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MECHANISM OF RESISTANCE TO BACTERICIDAL FATTY ACIDS IN STAPHYLOCOCCUS AUREUS

The Ohio State University

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MECHANISM OF RESISTANCE TO BACTERICIDAL FATTY ACIDS
IN STAPHYLOCOCCUS AUREUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Joel E. Mortensen, B.S.

* * * * * *

The Ohio State University
1983

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FAME was made possible by the mass spectrometry of Dr. Brian Andresen of the National Reye's Syndrome Foundation and The Ohio State University Department of Pharmacology.
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FIELD OF STUDY

Host-parasite interaction
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
</tr>
<tr>
<td>VITA</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
</tr>
<tr>
<td>INTRODUCTION</td>
</tr>
</tbody>
</table>

## Chapter One: The Effect of Encapsulation to Staphylocidal Fatty Areas
- Introduction | Page  5
- Materials and Methods | Page  6
- Results | Page  11
- Discussion | Page  13

## Chapter Two: Modification of Bactericidal Fatty Acids by an Enzyme of *Staphylococcus aureus*
- Introduction | Page  20
- Materials and Methods | Page  22
- Results | Page  26
- Discussion | Page  30

# BIBLIOGRAPHY
Page  44
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Biochemical characteristics of Strain M and Strain M-AL</td>
<td>18</td>
</tr>
<tr>
<td>II</td>
<td>Relative sensitivity to bactericidal lipids</td>
<td>19</td>
</tr>
<tr>
<td>III</td>
<td>Esterification of oleic acid by FAME</td>
<td>41</td>
</tr>
<tr>
<td>IV</td>
<td>Molar ratio for 30 percent conversion</td>
<td>42</td>
</tr>
<tr>
<td>V</td>
<td>In vitro FAME production vs survivability in abscesses</td>
<td>43</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Composite of electron micrographs</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Mass spectra of the modified lipid</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>The amount of oleic acid converted to ethyl oleate</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>Esterification of oleic acid to ethyl oleate</td>
<td>38</td>
</tr>
<tr>
<td>5</td>
<td>The effect of pH on the FAME reaction</td>
<td>39</td>
</tr>
<tr>
<td>6</td>
<td>The effect of temperature on the FAME reaction</td>
<td>40</td>
</tr>
</tbody>
</table>
INTRODUCTION

Over 80% of the suppurative diseases encountered in medicine are caused by Staphylococcus aureus. Usually, these infections occur in the skin but S. aureus may also invade and produce severe infection in any part of the body. Although numerous antistaphylococcal antibiotics have been introduced during the past 35 years, the control of staphylococcal infections remains a major medical problem (Joklik, Willet and Amos, 1980).

The study of the complex interactions between the host and this organism has been hampered by the lack of an adequate model system. Humans and most other animals are usually resistant to an experimental subcutaneous challenge with up to $10^6$ cocci (Elek, 1956; Elek and Cohen, 1957). As a result, experimental models have employed large inocula (Kapral, 1974), incisions (Möller and Rydberg, 1969), or foreign material to establish a lesion (James and MacLeon, 1961; Noble, 1965; Taubler and Kapral, 1966; Isenberg et al., 1976). None of these conditions parallels the course of a natural infection involving a few organisms that multiply and cause disease.

Kapral and others developed a peritoneal abscess model that has been useful in studying several facets of the host parasite interactions of staphylococci in localized lesions. When $10^9$ non-encapsulated S. aureus were injected into the peritoneal cavity of mice,
the organisms were rapidly clumped due to the interaction of staphylococcal surface clumping factor and soluble fibrinogen present in the peritoneal fluid. Within 3 hours, leukocytes entered the peritoneal cavity and formed a thick layer around the clumped staphylococci (Kapral, 1966). The leukocytes that first contacted the clumped cocci were quickly destroyed, but the leukocytes at the periphery remained viable. During the next 2-3 days, a connective tissue capsule developed around the lesion (Kapral et al., 1980). Histologically, the abscess then consisted of a core of densely packed cocci, a zone of acellular debris and destroyed leukocytes, a region of intact leukocytes, and the vascularized capsule (Kapral et al., 1980).

While studying the fate of staphylococci within abscesses, 3 basic patterns of survival were noted. Some strains persisted for weeks without a significant loss of viability. Other strains were eliminated rapidly and continuously. The third group demonstrated a decline in population during the first 4 days, then increased to approximately original inocula levels. After 10 days, these strains were continuously eliminated from the abscesses (Dye and Kapral, 1981; Kapral et al., 1980).

