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Staphylococcal (phospho)lipases promote biofilm formation and host cell invasion

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ABSTRACT

Most *Staphylococcus aureus* strains secrete two lipases SAL1 and SAL2 encoded by *gehA* and *gehB*. These two lipases differ with respect to their substrate specificity. *Staphylococcus hyicus* secretes another lipase, SHL, which is in contrast to *S. aureus* lipases Ca^{2+}-dependent and has a broad-spectrum lipase and phospholipase activity. The aim of this study was to investigate the role of staphylococcal (phospho) lipases in virulence. For this we constructed a *geh*A-*geh*B double deletion mutant in *S. aureus* USA300 and expressed SHL in agr-positive (accessory gene regulator) and agr-negative *S. aureus* strains. The lipases themselves have no hemolytic or cytotoxic activity. However, in agr-negative strains SHL-expression caused an upregulation of hemolytic activity. We further show that SHL-expression significantly enhanced biofilm formation probably due to an increase of extracellular DNA release. SHL-expression also increased host cell invasion 4–6-fold. Trioleate, a main triacylglycerol component of mammalian skin, induced lipase production. Finally, in the mouse sepsis and skin colonization models the lipase producing and mutant strain showed no significant difference compared to the WT strain. In conclusion, we show that staphylococcal lipases promote biofilm formation and host cell invasion and thereby contribute to *S. aureus* virulence.

1. Introduction

Since the first lipolytic activity has been described in staphylococci in 1901 by Eijkmann, various staphylococcal lipase were purified and their biochemical properties investigated (Götz et al., 1998). Lipases from various staphylococcal species were described such as from *S. epidermidis* (Bowden et al., 2002; Götz et al., 1998; Xie et al., 2012), *S. hyicus* (Götz et al., 1985; Leuveling Tjeenk et al., 1994; Simons et al., 1998; Tiesinga et al., 2007; van Oort et al., 1989), *S. saprophyticus* (Sakinc et al., 2005), *S. simulans* (Sayari et al., 2001), *S. warneri* (van Kampen et al., 2001; Yokoi et al., 2012), or *S. xylosus* (Mosbah et al., 2005).

The lipase precursor structure of all so far investigated staphylococcal lipases is always the same: they are organized as pre-pro-proteins, with pre-regions corresponding to a signal peptide of 35–38 amino acids, a pro-peptide of 207–321 amino acids with an overall hydrophilic character, and a mature peptide comprising 383–396 amino acids (Rosenstein and Götz, 2000). Normally, the lipases are secreted in the pro-form, which is subsequently processed by an extracellular protease (Götz et al., 1998; Wenzig et al., 1990). In *S. aureus* the processing enzyme is the metalloprotease aureolysin (Cadieux et al., 2014). There is evidence that the lipase pro-peptide contributes to secretion, stability and activity and is rather acting as an intramolecular chaperone (Demleitner and Götz, 1994).

The best biochemical characterized lipases are those from *S. hyicus* and *S. aureus*. The *S. hyicus* lipase, named SHL, was the first lipase that has been cloned and sequenced (Götz et al., 1985). This lipase, whose activity is Ca^{2+}-dependent, should be regarded as a phospho-lipase as its activity with phospholipids was higher than with triglycerides (van Oort et al., 1989). Triglycerides were fully hydrolyzed to free fatty acid

Abbreviations: agr, accessory gene regulator; CNS, coagulase negative staphylococci; e-DNA, extracellular DNA; *geh*A, gene encoding SAL1 lipase; *geh*B, gene encoding SAL2 lipase; Hla, alpha hemolysin; PMN, human polymorphonuclear leukocytes; *S*, *Staphylococcus*; shl, gene from *S. hyicus* encoding SHL phospho-lipase; TAGs, triacyl glycerides; TriO, glyceryl trioleate

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and glycerol and in phosphatidylcholines and lysophospholipids fatty acids were also completely hydrolyzed. Thus SHL is unique among staphylococcal lipases as it has both lipase and an even higher phospholipase A1 and lysophospholipase A activity. Structural analysis of the mature SHL showed that the substrate-binding cavity contains two large hydrophobic acyl chain-binding pockets and a shallow and more polar third pocket that is capable of binding either a (short) fatty acid or a phospholipid head-group, explaining the broad substrate specificity (Tiesinga et al., 2007).

