Biochimie 82 (2000) 1005−1014

S0300908400011809/REV

Staphylococcal lipases: Biochemical and molecular characterization

Ralf Rosenstein, Friedrich Götz*

Mikrobielle Genetik, Universität Tübingen, Waldhäuser Str. 70/8, 72076 Tübingen, Germany

(Received 15 May 2000; accepted 14 September 2000)

Abstract — To date, the nucleotide sequences of nine different lipase genes from six *Staphylococcus* species, three from *S. epidermidis*, two from *S. aureus*, and one each from *S. haemolyticus*, *S. hyicus*, *S. warneri*, and *S. xylosus*, have been determined. All deduced lipase proteins are similarly organized as pre-pro-proteins, with pre-regions corresponding to a signal peptide of 35 to 38 amino acids, a pro-peptide of 207 to 321 amino acids with an overall hydrophilic character, and a mature peptide comprising 383 to 396 amino acids. The lipases are secreted in the pro-form and are afterwards processed to the mature form by specific proteases. The pro-peptide of the *S. hyicus* lipase is necessary for efficient translocation and for protection against proteolytic degradation. Despite being very similar in their primary structures the staphylococcal lipases show significant differences in their biochemical and catalytic properties, such as substrate selectivity, pH optimum and interfacial activation. The lipase from *S. hyicus* is unique among the staphylococcal and bacterial lipases in that it has not only lipase activity, but also a high phospho-lipase activity. All staphylococcal lipases are dependent on Ca^{2+}, which is thought to have a function in stabilizing the tertiary structure of the lipases. Evidence exists that staphylococcal lipases like other bacterial lipases, possess a lid-like domain that might be involved in the interfacial activation of these enzymes. © 2000 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS

***Staphylococcus* / lipase / phospholipase / pre-pro-protein / substrate specificity**

1. Introduction

Lipolytic and esterolytic activities are frequently detected among the various exo-enzymatic activities of staphylococci. The corresponding enzymes, lipases or glycerol ester hydrolases (EC 3.1.1.3) are defined as enzymes that hydrolyze emulsions of lipids with long-chain fatty acids. Many of them show an interfacial activation, i.e., a sharp increase in activity when the solubility limit of the substrate is reached [1].

Some staphylococcal lipolytic enzymes also hydrolyze, and some preferably hydrolyze, water-soluble, monomeric substrates with short-chain fatty acids, such as tributyrin or Tween, and some of them show no interfacial activation, thus having typical features of esterases. Therefore, an uncertainty exists as to whether the staphylococcal lipolytic enzymes should be classified as lipases or as esterases. This dilemma also occurs with lipolytic enzymes from *Pseudomonas aeruginosa* and *Bacillus subtilis*, which react with monomeric substrates as well as with emulsions and show no interfacial activation [2]. The classification scheme for the distinction between lipases and esterases that works well for the eukaryotic enzymes seems to be less unambiguous when applied to the bacterial enzymes. A redefinition of both classes of acylester-hydrolyzing enzymes might therefore be helpful. Despite the lack of clarity, the designation 'lipase' for the staphylococcal lipolytic enzymes is commonly accepted in the literature and will also be used in this review.

The importance of staphylococcal lipases, like other microbial lipases, results from their significance in bacterial lipid metabolism and their involvement in pathogenic processes, and also because they are valuable tools in biotechnology [3]. Their potential as biocatalysts is based on enzymatic features, e.g., regio- and enantio-specificity, a broad substrate specificity, and the ability to catalyze not only the hydrolysis, but also the synthesis of fatty acid compounds. The increasing interest in lipases is reflected by the numerous reviews on this topic published during the past few years; some of the reviews cover a broad spectrum of bacterial lipases (see, for example, references [2–4]). The present review will concentrate on the lipases produced by the members of the Gram-positive genus *Staphylococcus* and will focus on their molecular and biochemical characterization.

2. Molecular biology of staphylococcal lipases

To date, the nucleotide sequences of nine lipase genes from six different staphylococcal species have been published. Three are derived from *S. epidermidis* (two from *S. epidermidis* 9 and one from *S. epidermidis* RP62A), two from *S. aureus* (from strains NCTC 8530 and PS54), and one each from *S. haemolyticus* L62, *S. hyicus* DSM

* *Correspondence and reprints.*
E-mail address: friedrich.goetz@uni-tuebingen.de (Friedrich Götz).

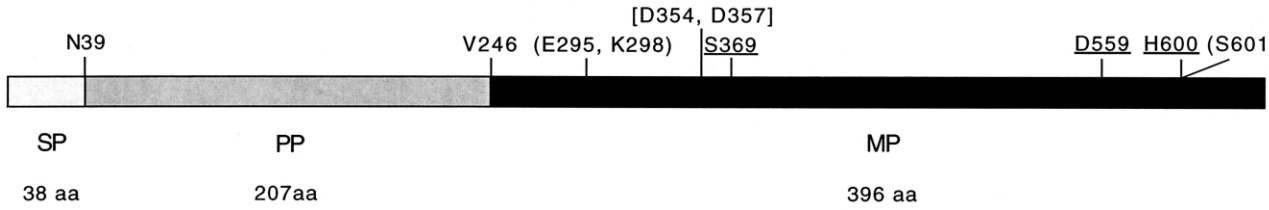

Figure 1. Organization of the *S. hyicus* lipase as an example for the pre-pro-structure of staphylococcal lipases. SP, signal peptide; PP, pro-peptide; MP, mature peptide. The first amino acids of the pro-peptide region and the mature peptide are given. The residues forming the catalytic triad are indicated by underlining. The amino acids responsible for the phospholipase activity are indicated by parentheses. Brackets indicate those amino acids that are involved in calcium binding.

20459, *S. warneri* 863, and *S. xylosus* DSM 20266 [5–13]. For convenience, the following abbreviations will be used for the lipases from these strains: *S. aureus* NCTC 8530, SAL-1; *S. aureus* PS54, SAL-2; *S. epidermidis* 9 (GehC), SEL-1; *S. epidermidis* 9 (GehD), SEL-2; *S. epidermidis* RP62A, SEL-3; *S. haemolyticus*, SHaL; *S. hyicus*, SHyL; *S. warneri*, SWL; and *S. xylosus*, SXL.

