of lipolytic enzymes in pathogenesis could be fruitful for new drug developments. While almost nothing is known about virulence factors that help *C. parapsilosis* to be infectious, most of *Candida* research has been focused on *C. albicans*. This yeast possesses a large spectrum of hydrolytic enzymes with relatively broad substrate specificities including proteases, phospholipases and lipases, which might be the reason for the outstanding position of this human pathogen [21]. As early as 1966 lipolytic activity of *C. albicans* was described by Werner [22]. Werner showed that the fungus could grow with different

Tween detergents as sole source of carbon. The data was refined by Ogawa et al. [23], who showed that induction of lipase by sodium taurocholate correlated with fungal growth in carbohydrate-restricted media. Interestingly, optimal enzyme activity was detected at a weakly acidic pH which coincides with the pH of the human skin. Five years later, Fu et al. [24] discovered the first lipase gene of *C. albicans*, *LIP1*, by accident while they were searching for phospholipases. Expression analysis revealed that *LIP1* is transcribed at 30 and 37 °C. The authors also detected expression of *LIP1* in media with different Tweens as sole source of carbon. However, they did neither detect any mRNA of *LIP1* in a complex medium (YPD), nor in Lee's medium, which is a defined amino acid medium used to induce yeast-to-hyphal transition via a pH- and temperature-transition. Southern blot analysis with *LIP1* as probe under low stringency conditions provided the first evidence that a whole lipase gene family might exist.

4. The lipase gene family of *Candida albicans*

Through subsequent cloning, sequencing, blast searches and sequence alignments in the *C. albicans* genome databases [25], we identified nine additional putative lipase genes (*LIP2–10*) with significant homologies to *LIP1* [26]. As an example, a Southern blot of genomic DNA digests of different *Candida* species probed with a DIG-labelled DNA-fragment of *LIP8* under low stringency conditions reveals several visible bands (Fig. 2) supporting the existence of a lipase gene family. Furthermore, other medically relevant *Candida* species seem to have homologous DNA sequences. Two bands from *C. parapsilosis* and *C. tropicalis* hybridized. No similar sequences were detected in *C. krusei* and *C. glabrata*. At least five bands were identified in the genome of *C. dubliniensis*. This is the first evidence that *C. dubliniensis* might have a lipase gene family that is highly related to the one of *C. albicans*. *C. dubliniensis*

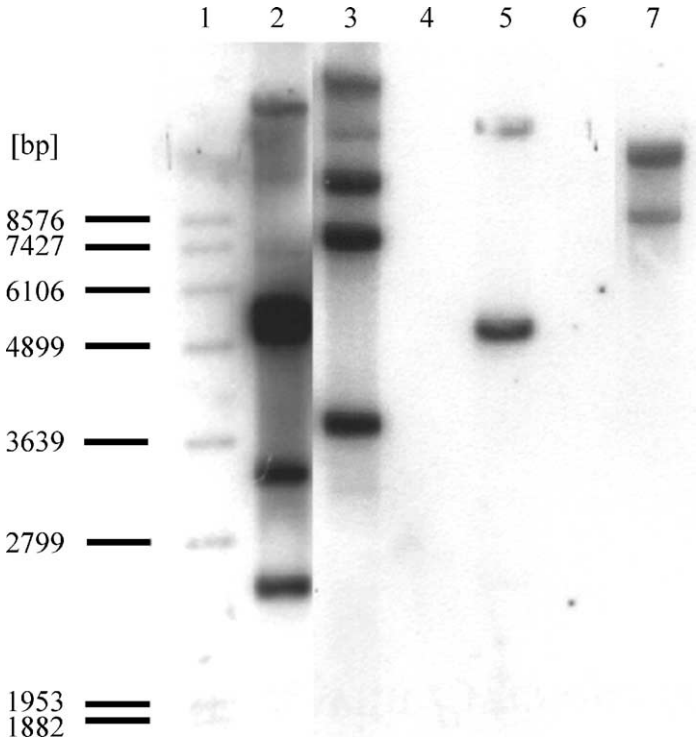
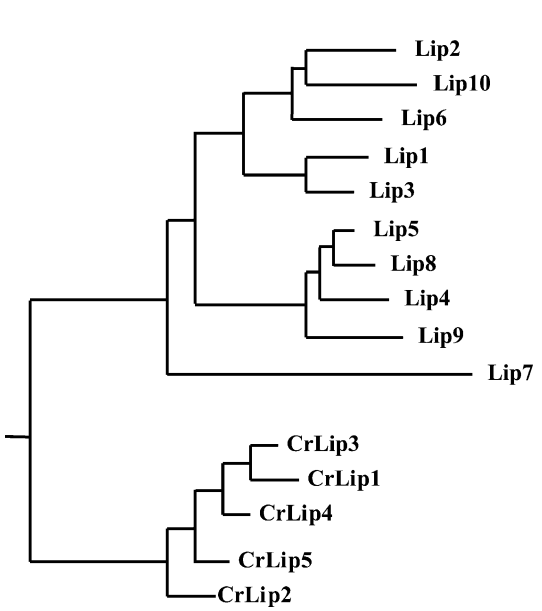


