BACTERIAL PATHOGENICITY

Characterisation and expression of fatty acid modifying enzyme produced by *Staphylococcus epidermidis*

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The production of fatty-acid modifying enzyme (FAME) – first identified as a possible virulence factor in *Staphylococcus aureus* – has also been identified in *S. epidermidis*. This extracellular enzyme inactivates bactericidal fatty acids by esterifying them to cholesterol. FAME may provide protection for *S. epidermidis* by inactivating these lipids present on the skin. Over 88% of 51 randomly collected *S. epidermidis* isolates produced FAME; 92.2% and 13.7% of the same strains produced lipase and slime, respectively. There appeared to be no correlation of lipase activity or slime production with FAME production. The temperature optimum for FAME was between 20°C and 35°C, and the pH optimum was 6.0. Optimal enzyme activity was present at NaCl concentrations of between 250 and 500 mM. FAME was not detected in culture filtrates until early stationary phase, indicating some regulatory control over enzyme production.

Introduction

Staphylococcus epidermidis is a commensal organism that colonises the skin of man [1]. Very often hospitalised patients undergo invasive procedures that compromise the protective skin layer; consequently *S. epidermidis* grows on foreign bodies (e.g., catheters, prosthetic devices, etc.) inserted into the patient [2]. With the increased use of invasive techniques, the incidence of nosocomial *S. epidermidis* infections has increased dramatically. There are *c.* 0.7 coagulase-negative staphylococcal bacteraemias /1000 hospital discharges, with an average mortality rate of 30% [3].

The skin is a very difficult niche for bacteria to grow on; *S. epidermidis* has developed several mechanisms to survive and grow on human skin. Sebaceous glands in the skin secrete several factors that are bactericidal to *S. epidermidis* [4–7]; these factors include neutral lipids such as the long-chain fatty acids and mono-glycerides. Gram-positive organisms like *S. epidermidis* are very susceptible to killing by fatty acids [4].

Bactericidal lipids are also present in murine abscesses induced by *S. aureus* [5–7]; therefore, to survive in an abscess, the bacteria must be able to inactivate the

lipids. Fatty acid modifying enzyme (FAME) is an extracellular protein produced by *S. aureus* that inactivates the bactericidal activity of fatty acids by esterification of these lipids to cholesterol [8, 9]. FAME may also protect *S. epidermidis* from killing by bactericidal lipids so that it can colonise the skin.

A previous study demonstrated that four of nine *S. epidermidis* isolates examined produced FAME [10]. If this factor is important for the survival of *S. epidermidis* on the skin, significantly more isolates would be expected to produce FAME. The present study used a much larger sample size (51 isolates) and also examined any correlation between the production of FAME and the production of lipase and slime.

Materials and methods

Isolation and identification of S. epidermidis isolates

The bacterial isolates were obtained from the nasopharynx or axilla of laboratory personnel and from medical students at Kirksville College of Osteopathic Medicine. The names of persons from whom the samples were obtained were not recorded. All isolates were grown initially on Trypticase Soy Agar (TSA; Difco) for 16 h at 37°C. Single non-pigmented colonies were then transferred to Mannitol Salt Agar (MSA; Difco) and incubated for 48 h at 37°C. A mannitol-

negative colony was then grown on TSA. An isolated colony was stained by Gram's method and tested for catalase production. All gram-positive cocci that were mannitol-negative and catalase-positive were then speciated by the API STAPH system (bioMérieux Vitek Inc., Hazelwood, MO, USA). Two *S. epidermidis* strains that produced slime (ATCC 35983 and ATCC 35984) were purchased from the American Type Culture Collection (Rockville, MD, USA) and were used as controls for the API STAPH identification kit and to determine slime production. Isolates were incubated in Trypticase Soy Broth (TSB; Difco) at 37°C for 16 h and stored at -75°C after the addition of sterile glycerol 10% v/v.

Assay for slime and lipase production

One ml of each isolate in glycerol 10% was thawed and placed in 10 ml of TSB in sterile 15-ml polypropylene disposable centrifuge tubes (FisherBrand; Fisher Scientific, Pittsburg, PA, USA). The samples were rotated for 12 h at 37°C and then placed in a test-tube rack for 4 h at 37°C. The tubes were inverted and visually inspected for slime production. The slime-producing ATCC strains were used as positive controls.