The destruction of staphylococci within abscesses involved neither phagocytosis nor the intraleukocytic myeloperoxidase or cationic protein systems, but instead was dependent upon the production of a certain staphylocidal free fatty acid (SFA) (Dye and Kapral, 1980, 1981a; Kapral and Dye, 1981).
A study by Shryock and co-workers of the accumulation of lipids during abscess development was carried out with frozen sections stained with Oil Red O. It was found that there was virtually no lipid accumulation during the first 8 hours. However, after 12 hours, cells containing lipid droplets were noted at the periphery of the neutrophil layer. By 24 hours, a continuous band of lipid containing cells could be demonstrated at the periphery of the abscess. As the vascularized connective tissue capsule developed, lipid accumulation increased and many extracellular droplets could be visualized. In addition, at this time, many extracellular droplets became evident in the core of the abscess near the clumped staphylococci. After 7 days, the basic distribution of the lipid droplets remained unchanged, although the average size of the droplets did increase slightly for the next 4 weeks (Shryock, Dye and Kapral, submitted for publication). Results of the studies measuring the amount of SFA that was present within developing abscesses indicated no measurable activity before 12 hours of development. Thereafter, the amount of SFA increased rapidly until 7-10 days when peak levels were obtained, then declined slowly and continuously after day 10 (Shryock et al., submitted for publication).

Strains differed markedly in sensitivity to this fatty acid in vitro, but this sensitivity correlated with survival in vivo. Strains which were rapidly destroyed in abscesses were the most sensitive strains, whereas strains destroyed in abscesses only after a delay were of intermediate sensitivity. The third group, organisms capable of long term survival, were the most resistant to
SFA (Dye and Kapral, 1981b). This differential sensitivity of the three groups of organisms was an important characteristic used to distinguish SFA from other cidal fatty acids. When representative strains from the three groups were tested against the more common saturated and unsaturated fatty acids such as palmitic, stearic, myristic, palmitoleic, linoleic and oleic acid, which were also present in abscesses, no differential sensitivity was noted (Dye and Kapral, 1981a). Due to these findings, attempts were made to determine the reasons for the differences in the sensitivity among these strains.

This study examines the role of encapsulation on the resistance to SFA and the characteristics of a factor capable of inactivating SFA and other bactericidal fatty acids.
CHAPTER 1

The Effect of Encapsulation of *Staphylococcus aureus* on Resistance to Bactericidal Fatty Acids

Since it appeared likely that the staphylocidal fatty acid acted at the membrane or intracellular level, it seemed reasonable to evaluate whether a capsule, by virtue of its hydrophilic nature, could confer protection to the organism by serving as a barrier to the lipid. This investigation examines the role of capsules in the resistance of *Staphylococcus aureus* strains to bactericidal fatty acids.
MATERIALS AND METHODS

Staphylococcus aureus strains.

Strains 303, 18Z, 18ZG, and TG have been described (Kapral and Miller, 1971; Kapral et al., 1980; Dye and Kapral, 1980). Strain M, an encapsulated organism, was isolated by Scott from a human infection (Scott, 1969), and has been characterized by Melly and others (Melly et al., 1978; Liau, Melly and Nash, 1974). Strain M-Al was derived from strain M as described below. Strain Smith mucoid (encapsulated) and Smith compact (a nonencapsulated variant) have been studied by Koenig and others and have been used extensively for pathogenicity studies in mice (Koenig, 1962; Melly et al., 1974; Yoshida and Elkstedt, 1968). Strain D, another encapsulated strain, was kindly provided by Dr. Karakawa. All S. aureus strains used produced soluble coagulase and clumping factor.

Quantitation of Bactericidal Activity.

The assay used to test the sensitivity of various strains has been previously described (Dye and Kapral, 1981a and b). Lipids dissolved in ethanol or the abscess homogenates were diluted serially (two-fold) in 2 M NaCl-2 mM EDTA. To each dilution was added 1000 indicator organisms in sufficient diluent to give a final volume of 1 ml. The mixtures were incubated at 37°C for one hour and the surviving organisms were enumerated by pour plate counts. The final
concentration of ethanol was less than 5% to avoid toxicity (Dye and Kapral, 1981a). All assays were done in duplicate.