Fig. 2. Southern blot analysis of different *Candida* species. Genomic DNA digests were hybridized with a *LIP8*-probe under low stringency conditions. DIG size marker (1); genomic DNA of *C. albicans* (2); *C. dubliniensis* (3); *C. krusei* (4); *C. parapsilosis* (5); *C. glabrata* (6); and *C. tropicalis* (7) was digested with *Bst*1107I.



	Orf	Sig	Pro	Chr
Lip2	1401	16	450	1
Lip10	1398	16	449	1
Lip6	1392	16	447	1
Lip1	1407	16	452	1
Lip3	1416	16	455	1
Lip5	1392	14	449	7
Lip8	1380	14	446	7
Lip4	1380	14	445	6
Lip9	1362	14	439	7
Lip7	1281	-	426	R

Fig. 3. Dendrogram of lipase gene families of *C. albicans* and *C. rugosa*. Lip1–10: deduced lipases of *C. albicans*; CrLip1–5: lipases of *C. rugosa*; Orf: open reading frame of *C. albicans* lipase genes in base pares; Sig: number of amino acids of the signal peptide according to von Heijne [28]; no signal sequence was identified for Lip7; Pro: number of amino acids of the mature protein; Chr: chromosomal location.

has just recently been identified as a unique yeast species, which is phylogenetically closely related to *C. albicans* [27]. So far, this opportunistic pathogen was implicated in oral candidosis, but almost nothing is known about its virulence factors. It will be promising to compare the isoenzyme families of both *Candida* species according to differential regulation and adaptations. Concerning *C. albicans*, it has been shown that ten lipase genes encode putative secreted lipases with open reading frames ranging from 1281 to 1416 bp (Fig. 3). They encode highly similar proteins with up to 80% identical amino acid sequences (*LIP5* and *LIP8*). Except for *LIP7*, all remaining *LIP* genes encode preproteins with an *N*-terminal signal peptide of approximately 15 amino acids according to the von Heijne rules [28]. This peptide destines the lipase for secretion or it will be delivered to vacuoles, lysosome, or cytoplasmatic membranes. The mature lipase isoenzymes are on average 449 amino acids. The predicted amino acid sequences contain a conserved pentapeptide “GXSXG”, which is found in most lipases and esterases. The catalytic center of lipases contains the so-called catalytical triad consisting of the nucleophilic serine, which is located in this pentapepide, a histidine and an acidic residue, namely aspartate or glutamate. There are also four conserved cysteine residues possibly involved in disulfide bridge formation. The number of putative *N*-glycosylation sites ranges from one (Lip5) to eight (Lip7), whereby each putative enzyme possesses one positionally conserved *N*-glycosylation site. Whether these sites participate in catalysis still needs to be elucidated via in vitro mutagenesis experiments.