Lipase production was determined qualitatively by incubating the isolates for 48 h at 37°C on Egg Yolk Enrichment Medium (Difco). Clearing of the medium around the bacterial colonies indicated that an isolate produced lipase.

Collection of culture filtrates and FAME assay

One ml of frozen *S. epidermidis* isolate no. 52 (FAME, lipase and slime positive) was added to 100 ml of TSB, pre-warmed to 37°C, and shaken (110 rpm) in a 250-ml Erhlenmeyer flask at 37°C for 16 h. One ml of this culture was then added to 100 ml of pre-warmed TSB and incubated as above. Culture filtrates were then obtained by passing the culture through a 0.2- μ m sterile filter (VacuCap 90 for large volumes, or Acrodisc for 1-ml volumes; Gelman Sciences, Ann Arbor, MI, USA). The culture filtrates were stored at -75°C in divided volumes until required. FAME assays were performed as described previously [11] except that 100- μ l samples of culture filtrate were used. Isolates were considered positive for FAME activity if the cpm of cholesterol ester/OD₆₀₀ was > 200.

Effect of proteinase K, NaCl concentration, temperature and pH on FAME activity

To determine protease sensitivity, 10 μ l of proteinase K (17 μ g) were added to 100 μ l of culture filtrate (60 μ g of protein) of *S. epidermidis* isolate no. 52; 10 μ l of TSB were added to 100 μ l of culture filtrate as a control. Samples were incubated at 37°C for 30 min before being assayed for FAME activity.

The effect of NaCl concentration on FAME activity was determined by dialysing 10 ml of culture filtrate in 10 000–12 000 mol. wt. cut-off tubing against 1 L of distilled H₂O (16 h, 4°C) to remove the salts. One-ml samples of the dialysed filtrate were brought to various NaCl concentrations (0 mM, 250 mM, 500 mM, 750 mM, 1 M, 1.25 M, 1.5 M, 1.75 M and 2.0 M) with solid NaCl. No detectable precipitation of protein occurred at any of the NaCl concentrations. Duplicate FAME assays were then performed at each NaCl concentration.

To determine the effect of temperature on FAME activity, culture filtrates were assayed in duplicate at temperatures ranging from 5°C to 60°C in 5°C increments. Culture filtrate was also assayed for FAME after boiling for 10 min.

To determine the pH optimum, culture filtrates were concentrated 10-fold with a Macrosep centrifugal concentrator (3000 mol. wt cut-off; Filtron, Northborough, MA, USA) and 15 μ l of concentrated culture filtrate were added to 85 μ l of the following buffers: 10 mM citrate at pH 4.0–5.5, 10 mM Hepes at pH 6.5–8.0 and 10 mM MES at pH 5.0–7.0. Duplicate FAME assays were performed at pH 4.0–7.0 at increments of 0.5.

FAME production during growth

S. epidermidis isolate no. 52 was incubated in TSB for 16 h at 37°C with agitation (110 rpm); 1 ml of the overnight culture was centrifuged (5 min, 12 000 g, 4°C) and the pellet was suspended in 1 ml of sterile TSB. This was repeated three times, after which 750 μ l of the washed cells were added to 100 ml of pre-warmed TSB (37°C) and the culture (OD₆₀₀ = 0.05 SD 0.005) was incubated at 37°C for 12 h with agitation (110 rpm). Samples (1 ml) were taken every hour for 12 h and OD₆₀₀ and FAME activity was determined.

Results

Identification and characterisation of S. epidermidis isolates

Fifty-one isolates from different individuals were identified as *S. epidermidis* and assayed for production of FAME, lipase and slime: 88.2% were positive for FAME, 92.2% were positive for lipase activity and 13.7% produced slime. All the slime-positive strains were lipase- and FAME-positive. Of the lipase-negative isolates, 7.8% were FAME-positive, while 11.8% of the lipase-positive isolates were FAME-negative.

Characterisation of FAME

Addition of proteinase K reduced FAME activity to background levels, whereas 9752.3 cpm of cholesterol

ester was recovered from the sample without the added protease.