2.1. Molecular organization of staphylococcal lipases

The nucleotide sequence of the gene encoding SHyL, published in 1985, was the first description of the primary structure of a bacterial lipase [7]. Since then, numerous studies on the molecular and biochemical properties of SHyL have been undertaken, making SHyL the best-studied staphylococcal lipase. From the nucleotide sequence a protein of 641 amino acids with a predicted molecular mass of 71 382 Da was deduced [7]. Analysis of the supernatant of the donor strain *S. hyicus* by SDS-PAGE revealed a lipase with an apparent size of 46 kDa. When the SHyL gene was cloned in *S. carnosus* TM300, a cloning host with low extracellular proteolytic activity, only an 86-kDa form was detected in the supernatant. The N-terminal amino acid sequences of the 86- and 46-kDa forms were determined, and a comparison with the SHyL sequence revealed that both forms were derived from the same precursor protein. The sequence of the 86-kDa form of SHyL secreted by *S. carnosus* starts with Asn39, immediately after a predicted cleavage site (-Ala36–Glu37–Ala38–) for signal peptidase I. The 46-kDa form produced by *S. hyicus* starts with Val246 [14]. It soon became clear that SHyL is secreted into the medium as a pro-form (pro-SHyL), which is subsequently processed to the mature lipase (mature SHyL). This processing apparently does not occur in the heterologous host *S. carnosus*. Based on the N-terminal sequences of the lipase forms found in the supernatants of *S. hyicus* and *S. carnosus*, it was evident that SHyL is translated as a 641-amino-acid precursor protein with a signal peptide of 38 amino acids and a pro-peptide of 207 amino acids, which is processed to the mature lipase of 396 amino acids *(figure 1)*.

A comparison of the SHyL amino acid sequence with the deduced amino acid sequences of other staphylococcal lipases reveals a very similar molecular organization of the proteins *(table I)*. All staphylococcal lipases are translated as a pre-pro-enzyme with a leader peptide of 35 to 38 amino acids, followed by a pro-sequence (207 to 321 amino acids) and the mature form, i.e., the active lipase that normally appears in the supernatant of the producing *Staphylococcus* strain (383 to 396 amino acids). A multiple alignment of the lipase sequences shows a remarkable sequence conservation in the region covering the signal peptides, with a motif containing the perfectly conserved residues Ser, Ile, Arg and Lys, designated as the SIRK-motif [9, 15]. It is still unclear whether the strong conservation of this motif in the signal peptide of the staphylococcal lipases reflects a biological function in the secretion process.

The various pro-peptides have no striking similarities at the sequence level, but are distinguished by their overall hydrophilic character. When separated in SDS-polyacrylamide gels, the pro-forms of the lipases often reveal molecular masses that are significantly higher than those calculated from their sequences [5, 7, 9]. This phenomenon most probably is caused by the hydrophilic pro-peptides since the molecular masses of the mature lipases show a much better agreement between theoretical and observed values.

All known staphylococcal lipases reveal the highest sequence similarity to each other in their mature parts, with identities ranging from 50 to 81% (*table II*; SEL-1 was excluded from the comparison since its mature form differs only in two residues, Y453F and E675D, from SEL-3). The serine, aspartic acid, and histidine residues that are presumably involved in the lipolytic catalytic triad (see below) are well conserved in all sequences *(figure 2)*. In addition, a P-loop consensus sequence, –[AG]–x4–G–K–[ST]– , is found in all staphylococcal lipases except SEL-2 and SHaL. The P-loop motif commonly occurs in ATP- or GTP-binding proteins [16]. In vitro assays with purified SAL-1 in the presence of 2 mM ATP or GTP have shown a decrease in lipase activity by

Table I. Strains and characteristics.

Strain	*Abbreviation*	*Gene*	*Length (amino acids)*			*Reference*
			Pre-protein (signal peptide)	*Pro-protein*	*Mature protein*	
S. aureus NCTC8530	SAL-1	*geh*	35	256	391[b]	[9]
S. aureus PS54	SAL-2	*geh*	37	259	394[a]	[8]
S. epidermidis 9	SEL-1	*geh*C	35	267	386[b]	[5]
S. epidermidis 9	SEL-2	*geh*D	37	216	390[a]	[6]
S. epidermidis RP62A	SEL-3	*geh*1	35	267	386[a]	[12]
S. haemolyticus L62	SHaL	–	37	259	392[b]	[13]
S. hyicus	SHyL	–	38	207	396[b]	[7]
S. warneri 863	SWL	*geh*A	35	313	384[a]	[11]
S. xylosus DSM20266	SXL	*geh*M	35	321	383[a]	[10]

[a] Start of mature protein was deduced from homology comparison.
[b] Start of mature protein was determined by Edman sequencing.

about one-third [17]. Attempts to mutagenize the P-Loop consensus sequence in SAL-1 failed since no viable transformants with the mutated gene could be obtained [17]. Taking these preliminary results together, one might speculate that binding of ATP/GTP to the P-loop could play a role in decreasing the intracellular lipase activity in order to protect the producing strain from detrimental effects. But further experiments are necessary to elucidate a putative functional role for the nucleotide binding motif in the staphylococcal lipases.

2.2. Processing of the pro-form

The proteolytic processing of pro-SHyL has been studied in more detail [15]. Since the 86-kDa pro-SHyL purified from *S. carnosus* is processed by culture supernatants of *S. hyicus*, it became apparent that the processing of the pro-form by an extracellular protease occurs after secretion. In a co-fermentation of *S. hyicus* with an *S. carnosus* recombinant strain that produces pro-SHyL, several intermediate degradation products of 72, 55, and 46 kDa were produced during the processing of the pro-form to the mature lipase [14]. The 86-kDa pro-form and all intermediary products had lipase activity. In the later stationary growth phase, products even smaller than 46 kDa that showed no lipolytic activity were observed, indicating that a degradation beyond Val246 affects the catalytic function. The pro-form and the 72- and 55-kDa products were predominant in the earlier growth phases, whereas the smaller forms dominated in the stationary phase. This indicates a stepwise processing starting at the N-terminus of pro-SHyL.

The proteolytic activities in the supernatant of *S. hyicus* were analyzed. Two proteases, ShpI and ShpII, were identified; ShpII proved to be involved in pro-SHyL processing [18, 19]. ShpII, a 34-kDa protein with the highest activities at pH 7.4 and 55 °C, is a neutral metalloprotease that is strongly inhibited by chelating compounds. In vitro, ShpII cleaves pro-SHyL between Thr245 and Val246. In accordance with the proposed role and the biochemical properties of this protease, the in vivo processing of the pro-lipase by *S. hyicus* could be inhibited in the presence of 300 μM EDTA [19]. A similar processing scheme seems to be involved in the maturation of the other staphylococcal lipases since in most cases 40- to 48-kDa lipase forms have been detected in the supernatants of the corresponding donor strains [5, 9, 13, 20, 21].