The lipase genes are located on four different chromosomes: 1, 6, 7, and R. According to homologies on the amino acid level it is possible to divide the lipase isoenzyme family into three subgroups. The first subgroup with *LIP1–3*, *LIP6*, and *LIP10* shows similarities of at least 74%. The second group consists of *LIP4*, *LIP5*, *LIP8*, and *LIP9* of at least 68% similarity. The sequence homologies on the amino acid level also reflect the location of the respective lipase gene. The members of the first subgroup are all located on chromosome 1, in contrast to the second group, whose members are situated on chromosome 7 except *LIP4*, which is located on chromosome 6. *LIP7* forming a group of its own shows the greatest divergence from the rest of the whole lipase family. It does not encode

any signal peptide according to the von Heijne rules and it is the only lipase gene that is located on chromosome R, the largest chromosome of the *C. albicans* genome. Another point to be stressed is that both subgroups have differently sized signal peptides of 16 and 14 amino acids, respectively, fortifying the subdivision. This data suggests that at least two ancestor lipase genes existed and were duplicated. There are many repetitive sequences in the *C. albicans* genome, which ease recombinational processes and, thus, support gene duplications. It has also been suggested that the high genetical flexibility is due to translocations of chromosomes [29]. For spontaneous morphological *C. albicans* mutants it has been shown that a broad spectrum of multiple chromosomal rearrangements occurs. This enables the fungus to cope with changing nutritional environments of the host [30]. It may be that the development of a lipase gene family is an adaptive mechanism of *C. albicans* to its human host.

After identifying a family of lipase genes, questions arose whether every member is transcriptionally active and whether there might be a differential regulation of the family.

5. Expression of lipase genes in vitro

To examine whether there is a differential regulation of the lipase gene family expression, Northern blots and RT-PCR analysis have been performed. All members of this new gene family are expressed in several media with and without lipids. *C. albicans* is able to hydrolyse Tween 40 and utilise it for growth. Six *LIP* genes are expressed in medium with Tween 40 as a sole source of carbon [26], suggesting that these putative isoenzymes are involved in the hydrolysis of lipids. However, most *LIP* genes are expressed in media lacking lipids including protein medium, which simultaneously induced transcription of secreted proteinase genes. This indicates that lipases might have additional roles besides nutritional aspects. The release of fatty acids may change the pH in the microenvironment of the cells resulting in an optimisation for other fungal enzymes such as the secreted proteinases. In order to test whether there is a regulation of lipase genes due to the state of morphology, different inducers of yeast-to-hyphal transition were used. There was no apparent regulation detectable, because transcripts of various *LIP*s were found in both types of morphology.

The analysis of the expression pattern of all *Candida* lipase genes revealed that some of these genes might be constitutively expressed, while the expression of others is regulated depending on the environment. Fungi can respond rapidly to environmental changes. This flexibility could allow these organisms to take advantage of impaired immunity in patients and, therefore, facilitate establishment of disease [31].

For *C. lipolytica* [32] and *C. rugosa* [33] it has been shown that the expression of lipases depends not only on the type of energy source, but also on the oxygen partial pressure. *C. rugosa* possesses a lipase gene family with at least seven members [34]. They are subdivided into two groups, one constitutive and one regulative group. The regulated lipases are inducible by FFA. Lotti et al. [33] demonstrated that *C. rugosa* growing in Tween medium accumulated lipases during the mid-logarithmic phase and secreted them at a later point of time. These observations suggest that along with the nutritional source, the growth phase also influences lipolytic activity. Likewise, lipolytic activity of the human pathogenic bacteria *S. aureus*, *S. epidermidis*, and *P. aeruginosa* could only be detected once stationary phase was reached. The lipolytic system of *Acinetobacter calcoaceticus* consisting of two esterases and one lipase is induced at the end of the logarithmic growth phase [35].

After showing that the *LIP* gene family is differentially expressed in vitro there still remained the question whether these genes are expressed during infection and support virulence of *C. albicans*.