A crude estimate of the protein mol. wt was obtained by placing culture filtrates in centrifugal microconcentrators with various mol.-wt cut-off filters. Assays of the retentates and filtrates revealed that the protein was between 30 000 and 50 000 in mol. wt (data not shown).

The temperature optimum for FAME activity was between 20°C and 35°C (Fig. 1). At 60°C there was little detectable FAME activity. Even though the assay was temperature sensitive, the enzyme itself was resistant to inactivation at high temperatures. Boiling a culture filtrate for 10 min did not affect FAME activity when compared to a non-boiled control (data not shown).

The pH optimum was between 5.5 and 6.5 (Fig. 2). A peak of FAME activity was observed at pH 6.0. Very little FAME activity was observed at pH 4.5 or 7.5 (Fig. 2).

Although FAME activity was still detectable in the absence of NaCl, a significant increase in activity was observed when NaCl was added (Fig. 3). Reductions in FAME activity did not occur until salt concentrations were > 500 mM. Salt concentrations > 500 mM resulted in a rapid decline in enzyme activity.

Production of FAME during the growth cycle

Detectable levels of FAME activity were not obtained until 3 h of growth (Fig. 4). Activity then increased

Fig. 1. The effect of temperature of FAME activity. FAME assays were performed in duplicate; bar, SD.

Fig. 2. The effect of pH on FAME activity. FAME assays were performed in duplicate; bar, SD.

Fig. 3. The effect of NaCl concentration on FAME activity. FAME assays were performed in duplicate; bar, SD.

steadily, peaking at the commencement of the stationary phase (5–6 h). Activity then decreased steadily during the stationary phase (6–12 h).

Discussion

This study characterised FAME produced by *S. epidermidis* and showed that enzyme production was not detectable in culture filtrates until the early logarithmic phase. A collection of *S. epidermidis*

Fig. 4. FAME activity during growth. ■, OD₆₀₀; ●, average cpm/average OD₆₀₀. Results are the average of three experiments; bar, SD.

isolates was assayed for FAME activity, lipase activity and slime production. Treatment of FAME-containing culture filtrate with proteinase K destroyed FAME activity, providing evidence that FAME is an extracellular protein with a mol. wt between 30 000 and 50 000. Previous work in our laboratory indicated that *S. aureus* FAME was between 50 000 and 100 000 mol. wt with similar mol. wt cut-off filters (unpublished data). Thus it appears that, whatever the native conformation, FAME expressed by *S. aureus* is larger than FAME expressed by *S. epidermidis*.

The previous study also demonstrated that *S. epidermidis* produced FAME; 44% of nine isolates investigated in that study produced the enzyme [10]. The current study used a larger sample (51) and demonstrated that 88.2% of *S. epidermidis* isolates produced FAME. If FAME is useful to the organism in establishing a niche on the skin a large number of isolates would be expected to produce this enzyme. Furthermore, 92.2% of isolates produced lipase and 13.2% produced slime. No correlation between lipase activity and FAME activity was observed, indicating that although the enzymic functions of the two proteins are similar, they are probably not expressed by the same genetic locus.

All slime-producing isolates also produced lipase and FAME; however, only seven of the isolates produced slime. More slime producers would have to be examined to determine the validity of this correlation. The prevalence of slime-producing isolates in this study was lower than in other studies of *S. epidermidis* from healthy individuals [12].

The temperature optimum, pH optimum and salt sensitivity for FAME activity were in keeping with the environment of the skin. The temperature range over which FAME was active was relatively wide

(20–37°C). This may be useful to the bacterium, as it dwells at or near room temperature on the skin and when causing infections is at or near 37°C. However, the enzyme appears to be very resistant to denaturing by elevated temperatures (100°C for 10 min). The optimum temperature range of FAME activity for *S. aureus* was not as wide and was *c.* 10°C higher than that for *S. epidermidis* [8]. *S. aureus* FAME activity was inactivated by boiling and was decreased by extended incubation at room temperature (unpublished data).