Median lethal endpoints were determined by probit analysis (Finney, 1971). The 95% confidence interval for the endpoint was previously estimated to be the mean LD50 ± 20%. This interval however, does not necessarily apply when comparing endpoints obtained with different lipid suspensions (Dye and Kapral, 1981a).

Isolation of Non-encapsulated Mutants.

After finding significant differences in surface charge and surface hydrophobicity, Wadstrom and co-workers suggested that hydrophobic interaction chromatography should be a suitable method for identifying S. aureus strains producing capsules (Wadstrom, et al., 1981). Based on their observations, the following procedure was developed to isolate a non-encapsulated mutant of the encapsulated mutant of the encapsulated strain M.

S. aureus strain M was grown overnight in Trypticase Soy Broth (TSB) (BBL, Cockeysville, MD, USA) at 37°C with constant agitation. Ethylmethane sulphonate (0.08 ml) was added to 2 ml culture as a mutagen and the mixture incubated for 8 minutes at 37°C. The organisms were removed by centrifugation, washed twice in saline, resuspended in TSB and incubated overnight at 37°C. The organisms were removed by centrifugation, washed twice in saline, resuspended in TSB and incubated overnight at 37°C. Following incubation, the organisms were centrifuged and suspended in 4 M NaCl to give a concentration of 10^9 staphylococci/ml. The suspension was then added to a sterile 5 x 30 mm phenyl-sepharose (Pharmacia Fine Chemicals,
Uppsala, Sweden) column equilibrated with 4M NaCl. After the staphylococci were added, the column was washed with 12 bed volumes of 4 M NaCl to remove the non-attached encapsulated organisms. This fraction was discarded. The organisms adsorbed to the column were then eluted with 4 bed volumes of 10 mM phosphate buffer, pH 7. This entire fraction was incubated overnight in TSB. Spread plates were made and screened for non-mucoid colonies.

Potential non-encapsulated mutants were tested for the presence of protein A, soluble clumping factor and coagulase, and the production of alpha, beta and delta toxins. They were also tested for production of acid from various alcohols and carbohydrates, DNase production, tellurite reduction, and antibiotic sensitivity patterns, to verify that isolates did not differ from the parent strain in these respects.

Strain Smith mucoid produces non-encapsulated variants spontaneously on agar plates.

Determination of Encapsulation.

Five techniques were used to distinguish encapsulated from non-encapsulated strains. Organisms were mixed with India Ink (Pelican) and examined for negative staining using light microscopy (Duguid, 1951). Organisms were also mixed with India Ink (Pelican), embedded in Poly/Bed 812 (Polyscience, Inc., Paul Valley Industrial Park, Warrington, PA, USA), thin sectioned, and examined by electron microscopy (Melly et al., 1979).

Colonial morphology in serum soft agar was examined using the technique of Finkelstein and Sulkin (Finkelstein and Sulkin, 1957).
Appropriate concentrations of organisms were added to brain heart infusion broth containing 0.15% agar (w/v) and 1% normal rabbit serum (v/v) and incubated overnight at 37°C.

The presence of Protein A was demonstrated by mixing whole staphylococcal cells with chicken RBCs sensitized with anti-RBC IgG. If present, Protein A interacted with the Fc portion of the IgG molecule and agglutinated the RBCs (Windblad and Ericson, 1973).

The presence of clumping factor on the cell surface was demonstrated by mixing 0.01 ml of saline washed whole organisms with rabbit plasma (Difco Laboratories, Detroit, Michigan, USA). Rapid clumping of organisms was considered a positive reaction (Duthie, 1954).

Abscess Homogenates.

The preparation of abscess homogenates and the isolation of neutral lipid fractions from these homogenates was previously described (Dye and Kapral, 1980; Dye and Kapral, 1981b). In brief, seven day peritoneal abscesses were harvested from ICR swiss white mice and homogenized. Water soluble non-lipid contaminants were removed from the homogenate by partition chromatography on Sephadex G-25 (Rouser, Kritcheusky and Yanamoto, 1967). Lipids were extracted using the Bligh-Dyer modification of the Folch procedure (Bligh and Dyer, 1959), and separated into major lipid classes by fractionation on silicic acid (Kates, 1972).

Chemicals.

All solvents were reagent grade and were distilled in glass just prior to use.
Lipid standards were purchased from Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, U.S.A.
RESULTS

Figure 1 is a composite of electron micrographs of three encapsulated organisms, strain M, strain Smith mucoid and strain D (top three panels) and three non-encapsulated organisms, strain 18ZG, strain Smith compact and strain TG (bottom three panels). The particle-free zone around the organisms is indicative of the presence of a capsule (Melly, 1979). Strain M-Al (not shown) demonstrated no capsule when examined with electron microscopy.