6. In vivo expression of lipase gene family during mouse infection

The fact that a family of genes encodes these secreted lipases may indicate that different lipase genes are needed during different stages or types of infection. Such a divergent importance, depending on the specific host environment, has been shown for the secreted aspartyl proteinase (*SAP*) gene family. According to this model of tissue- and infection-specific roles, different *SAP* genes were differentially regulated in vitro and in vivo, depending on the environment and the morphological form [36]. Using the

RT-PCR technique, the in vivo expression pattern of lipase genes was analysed by intraperitoneal infection of mice. After 3 days of infection the liver was taken and analysed for lipase gene expression. According to the obtained results *LIP5*, *LIP6*, *LIP8*, and *LIP9* were expressed [26]. This is the first evidence that secreted lipases of a human pathogenic fungus are involved in the infection process.

7. Putative roles of extracellular microbial lipases

In the following, we will discuss the putative roles of extracellular microbial lipases as shown in Fig. 4. The most prominent role of extracellular lipases for a micro-organism is the digestion of lipids for nutrient acquisition. These enzymes might help bacteria and fungi to grow in a carbohydrate-restricted area or environments where lipids are the sole carbon source. As mentioned before, extracellular lipolytic activity supports growth of pathogenic *Staphylococcus* species [3,4]. For *C. albicans*, it is known to proliferate in emulsions that are used for parenteral nutrition, which contain lipids as supplement, in contrast to standard preparations. *C. albicans* has been shown to proliferate better in the lipid-containing emulsions [37]. Moreover, patients in need of nutritional support are often immunodeficient. Therefore, they run a higher risk of infection from such an opportunistic organism like *C. albicans*. Another function of lipases may be to support the micro-organism to stick to host tissue and/or neighbouring cells. For *S. aureus* it has been postulated that its lipase enhances adhesion by degrading host surface molecules and thereby liberating new receptors. Additionally, released FFAs might increase