Table II. Identities (similarities) of the mature lipases, determined by using the 'BLAST 2 sequences' program at the NCBI-server [61].

	SAL-2	*SEL-2*	*SEL-3*	*SHaL*	*SHyL*	*SWL*	*SXL*
SAL-1	55 (68)	51 (67)	81 (88)	53 (67)	53 (66)	66 (78)	71 (83)
SAL-2		58 (74)	54 (67)	66 (79)	51 (64)	53 (68)	53 (66)
SEL-2			53 (71)	70 (83)	50 (67)	50 (67)	50 (66)
SEL-3				54 (69)	56 (70)	66 (79)	77 (87)
SHaL					51 (67)	54 (66)	53 (66)
SHyL						50 (64)	53 (69)
SWL							65 (79)

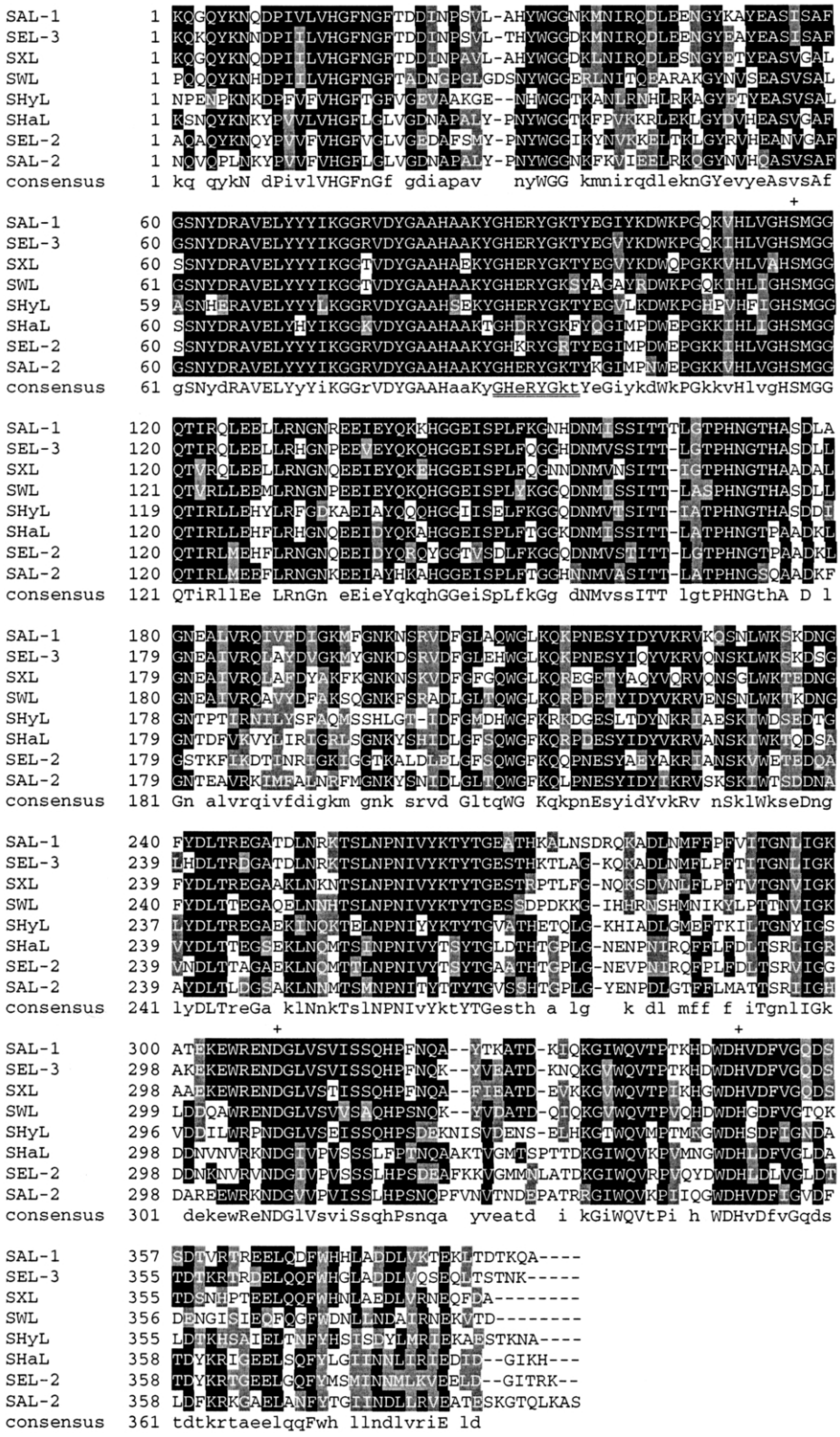

Figure 2. Multiple alignment of the mature forms of the staphylococcal lipases. The sequences were aligned by using CLUSTAL W 1.7 at the BCM Search Launcher (http://dot.imgen.bcm.tmc.edu:9331). Some amino acids from the N-termini of the mature portions of SAL-1, SHaL, and SHyL were omitted. SEL-1 is not included since it is almost identical to SEL-3. The conserved residues (+) which probably form the catalytic triad are indicated above the aligned sequences. The consensus sequence is given; capital letters indicate conserved residues. The 'P-loop' motif is marked by underlining.

2.3. Role of the pro-peptide region

According to the sequence comparisons, all staphylococcal lipases are predicted to be primarily synthesized as pre-pro-lipases. While the function of the leader peptide in secretion is obvious, the role for the pro-peptide remained unclear. One hypothesis proposed a function in masking the enzyme activity until the secretion process is completed in order to protect the producing cell from detrimental effects of the lipase activity. However, the pro-form of SHyL is almost as active as the mature protein, and the pro-lipase can be synthesized intracellularly without hampering the vital functions of the producing strain (G. Thumm, personal communication). Another possible function is the involvement in secretion, probably as an intramolecular chaperone, as has been described for the pro-peptide of *Bacillus subtilis* subtilisin E [22].