Most *S. aureus* strains contain two lipase genes. The first analyzed *S. aureus* lipase gene (*geh*) encodes a 682 aa lipase (Nikoleit et al., 1995); according to the new nomenclature it is named SAL1 encoded by *gehA*. The activity of SAL1 is Ca^{2+}-independent and primarily reacts with short-chain glycerides such as tributyrylglycerol or *p*-nitrophenyl octanoate and does hydrolyze Tween 20 or 80 (Nikoleit et al., 1995). The second lipase in *S. aureus* is SAL2 encoded by *gehB*. SAL2 hydrolyzes both short- and long-chain triglycerides (Cadieux et al., 2014). Both SAL1 and SAL2 show a high degree of similarity particularly in the mature lipase part and are organized as pre-pro-enzymes (Götz et al., 1998).

There are only few reports as to the possible contribution of lipases in virulence. It has been described that pretreatment of human polymorphonuclear leukocytes (PMN) with only 1 nM purified *S. aureus* lipase, strongly enhanced chemotactic response toward casein, inhibits migration of PMN and the lipase bound the leukocyte surface (Tyski et al., 1987). It has also been described that low concentrations of lipase were strongly chemotactic while higher concentrations of lipase (12 µg/ml) granulocytes were immobilized and phagocytic killing of *S. aureus* was almost abolished because of decreased bacterial uptake (Rollof et al., 1988). The lipase interfered with the membrane functions, as there were marked changes at the granulocyte surface structure observed which lacked normal microvilli (Rollof et al., 1988). It was also reported that 30% of patients with *S. aureus* endocarditis or complicated septicemia had lipase antibodies, while patients with uncomplicated *S. aureus* septicemia were negative (Ryding et al., 1992). Coagulase negative staphylococci (CNS) are common inhabitants of human skin and mucous membranes, but sometimes they can cause severe infections, particularly *S. epidermidis*, which is frequently associated with eye infections. In a study where CNS were isolated from ocular specimens all isolates expressed lipolytic activity suggesting a role in virulence (Priya et al., 2014). Investigations have also been carried out with a lipase deletion mutant in *S. aureus* RN4220 (Hu et al., 2012). This mutant showed a lower biofilm formation and abscess formation and bacterial load in various organs in a mouse model was decreased; furthermore, active immunization with lipase protected mice against a lethal challenge with *S. aureus* (Hu et al., 2012).

To better understand the role of lipases in virulence we focused on the *S. aureus* lipases SAL1 and SAL2 and the *S. hyicus* phospho-lipase SHL. To evaluate the effect of lipases on virulence, we created a *gehA* and *gehB* double mutant in *S. aureus* USA300 and for evaluating the effect of SHL we expressed the corresponding gene in various *S. aureus* strains. Here we show that the lipases play rather a subtle than aggressive role in pathogenesis by enhancing biofilm formation and host cell invasion.

2. Materials and methods

2.1. Bacterial strains and growth

List of bacteria strains and plasmids used in this study are listed in Table 1. Staphylococcal strains were aerobically grown in Tryptic Soy Broth (TSB) liquid medium at 37 °C with the supplement of chloramphenicol (20 µg/ml) if necessary. For feeding with glyceryl trioleate (TriO) (Sigma, Germany) the staphylococcal strains were first pre-cultivated aerobically in TSB at 37 °C for 8 h; this culture was used to inoculate fresh TSB (OD_{578nm} 0.1) supplied with 100 µM TriO from 0.1 M stock solution. The samples were harvested after 16 h cultivation at 37 °C under constant shaking at 150 rpm.