The pH optimum (Fig. 2) for *S. epidermidis* FAME was much more restricted than that seen with *S. aureus*. Enzyme activity produced by *S. epidermidis* was greatest at pH 6.0 with a significant reduction in activity at pH 5.5 and 6.5. *S. aureus* FAME activity, on the other hand, had an optimal pH range of 5.5–6.5 and was less sensitive to changes in pH [8].

The optimum salt concentration for *S. epidermidis* FAME activity was between 250 and 500 mM (Fig. 3), with higher salt concentrations resulting in less activity. Unlike *S. aureus*, which does not appear to require salt for maximal activity [8], *S. epidermidis* FAME requires salt for optimum activity. Therefore, these studies have identified several differences between FAME produced by *S. epidermidis* and *S. aureus*.

Determination of when FAME is produced during the growth of *S. epidermidis* revealed some interesting findings. First, no detectable amounts of FAME were present in the culture filtrates until early logarithmic phase (Fig. 4). FAME activity then increased rapidly, peaking after 5–6 h of growth and then decreasing. These results are very similar to those obtained by Farrell *et al.* [13] for lipase production by *S. epidermidis*. They found that lipase was not detectable until early stationary phase and that there was a peak of activity around 6 h of growth, followed by decreasing lipase activity. These findings are also very similar to those observed for FAME produced by *S. aureus* [11].

Other studies with *S. aureus* have demonstrated that several extracellular virulence factors (e.g., α -toxin, toxic shock syndrome toxin, lipase, β -toxin) are not produced until early stationary phase. However, the expression of some cell-associated factors (e.g., protein A, fibronectin-binding protein) is inhibited during early stationary phase. Mutation in one genetic locus could cause complete loss or significant reduction of the extracellular virulence factors and increased production of the cell-associated factors [14].

Later studies revealed that those factors were being regulated by a two-component global regulator called the accessory gene regulator (Agr) [14]. An RNA transcript is involved in increasing and decreasing the expression of the factors described above [15]. The transcript for δ toxin is on the 3' end of this RNA

molecule, and this has been used as an indirect measure of Agr activity [16]. If δ toxin is detectable, then the Agr regulon is active. Previous work in our laboratory determined that production of FAME by *S. aureus* was regulated by Agr [11]. Other investigators have shown that lipase expression is also regulated by Agr [14]. Recently an *agr*-like locus was identified and cloned from *S. epidermidis* [17]. The *agr*-like locus was similar (80–90% DNA homology) to the two-component (*agr* A and B) regulator *agr* locus in *S. aureus* [17]. If the *agr*-like locus in *S. epidermidis* controls lipase and FAME production, this could explain why these extracellular enzymes are not produced until early stationary phase. It is interesting to note that the δ toxin gene is also located at the 3' end of the proposed RNA III transcript in *S. epidermidis* [17, 18]. Therefore, the production of δ toxin by a *S. epidermidis* isolate may indicate that Agr regulates gene expression in *S. epidermidis* [18].

If δ toxin is a good indicator of Agr regulatory control of gene expression, then mutations in the *agr* locus of *S. epidermidis* may provide one explanation for the recovery of phenotypic variants. Deighton *et al.* [19] recovered two variants of *S. epidermidis* from a vegetation of an aortic heart valve. The δ -toxin-producing variant (A-205) was able to produce slime, lipase, lecithinase and protease and to adhere to plastic. However, the other variant (A-204) was unable to express δ toxin and did not produce slime, lecithinase or protease, nor was it able to adhere to plastic. Variant A-204 is very similar to the *agr* mutants obtained in *S. aureus* [14] in that several extracellular products are missing. Two very important phenotypes are missing in A-204; the ability to bind to plastic [2, 20, 21] and to produce slime [2, 3, 12, 22]. If Agr controls the expression of these extensively studied virulence factors, then further study of this particular global regulator is essential. Developing ways to shut down Agr may provide new and exciting means to control infections caused by *S. epidermidis*.

In future studies we hope to determine if the *agr*-like locus in *S. epidermidis* controls FAME production. With this enzyme as a marker, we hope to determine whether mutants of regulatory elements such as Agr also control the expression of polysaccharide adhesin [2, 20, 21] and slime [2, 3, 12, 22].

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