The role of the pro-peptide region of SHyL was more thoroughly investigated. An expression vector carrying the cloned gene encoding SHyL was used for the construction of various secretion vectors [23]. The *Escherichia coli* β-lactamase gene (*bla*) lacking its own signal sequence was fused at various sites along the gene regions encoding the pro-peptide and mature forms of SHyL. The amount of β-lactamase secreted into the supernatant of *S. carnosus* clones harboring the recombinant plasmids was measured; only those constructs having at least 160 aa of the SHyL pre-pro-region fused to β-lactamase secreted an amount of fusion protein comparable to that of native lipase. All hybrid proteins having a smaller portion of the lipase fused to β-lactamase remained in the membrane fraction, which indicates a defect in the translocation, and were prone to extensive proteolytic degradation, which indicates an instability higher than those fusion proteins with an SHyL portion >160 aa. These results support a dual role for the SHyL pro-peptide: an involvement in protein translocation and a role in stabilization against proteolytic degradation. Using the 160-aa pre-pro-portion of SHyL, several other heterologous proteins were successfully secreted by *S. carnosus*, such as pro-insulin and malaria antigen [24, 25], thereby showing that the results obtained with *E. coli* β-lactamase are generally applicable.

In order to evaluate whether the two functions of the pro-peptide are represented by different sections of the pro-peptide, several derivatives of SHyL with deletions in the pro-peptide region were constructed and tested for lipase production in *S. carnosus* [26]. The results obtained with these lipase mutants indicate that the SHyL pro-peptide may have two functional domains with each one located in one half of the pro-region. The N-terminal part seems to be important for lipase activity and the C-terminal part for translocation and stability. A stabilizing effect of the SHyL pro-peptide has also been observed in an experiment where OmpA of *E. coli* is fused to the pre-pro portion of SHyL; in contrast to the construct without the lipase secretion signals, no proteolytic degradation occurred after secretion by *S. carnosus* [27]. A number of experiments designed to address the question whether the pro-peptide could act also in *trans* indicated that the pro region has to be covalently attached to the mature protein in order to exert its beneficial effects on translocation, stability, and activity (G. Thumm, personal communication).

3. Biochemical characterization of staphylococcal lipases

Lipolytic activity exerted by *S. aureus* strains was described by C. Eijkman as early as 1901 [28]. In 1963, it was discovered that *S. aureus* lipases are responsible for the release of fatty acids, mainly octadecenoic acid, in human plasma [29]. In the 1980s, lipases from various *S. aureus* strains were purified and only some of them were more thoroughly characterized (see also [30]). The activity of most of these lipases increases in the presence of Ca^{2+}, with EDTA correspondingly acting as an inhibitor. One of the lipases hydrolyzed mono-, di-, and trioleylglycerols at similar rates, thus revealing no positional specificity [31]. To date, lipases from six different *Staphylococcus* species are known, but their biochemical characterization is still far from being complete. The best-characterized lipases are SHyL, SAL-1, and SEL-3 and, to a lesser degree, SHaL and SWL. Few data are available for SEL-1, but the mature form of this lipase differs from SEL-3 only at two amino acid positions, and therefore it is conceivable that the two lipases have the same biochemical properties. Very few biochemical data are available for the remaining staphylococcal lipases.

3.1. Ca^{2+}-dependency

SAL-1, SEL-3, SHyL, and SWL require Ca^{2+} for full enzymatic activity. Correspondingly, chelating compounds, e.g., EDTA or EGTA, act as inhibitors of these lipases. For SAL-1, Ca^{2+} can be replaced by strontium or barium without loss of activity [32]. Various roles for Ca^{2+} in the lipolytic activity of staphylococcal lipases have been discussed. One proposed function is the formation of calcium salts of the released fatty acids in order to remove them from the reaction equilibrium to circumvent product inhibition [33]. It has also been hypothesized that Ca^{2+} might be directly involved in catalysis [34]. Recently, however, it was shown that Ca^{2+} is most probably necessary for stabilizing the three-dimensional structure of the lipase during catalysis [32]. In the crystal structure of *Burkholderia glumae* lipase, the calcium binding site was localized far from the active site [35]. An alignment of the *B. glumae* lipase sequence with the sequences of the other lipases led to the identification of two aspartate residues responsible for calcium binding in SHyL *(figure 1)*. Site-directed mutagenesis of these residues results in a

loss of calcium binding, rendering the corresponding mutant lipases still active at room temperature, but inactive at higher temperatures [36]. Also for SHaL, an increased stability at temperatures up to 50 °C in the presence of Ca^{2+} has been reported [13]. Thus, a structural role for calcium in enhancing the lipolytic activity of staphylococcal lipases became evident.

3.2. pH optimum

SAL-1 and SEL-3 are active over a broad pH range, with an optimum around pH 6 [12, 32]. Accordingly, both lipases are stable under acidic conditions, whereas they are inactivated at pH values above 10. This preference of acidic conditions is quite unusual among bacterial lipases, which in most cases exert their highest activities at alkaline pH. For SHyL, a pH optimum of 8.5 has been reported [37]. In contrast to SAL-1 and SEL-3, the enzyme is unstable at acidic conditions and undergoes a complete inactivation below pH 5. SHaL and SWL exhibit a pH profile similar to that of SHyL, with an optimum around pH 9 and strongly decreased activities at acidic pH values [13, 21].

3.3. Substrate preferences

The differences in pH dependency of SAL-1, SEL-3, and SHyL are also reflected in the substrate preferences of these lipases. SAL-1 and SEL-3 exhibit a strong preference for glycerides with short-chain fatty acids. Both lipases have a significant bias towards substrate molecules with butyric acid esterified to glycerol, *p*-nitrophenol, or umbelliferone. Corresponding ester compounds with an acyl chain length of one methyl group above or below this size, e.g., triacetylglycerol or tripentanoylglycerol, are poorly hydrolyzed by these enzymes [12, 32]. A similar chain-length preference has been found for SHaL and SWL, which also exhibit the highest lipolytic activities with tributyrylglycerol [13, 21]. Compared to other staphylococcal lipases, SWL is the most active enzyme, exceeding the lipolytic activity of SAL-1 and SEL-3 on tributyrin by about 50-fold [12]. It should be stressed that different assays for the characterization of the specificities of the lipases have been used by the various research groups and thus the published data are barely comparable.

SHyL differs from all these lipases in being very tolerant towards lipid compounds with different chain lengths. The enzyme activities of this lipase towards triacylglycerol, tripropionylgylcerol, and tributyrylglycerol, substrates with different fatty acid chain lengths, are almost identical, while the lipolysis of tripentanoyl- and trihexanoylglycerides is slightly reduced. Trioctanoylglycerol, which is hardly hydrolyzed by SAL-1 and SEL-3, seems to be preferably degraded by SHyL [32]. Furthermore, SHyL is distinguished in that it readily recognizes phospholipids of different chain lengths as substrates and thus can also be regarded as a phospholipase [37]. To date, SHyL is unique among bacterial lipases in having a very broad substrate spectrum ranging from lipids of various chain lengths to phospholipids and lysophospholipids [12].