2.2. Construct of lipase mutants in SA113

For the deletion of *geh*A and *geh*B in SA113 a knockout plasmid pBT2 containing the upstream and downstream flanking regions of *geh*A and *geh*B were constructed. The flanking regions were amplified by PCR from the genome of SA113. To construct pBT2-*geh*A:*erm* plasmid, the upstream region was amplified by using primer pairs F*geh*A-up (BamHI) and R*geh*A-up (HindIII) and the downstream region using primer pairs F*geh*A-down (*Bgl*II) and R*gehA*-down (EcoRI). To construct pBT2-*geh*B:*spc* plasmid, the upstream region was amplified by using primer pairs F*geh*B-up (*Bgl*II) and R*geh*B-up (EcoRI) and the downstream region using primer pairs F*geh*B-down (Eco47III) and R*geh*B-down (ScaI). These fragments were cut by specific restriction enzymes mentioned in the primers, which were listed in Table S1. These plasmids were transformed into *E. coli* DH5α and subsequently transformed by electroporation into RN4220 and then SA113. The deletion of *geh*A and *geh*B was followed as described previously (Brückner, 1997). The positive clones were confirmed by PCR using primer pairs (F1-*geh*A and R1-*geh*A) for SA113 Δ*geh*A:*erm* and primer pairs (F1-*geh*B and R1-*geh*B) for SA113 Δ*geh*B:*spc* and sequencing.

2.3. Phage transduction

The deletion mutant of *geh*A (SAUSA300_2603) and *geh*B (SAUSA300_0320) genes in *S. aureus* USA300 JE2 was carried out by phage transduction using ø11 with *S. aureus* 113 Δ*geh*A:*erm*B and *S. aureus* 113 Δ*geh*B:*spc* as donor strains. *S. aureus* 113 Δ*geh*A:*erm*B and *S. aureus* Δ*geh*B:*spc* were resuspended in 500 µl phage buffer (100 mM $MgSO_4$, 100 mM $CaCl_2$, 1 M Tris/HCl pH 7.8, 100 mM NaCl, 0.1% (w/v) gelatin) and 50 µl of phage ø11. Pre-warmed phage-Top-Agar (TSB medium containing 0.5% (w/v) agar) was added into the suspension and poured on the 100 mM $CaCl_2$ containing agar plate. The plates were then incubated at 37 °C for 5–6 h. The upper part of the agar plate was vortex-mixed for 5 min and incubated at 50 °C for 10 min. The supernatant was obtained by centrifugation (5000 x g) for 10 min at 4 °C. The filtered supernatant containing phage lysate was stored at 4 °C. An overnight colony of S. *aureus* JE2 on TSB agar plate was suspended in 200 µl phage buffer. 100 µl phage lysate was added to the suspension and incubated at 37 °C for 10 min. The suspension was then mixed with phage-top-agar and poured on the corresponding antibiotic agar plate (10 µg/ml for erythromycin and 450 µg/ml for spectinomycin). The plates were incubated at 37 °C for 1–2 days and the grown colonies were picked and checked by PCR using the primer pairs (F1-*geh*A and R1-*geh*A) for the *geh*A deletion and the primer pairs (F1-*geh*B and R1-*geh*B) for the *geh*B deletion. The primer sequences are listed in Table S1.

Construction of pCtuf-*gehAgehB*

To construct a complementation plasmid, *geh*A and *geh*B were inserted into plasmid pCtuf-amp by Gibson Assembly as described previously (Ebner et al., 2015). For the polymerase chain reaction (PCR), *geh*A and *geh*B were amplified from *S. aureus* USA300 using two primer pairs (F2-*geh*A and R2-*geh*A) and (F2-*geh*B and R2-*geh*B), respectively (Table S1). Linearized pCtuf-amp with *HindIII* and *PacI*, was assembled with PCR fragments of *gehA* and *gehB* by using HiFi DNA assembly mix (New England Biolabs) and transformed into *E. coli* DC10B. The resulting plasmid, pCtuf-*gehAgehB,* was verified by DNA sequencing and transformed into *S. aureus* JE2 Δ*gehA*Δ*gehB* via electroporation.

2.4. Agar diffusion assay

The TSB agar plates containing 1% Tween 20 were used to monitor lipase activity of different *S. aureus* strains and mutants. Lipase activity was visibly as a halo due to the precipitation of liberated fatty acids. This test was also applied to monitor lipase activity in the supernatants.