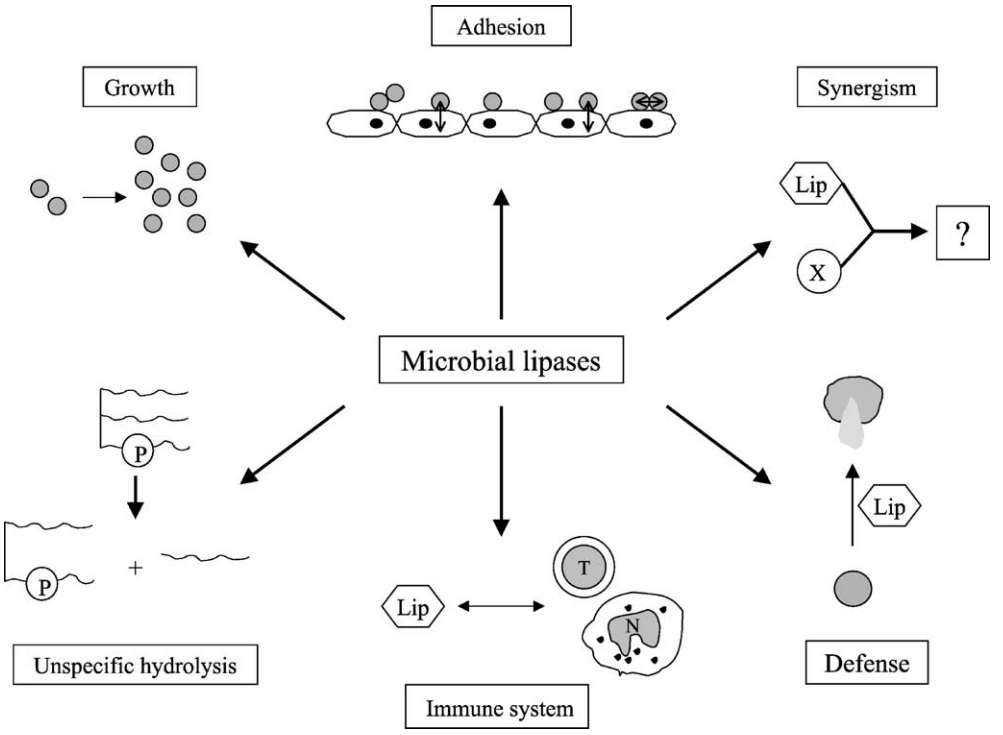


Fig. 4. Putative roles of microbial extracellular lipases. Growth: lipolysis might provide carbon sources that the micro-organism could use for growth; adhesion: released FFA due to lipolytic activity could support cell-to-cell and/or cell-to-host tissue adhesion; synergism: a lipase might work hand in hand with another enzyme or it might optimize conditions for other enzymes; unspecific hydrolysis: lipases might possess additional phospholipolytic activity; immune system: lipases and their catalytical end products may have an effect on different immune cells and might initiate inflammatory processes; defense: micro-organisms that secrete lipolytic enzymes might have a selection advantage by lysing competing microflora.

unspecific hydrophobic interactions, as it is assumed for *P. acnes* [8].

As mentioned above, it is important not to focus on just one enzyme, but to keep in mind that many different components fit into this puzzle of the development of infection. Jaeger and co-workers [11] have shown that phospholipases and lipases may act in concert. The combined action of lipases and phospholipases may occur also during *C. albicans* infections, as both activities have been detected in this fungus. Another synergism could be that lipases lower the pH of a microniche by liberating FFA and thus optimising the pH for the secreted aspartyl proteinases, which are well known virulence factors [38]. Another possible role of the lipase isoenzyme family is that it may perform more general functions by acting as a survival factor by optimising conditions for other enzymes. The use of a combination of various inhibitors and the generation of multi-gene knock-out strains will be helpful to explore this hypothesis. Besides the lipolytic activity, microbial lipases might have ancillary enzyme activities. In *Staphylococcus warneri*, it has been shown that lipase 2 has additional phospholipolytic activity [39], which adds another putative pathogenic trait to lipases. Through cleavage of phospholipids, they may have the ability to actively degrade host tissue and lyse cells, since phospholipids are the major component of cell membranes. It might also be possible that lipases act against other micro-organisms of the host microbial flora providing the pathogen with a selection advantage. Along with the direct involvement in actively degrading host tissue, there might be an indirect involvement in virulence by modifying signal transduction pathways.

Lipids, the main target of lipases, have diverse functions in humans. In addition to being energy stores and structural components of cell membranes, they are also very important biological effectors. They play roles in signal transduction, intracellular transport, and gene transcription. The hydrolysis of triacylglycerols by a lipase generates diacylglycerol and one FFA. Both end products of lipolytic activity can initiate many different processes. Diacylglycerol is a common second messenger and triggers various signal transduction cascades. Also FFAs are bioactive substances. For example, FFAs can act as second messengers influencing activity of phospholipases, ionic channels, ATPases, G-proteins, and protein kinases. They regulate the phosphoinositide and sphingomyelin cycle, hormone signal transduction, and can even modulate gene transcription. Furthermore, they are known to impair several immune system functions [40]. It is well known that FFAs have an inhibitory impact on T-cell proliferation and that cell-mediated cytotoxity and receptor-mediated phagocytosis are suppressed by saturated fatty acids [41]. This might be the reason why purified lipase of *S. aureus* had several effects on immune cells. The enzyme decreased phagocytotic killing by granulocytes [6] and induced chemotaxis of neutrophils [5] and granulocytes [6] alike. FFAs could also disturb balanced networks in the host. Hanley et al. [42] have shown that the generation of cholesterol and FFA in a proper ration is critical for the maintenance of the epidermal permeability barrier. The uncontrolled release of FFA might, therefore, facilitate the invasion of pathogens.

Hence, it is important to note that the combined effect of lipase itself and the products of its enzyme activity might contribute to microbial virulence.

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