3.4. Molecular basis of the phospholipase activity of SHyL

Despite the strong similarities in the primary structures of the mature staphylococcal lipases, SHyL differs significantly from the other lipases in biochemical and catalytic features. Several studies have been undertaken to identify elements in the primary structure of SHyL that are responsible for its exceptional enzymological properties [9, 38–40]. By construction of a hybrid lipase in which the C-terminal 146 amino acids of SHyL are replaced by 145 amino acids from the C-terminus of SAL-1, it was demonstrated that the structural elements providing the phospholipase activity must reside within the exchanged element [9]. Attempts were then made to more narrowly define the regions involved in phospholipase activity and chain-length selectivity of SHyL by van Kampen et al. [38–40]. Various chimeras between SHyL and SAL-1 were generated by in vivo recombination and were tested for activity on phospholipids and *p*-nitrophenyl esters of different chain lengths. Three elements in the C-terminal region of SHyL necessary for phospholipase activity were identified. Furthermore, a central element of about 70 amino acids was shown to be essential for the chain-length selectivity of this enzyme [39]. In a more recent study, small stretches of amino acids were exchanged between SAL-1 and a synthesized part of SHyL comprising the previously identified elements to localize single amino acid residues involved in phospholipase activity. A serine immediately following the catalytically active histidine had already been shown to be involved in hydrolysis of phospholipids [38]; van Kampen et al. could now identify a stretch of polar amino acids (position 535–542) which, when exchanged for the corresponding, more hydrophobic region of SAL-1, led to a drastic decrease of phospholipase activity. Two essential residues, E537 and K540, were identified within this stretch by introducing point mutations; K540 was shown to be the major determinant for phospholipase activity [40]. Interestingly, SAL-1 was made 23-fold more active towards phospholipids by the introduction of the reverse mutations, thus supporting the determined role for these amino acid residues. The authors concluded from their results that the polar stretch between amino acids 535 and 542 lies within a substrate binding pocket and is involved in the interaction with the polar head group of phospholipids [40].

3.5. The catalytic mechanism

Several efforts have been made to crystallize staphylococcal lipases, but the resulting crystals have been of poor

quality [41]. Therefore, no 3-D structures for staphylococcal lipases have been determined. The first 3-D structure of a bacterial lipase, from *Burkholderia glumae*, was resolved in 1993 [35]. Since then, the structures of several other lipases have been published [3]. A comparison of the known lipase structures reveals a common fold structure, known as the α/β hydrolase fold, and the lipases are therefore classified as α/β hydrolases [42]. The α/β fold contains the conserved amino acid residues that form the active site of the lipases.

3.5.1. The catalytic triad

The active site of lipases consists of three amino acid residues, Ser-Asp(Glu)-His, which form the catalytic triad. These amino acids always appear in this order in the amino acid sequence of the lipases, but they are distantly located from each other. In the tertiary structure, however, they are arranged near each other and constitute the active site. The nucleophilic serine is located in a highly conserved sequence motif that consists of the pentapeptide Gly–X–Ser–X–Gly [42]. The catalytic mechanism involves a nucleophilic attack of the serine hydroxyl group on the carbonyl carbon of the lipid ester bond and a proton transfer from the hydroxyl group to the ester oxygen of the substrate. This results in breakage of the ester bond and formation of an intermediate fatty acid ester with the nucleophilic serine. In a second step, the transient ester linkage is attacked by a water molecule, and the fatty acid and the regenerated catalytic triad are released [3].

By comparison of staphylococcal lipase sequences, it became obvious that they have conserved serine, aspartate, and histidine residues at positions corresponding to those of the catalytic triad of other lipases. In addition, the conserved pentapeptide motif that surrounds the nucleophilic serine in other lipases is also highly conserved in the staphylococcal sequences. Therefore, despite the lack of structural data for staphylococcal lipases, it is tempting to speculate that these conserved amino acids are involved in lipolysis. This hypothesis is supported by results of site-directed mutagenesis of the putative catalytic triad of SHyL [43]. The candidates for the amino acids involved in lipolysis, as revealed by sequence similarity, are Ser369, Asp559, and His600 of the SHyL sequence. Site-directed mutagenesis of any of these amino acids results either in a drastically reduced lipase activity or in a complete loss of lipolysis. Since secretion or substrate specificity of SHyL is not hampered by the mutations, these amino acids are directly involved in catalysis [43]. This supports the hypothesis that SHyL is a serine hydrolase with the catalytic triad comprised of Ser369-His600-Asp559. The corresponding amino acids are conserved in all known staphylococcal lipases (see *figure 2*). Therefore, it is conceivable that the results obtained with SHyL are applicable to the lipases from other staphylococci.

3.5.2. Interfacial activation

One of the basic criteria for the classification of lipases is a significant increase in activity when the substrate concentration reaches the solubility limit. At this point, neutral lipids such as tributyrin form emulsified droplets, whereas polar phospholipids such as lecithin aggregate in micelles when the critical micellar concentration (cmc) is passed. It was recognized early that true lipases are dependent on the lipid-water interface in substrate emulsions to obtain their full activity, a phenomenon which has been described as interfacial activation [1]. With tributyrin as a substrate, SHyL exhibits a sharp increase in activity when the solubility limit is exceeded. In contrast, with phospholipids such as lecithin, no corresponding rise in activity is observed [37]. However, as discussed by the authors, this does not completely rule out the possibility that SHyL is activated by micelles of phospholipids. The phospholipase of *Naja melanoleuce* lacks interfacial activation, which can be explained by the formation of lipid-protein aggregates via a co-micellization pathway at concentrations below the cmc [37, 44]. In contrast to SHyL, SAL-1 does not exhibit any interfacial activation with neutral lipids [45].

The structural basis for the activation of lipolytic activity at the lipid-water interface was elucidated when 3-D structures of lipases became available. Most of the bacterial lipases have a lid-like structure covering the active site [3]. In the absence of a water-substrate interface, this lid covers the active site completely. When the substrate is present in emulsified droplets or micelles, a structural change is initialized that causes the lid to open and a hydrophobic surface with the active site is presented. This hydrophobic surface presumably interacts with the water-lipid interface. The opening of the lid was observed in the 3-D structures of the lipase from *Burkholderia glumae*, which was analyzed in the closed configuration, and from *Burkholderia cepacia*, which was crystallized in the open form [35, 46–48].

As mentioned above, no 3-D structures are yet available for staphylococcal lipases, and therefore the evaluation of structure-function relationships is severely hampered. The first step towards the structural characterization of staphylococcal lipases was made by Simons et al. [49] who made an extensive sequence comparison of various staphylococcal lipases with those of *B. glumae* based on the assumption that lipases of staphylococci belong to the class of α/β-hydrolases and therefore have a 'core' similar to those lipases for which a 3-D structure is already known. The sequences of SHyL, SAL-1, SAL-2, and SEL-3 were aligned with the sequence of the *B. glumae* lipase, and the alignment was optimized manually taking the positions of (predicted) secondary structures into consideration. Putative hinge regions in the SHyL sequence as well as a conserved tryptophan residue, known to be also present in the lid regions of lipases with evaluated 3-D structures, were localized [49]. Experi-

ments with covalent inhibitors that inhibit SHyL only in the presence of micelles and results obtained with the substrate analogue *p*-nitrophenyl *N*-alkylcarbamate further support the hypothesis that SHyL has a lid covering its active site [45, 50]. It should be noted, however, that not all bacterial lipases reveal a lid in their 3-D structure [3]. Furthermore, not all lipases with lids show interfacial activation. It is thus conceivable that the model assuming only two configurations of lipases, open and closed, is far too simple to explain the lack of an absolute correlation between the presence of lid-like structures in lipases and their interfacial activation [49].

4. Further topics of research on staphylococcal lipases

Although our knowledge of staphylococcal lipases has steadily increased during the past years, many aspects of these interesting enzymes remain to be investigated. Besides a further need for biochemical studies, especially on the most recently discovered lipases, there is also a necessity for data on the regulation of lipase synthesis and the involvement of lipases in staphylococcal pathogenesis. To date, the substrate specificities and enzymological features have been studied only with SHyL, SAL-1, and SEL-3. The biochemical characterization of other staphylococcal lipases, such as SWL and SHaL, is only at a preliminary stage [13, 21], and because of different substrates, preparations of substrates, or methods for measuring the enzyme activity used in the different laboratories, the results are not undoubtedly comparable to those obtained with SHyL, SAL-1, and SEL-3. This problem has also been reported for other bacterial lipases [3]. Standardized methods for the determination of the enzyme properties of all staphylococcal lipases are greatly needed to allow a true comparison of the biochemical features of the staphylococcal lipases.

Very little is known about the regulation of lipase synthesis in staphylococci. For some of the staphylococcal lipases, a maximum level of synthesis during the stationary growth phase has been reported [5, 21]. Therefore, the staphylococcal lipases are likely to be regulated by the global regulatory system *agr* (accessory gene regulator), which is known to regulate the expression of the genes of several exoproteins and cell-wall-associated proteins in staphylococci [51–53]. Furthermore, another global regulator of gene expression, the alternative sigma factor σ^B, seems to be involved in lipase gene expression [54]. It should be noted that only the influence of global regulatory systems on lipase synthesis has been reported. No studies have yet been undertaken to elucidate regulation of lipase gene expression by substrate molecules or products of lipolysis that might also been involved in the fine regulation of expression.

Staphylococcal lipases could be potentially applied in biotechnology, but it is first extremely important to know how these exoenzymes are translocated across the cytoplasmic membrane and which factors influence the secretion process. The secretion of only SHyL has been investigated in some more detail. The pro-peptide of SHyL is important for an efficient translocation of the lipase, and it can also be used for the secretion of heterologous proteins, such as β-lactamase, OmpA, or pro-insulin [23, 24, 27]. Since the pro-peptides of the staphylococcal lipases differ greatly from each other, it will be necessary to evaluate whether all pro-peptides play a role in translocation comparable to that of the SHyL pro-region. Furthermore, it is conceivable that the conserved SIRK-motif in the signal peptides of the staphylococcal lipases has a function in the secretion process.

Most of the known staphylococcal lipases are produced by pathogenic members of the genus, i.e., *S. aureus* and *S. epidermidis*. While it is possible that lipases might support the persistence of these strains in the fatty secretions in mammalian skin and thus have an indirect influence on their pathogenic potential, a direct involvement of lipases in pathogenesis remains to be demonstrated. *S. aureus* causes a variety of infectious diseases such as furuncles, abscesses, pneumonia, osteomyelitis, and several forms of carditis and meningitis [55]. *S. aureus* produces lipase in infected patients [56]. Furthermore, lipase interferes with the phagocytosis of the infectious lipase-producing *S. aureus* cells by host granulocytes [57], thus indicating a direct involvement of lipase in pathogenesis. *S. epidermidis* can cause infections by forming biofilms on implants made of synthetic material. The formation of biofilms is thought to occur in two steps: a primary attachment to the polymer surface, followed by intercellular adhesion of the *S. epidermidis* cells, leading to multilayer cell clusters [58]. The intercellular adhesion is dependent on the biosynthesis of a long-chain polysaccharide, the polysaccharide intercellular adhesin (PIA) [59]. The genes involved in PIA biosynthesis (*ica* genes) are organized in an operon. *ica* genes have been identified in *S. epidermidis* RP62A and *S. aureus* ATCC 35556 [58, 60, 61]. Interestingly, lipase genes are encoded in the vicinity of the *ica* operons, but a role for lipases in biofilm formation and hence in pathogenesis has yet to be determined.

References

[1] Sarda L., Desnuelle P., Action de la lipase pancréatique sur les esters en émulsion, Biochim. Biophys. Acta 30 (1958) 513–521.

[2] Jaeger K.E., Ransac S., Dijkstra B.W., Colson C., van Heuvel M., Misset O., Bacterial lipases, FEMS Microbiol. Rev. 15 (1994) 29–63.

[3] Jaeger K.E., Dijkstra B.W., Reetz M.T., Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases, Annu. Rev. Microbiol. 53 (1999) 315–351.

[4] Arpigny J.L., Jaeger K.E., Bacterial lipolytic enzymes: classification and properties, Biochem. J. 343 (1999) 177–183.

[5] Farrell A.M., Foster T.J., Holland K.T., Molecular analysis and expression of the lipase of *Staphylococcus epidermidis*, J. Gen. Microbiol. 139 (1993) 267–277.
[6] Longshaw C.M., Farrell A.M., Holland K.T., Identification of a second lipase gene, *geh*D, in *Staphylococcus epidermidis*: comparison of sequence with those of other staphylococcal lipases, Microbiology 146 (2000) 1419–1427.
[7] Götz F., Popp F., Korn E., Schleifer K.H., Complete nucleotide sequence of the lipase gene from *Staphylococcus hyicus* cloned in *Staphylococcus carnosus*, Nucleic Acids Res. 13 (1985) 5895–5906.
[8] Lee C.Y., Iandolo J.J., Lysogenic conversion of staphylococcal lipase is caused by insertion of the bacteriophage L54a genome into the lipase structural gene, J. Bacteriol. 166 (1986) 385–391.
[9] Nikoleit K., Rosenstein R., Verheij H.M., Götz F., Comparative biochemical and molecular analysis of the *Staphylococcus hyicus*, *Staphylococcus aureus* and a hybrid lipase. Indication for a C-terminal phospholipase domain, Eur. J. Biochem. 228 (1995) 732–738.
[10] Rosenstein R., Götz F., GenBank entry, accession no. AF208229, 1999.
[11] Rosenstein R., Götz F., GenBank entry, accession no. AF208033, 1999.
[12] Simons J.W., van Kampen M.D., Riel S., Götz F., Egmond M.R., Verheij H.M., Cloning, purification and characterisation of the lipase from *Staphylococcus epidermidis* – comparison of the substrate selectivity with those of other microbial lipases, Eur. J. Biochem. 253 (1998) 675–683.
[13] Oh B., Kim H., Lee J., Kang S., Oh T., *Staphylococcus haemolyticus* lipase: biochemical properties, substrate specificity and gene cloning, FEMS Microbiol. Lett. 179 (1999) 385–392.
[14] Wenzig E., Lottspeich F., Verheij B., De Haas G.H., Götz F., Extracellular processing of the *Staphylococcus hyicus* lipase, Biochem. Life Sci. Adv. 9 (1990) 47–56.
[15] Götz F., Verheij H.M., Rosenstein R., Staphylococcal lipases: molecular characterisation, secretion, and processing, Chem. Phys. Lipids 93 (1998) 15–25.
[16] Saraste M., Sibbald P.R., Wittinghofer A., The P-loop – a common motif in ATP- and GTP-binding proteins, Trends Biochem. Sci. 15 (1990) 430–434.
[17] Nikoleit K., Biochemische und molekularbiologische Charakterisierung zweier (Phospho-) Lipasen aus Staphylokokken und einer Esterase aus *Streptomyces diastatochromogenes*, PhD thesis, Eberhard-Karls-Universität, Tübingen, 1995.
[18] Ayora S., Götz F., Genetic and biochemical properties of an extracellular neutral metalloprotease from *Staphylococcus hyicus*, Mol. Gen. Genet. 242 (1994) 421–430.
[19] Ayora S., Lindgren P.E., Götz F., Biochemical properties of a novel metalloprotease from *Staphylococcus hyicus* subsp. *hyicus* involved in extracellular lipase processing, J. Bacteriol. 176 (1994) 3218–3223.
[20] Rollof J., Normark S., In vivo processing of *Staphylococcus aureus* lipase, J. Bacteriol. 174 (1992) 1844–1847.
[21] Talon R., Dublet N., Montel M.C., Cantonnet M., Purification and characterization of extracellular *Staphylococcus warneri* lipase, Curr. Microbiol. 30 (1995) 11–16.
[22] Hu Z., Zhu X., Jordan F., Inouye M., A covalently trapped folding intermediate of subtilisin E: spontaneous dimerization of a prosubtilisin E Ser49Cys mutant in vivo and its autoprocessing in vitro, Biochemistry 33 (1994) 562–569.
[23] Liebl W., Götz F., Studies on lipase directed export of *Escherichia coli* β-lactamase in *Staphylococcus carnosus*, Mol. Gen. Genet. 204 (1986) 166–173.
[24] Knorr R., Produktion von rekombinantem Proinsulin in einem *Staphylococcus carnosus* Sekretionssystem, PhD thesis, Eberhard-Karls-Universität, Tübingen, 1992.
[25] Samuelson P., Hansson M., Ahlborg N., Andreoni C., Götz F., Bachi T., Nguyen T.N., Binz H., Uhlen M., Stahl S., Cell surface display of recombinant proteins on *Staphylococcus carnosus*, J. Bacteriol. 177 (1995) 1470–1476.
[26] Demleitner G., Götz F., Evidence for importance of the *Staphylococcus hyicus* lipase pro-peptide in lipase secretion, stability and activity, FEMS Microbiol. Lett. 121 (1994) 189–197.
[27] Meens J., Herbort M., Klein M., Freudl R., Use of the pre-pro part of *Staphylococcus hyicus* lipase as a carrier for secretion of *Escherichia coli* outer membrane protein A (OmpA) prevents proteolytic degradation of OmpA by cell-associated protease(s) in two different gram-positive bacteria, Appl. Environ. Microbiol. 63 (1997) 2814–2820.
[28] Eijkman C., Über Enzyme bei Bakterien und Schimmelpilzen, Zbl. Bakt. Parasitenk. Infektionskr. 29 (1901) 841–848.
[29] Weld J.T., Kean B.H., O'Leary W.M., Production of octadecenoic acid in plasma by *Staphylococcus aureus*, Proc. Soc. Exp. Biol. Med. 112 (1963) 448–451.
[30] Brune K.A., Götz F., Degradation of lipids by bacterial lipases, in: Winkelmann G. (Ed.), Microbial degradation of natural products, VCH, Weinheim, 1992, pp. 243–266.
[31] Rollof J., Hedstrom S.A., Nilsson-Ehle P., Positional specificity and substrate preference of purified *Staphylococcus aureus* lipase, Biochim. Biophys. Acta 921 (1987) 370–377.
[32] Simons J.W., Adams H., Cox R.C., Dekker N., Götz F., Slotboom A.J., Verheij H.M., The lipase from *Staphylococcus aureus*. Expression in *Escherichia coli*, large-scale purification and comparison of substrate specificity to *Staphylococcus hyicus* lipase, Eur. J. Biochem. 242 (1996) 760–769.
[33] Shah D.B., Wilson J.B., Egg yolk factor of *Staphylococcus aureus*, J. Bacteriol. 89 (1965) 949–953.
[34] Verheij H.M., Volwerk J.J., Jansen E.H.J.M., Pujik W.C., Dijkstra B.W., Drenth J., de Haas G.H., Methylation of histidine-48 in pancreatic phospholipase A2. Role of histidine and calcium ion in the catalytic mechanism, Biochemistry 19 (1980) 743–750.
[35] Noble M.E.M., Cleasby A., Johnson L.N., Egmond M.R., Frenken L.G.J., The crystal structure of triacylglycerol lipase from *Pseudomonas glumae* reveals a partially redundant catalytic aspartate, FEBS Lett. 331 (1993) 123–128.
[36] Simons J.W., van Kampen M.D., Ubarretxena-Belandia I., Cox R.C., Alves dos Santos C.M., Egmond M.R., Verheij H.M., Identification of a calcium binding site in *Staphylococcus hyicus* lipase: generation of calcium-independent variants, Biochemistry 38 (1999) 2–10.
[37] van Oort M.G., Deveer A.M., Dijkman R., Tjeenk M.L., Verheij H.M., de Haas G.H., Wenzig E., Götz F., Purification and substrate specificity of *Staphylococcus hyicus* lipase, Biochemistry 28 (1989) 9278–9285.
[38] van Kampen M.D., Simons J.W., Dekker N., Egmond M.R., Verheij H.M., The phospholipase activity of *Staphylococcus hyicus* lipase strongly depends on a single Ser to Val mutation, Chem. Phys. Lipids 93 (1998) 39–45.
[39] van Kampen M.D., Dekker N., Egmond M.R., Verheij H.M., Substrate specificity of *Staphylococcus hyicus* lipase and *Staphylococcus aureus* lipase as studied by in vivo chimeragenesis, Biochemistry 37 (1998) 3459–3466.
[40] van Kampen M.D., Verheij H.M., Egmond M.R., Modifying the substrate specificity of staphylococcal lipases, Biochemistry 38 (1999) 9524–9532.
[41] Ransac S., Blaauw M., Dijkstra B.W., Slotboom A.T., Boots J.W., Verheij H.M., Crystallization and preliminary X-ray analysis of a lipase from *Staphylococcus hyicus*, J. Struct. Biol. 114 (1995) 153–155.
[42] Ollis D.L., Cheah E., Cygler M., Dijkstra B., Frolow F., Franken S.M., Harel M., Remington S.J., Silman I., Schrag J., The alpha/beta hydrolase fold, Protein Eng. 5 (1992) 197–211.
[43] Jäger S., Demleitner G., Götz F., Lipase of *Staphylococcus hyicus*: analysis of the catalytic triad by site-directed mutagenesis, FEMS Microbiol. Lett. 79 (1992) 249–254.
[44] Van Eijk J., Verheij H.M., Dijkman R., de Haas G.H., Interaction of phospholipase A2 from *Naja melanoleuca* snake venom with monomeric substrate analogs. Activation of the enzyme by protein- protein or lipid-protein interactions? Eur. J. Biochem. 132 (1983) 183–188.

[45] Simons J.W., Boots J.W., Kats M.P., Slotboom A.J., Egmond M.R., Verheij H.M., Dissecting the catalytic mechanism of staphylococcal lipases using carbamate substrates: chain length selectivity, interfacial activation, and cofactor dependence, Biochemistry 36 (1997) 14539–14550.

[46] Schrag J.D., Li Y., Cygler M., Lang D., Burgdorf T., Hecht H.J., Schmid R.D., Schomburg D., Rydel T., Oliver J., Strickland L., Dunaway M., Larson S., Day J., McPherson A., The open conformation of a *Pseudomonas* lipase, Structure 5 (1997) 187–202.

[47] Kim K.K., Song H.K., Shin D.H., Hwang K.Y., Suh S.W., The crystal structure of a triacylglycerol lipase from *Pseudomonas cepacia* reveals a highly open conformation in the absence of a bound inhibitor, Structure 5 (1997) 173–185.

[48] Noble M.E.M., Cleasby A., Johnson L.N., Egmond M.R., Frenken L.G.J., Analysis of the structure of *Pseudomonas glumae* lipase, Prot. Eng. 7 (1994) 559–562.

[49] Simons J.W., Götz F., Egmond M.R., Verheij H.M., Biochemical properties of staphylococcal (phospho)lipases, Chem. Phys. Lipids 93 (1998) 27–37.

[50] Leuveling Tjeenk M., Bulsink Y.B., Slotboom A.J., Verheij H.M., de Haas G.H., Demleitner G., Götz F., Inactivation of *Staphylococcus hyicus* lipase by hexadecylsulfonyl fluoride: evidence for an active site serine, Protein Eng. 7 (1994) 579–583.

[51] Janzon L., Arvidson S., The role of the δ-lysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*, EMBO J. 9 (1990) 1391–1399.

[52] Peng H.L., Novick R.P., Kreiswirth B., Kornblum J., Schlievert P., Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*, J. Bacteriol. 170 (1988) 4365–4372.

[53] Recsei P., Kreiswirth B., O'Reilly M., Schlievert P., Gruss A., Novick R.P., Regulation of exoprotein gene expression in *Staphylococcus aureus* by agar, Mol. Gen. Genet. 202 (1986) 58–61.

[54] Kullik I., Giachino P., Fuchs T., Deletion of the alternative sigma factor σ^B in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes, J. Bacteriol. 180 (1998) 4814–4820.

[55] Kloos W.E., Schleifer K., -H., Götz F., The genus *Staphylococcus*, in: Balows A., Trüper H.G., Dworkin M., Harder W., Schleifer K.H. (Eds.), The prokaryotes, Springer-Verlag, New York, 1991, pp. 1369–1420.

[56] Christensson B., Fehrenbach F.J., Hedstrom S.A., A new serological assay for *Staphylococcus aureus* infections: detection of IgG antibodies to *S. aureus* lipase with an enzyme-linked immunosorbent assay, J. Infect. Dis. 152 (1985) 286–292.

[57] Rollof J., Braconier J.H., Soderstrom C., Nilsson-Ehle P., Interference of *Staphylococcus aureus* lipase with human granulocyte function, Eur. J. Clin. Microbiol. Infect. Dis. 7 (1988) 505–510.

[58] Heilmann C., Schweitzer O., Gerke C., Vanittanakom N., Mack D., Götz F., Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*, Mol. Microbiol. 20 (1996) 1083–1091.

[59] Mack D., Fischer W., Krokotsch A., Leopold K., Hartmann R., Egge H., Laufs R., The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear β-1,6-linked glucosaminoglycan: purification and structural analysis, J. Bacteriol. 179 (1996) 175–183.

[60] Cramton S.E., Gerke C., Schnell N.F., Nichols W.W., Götz F., The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation, Infect. Immun. 67 (1999) 5427–5433.

[61] Tatusova T.A., Madden T.L., BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences, FEMS Microbiol. Lett. 174 (1999) 247–250 [erratum: FEMS Microbiol. Lett. 177 (1999) 187–188].