The organisms deemed to be encapsulated by electron microscopy demonstrated no detectable surface protein A or clumping factor. They grew in streaming colonies in serum soft agar (Finkelstein and Sulkin, 1957) and demonstrated the characteristic halo under light microscopy with India Ink.

Strain M-Al, derived from the parent strain M after treatment with ethylmethane sulphonate, was indistinguishable from the parent strain with respect to the fermentation of various carbohydrates (Table 1). In addition, both parent and the mutant produced soluble coagulase, DNase and alpha and delta hemolysin. The antibiotic sensitivity patterns, as determined by The Ohio State University Hospitals laboratory, were also identical. The parent strain M did not demonstrate the presence of Protein A or clumping factor whereas the mutant exhibited both factors. No capsule could be visualized on the mutant when examined by light microscopy with India Ink.
To determine whether the presence of a capsule confers increased resistance to bactericidal fatty acids, various encapsulated and non-encapsulated organisms were evaluated for sensitivity to crude abscess homogenate, the neutral lipid fraction of abscess homogenate and a mixture of free fatty acids in the same proportions found in abscess homogenates.

It was found that the encapsulated organisms were more resistant than were most non-encapsulated organisms to the action of all of the lipid preparations. The non-encapsulated strain TG was an exception in that it demonstrated sensitivities similar to that of the encapsulated strains (Table 2). When comparing the encapsulated parent strains with their non-encapsulated mutants, the Smith mucoid strain was 2 to 6 fold more resistant than the Smith compact strain. Strain M was 3.5 to 9 fold more resistant than strain M-Al (Table 2).
DISCUSSION

Previous work has described a model system that involved the destruction of \textit{S. aureus} in intraperitoneal abscesses by certain specific staphylocidal lipids produced by the host (Dye and Kapral, 1980, 1981). Because strains of \textit{S. aureus} differ in their sensitivity to the fatty acids, attempts were made to determine the role of capsules in this interaction.

The antimicrobial activity of long chain fatty acids has been reported by numerous investigators in various systems. Kanai and Kondo presented an extensive review of the subject of antibacterial effects of fatty acids (Kanai and Kondo, 1979). It is known that chain length (Sheu and Freese, 1973), unsaturation (Heczko et al., 1979; Butcher, King and Dyke, 1976; Field and Parker, 1979), position of the unsaturation (Kabara and Vrable, 1977), presence of a free carboxyl group (Butcher et al., 1976; Kodiek and Worden, 1945), and stereochemical configurations (Butcher et al., 1976; Kabara and Vrable, 1977; Nieman, 1954) are factors that are important in determining the antimicrobial activity of fatty acids. In general, gram positive organisms are more sensitive to the bactericidal activity of long chain fatty acids than gram negative organisms (Sheu and Freese, 1973; Kabara and Vrable, 1977; Kodiek and Worden, 1945).
This study examined the effect of encapsulation on the resistance of \textit{S. aureus} to the bactericidal fatty acids produced in intraperitoneal abscesses. It has been suggested that the probable site of action of long chain unsaturated fatty acids is the membrane (Greenway and Dyke, 1979; Nieman, 1945) and that the difference in sensitivity to unsaturated fatty acids in gram negative and gram positive organisms is related to the barrier effect of the hydrophilic lipopolysaccharide outer membrane of the gram negative organisms (Sheu and Freese, 1973). In our study, encapsulation was found to offer protection from the bactericidal action of the lipid preparations tested. This is in keeping with the concept that a hydrophilic layer would impede lipid access to the membrane of \textit{S. aureus} and would result in reduced bactericidal activity upon the organism.

Previous concepts have viewed the role of a capsule as an antiphagocytic mechanism (Rogers and Melly, 1962; Koenig, 1962; Peterson et al., 1978); our findings suggest another possible role for the capsule. It now seems reasonable that a capsule could offer survival advantage as a protection from bactericidal lipids produced by the host to eliminate these organisms.

As noted previously, \textit{S. aureus} strain TG is unencapsulated and yet is very resistant to the bactericidal lipids examined. This indicates that a number of other mechanisms may determine resistance to bactericidal fatty acids. One possibility is the production of a slime layer. Absence of a capsule does not exclude the production of other extracellular polymers (Caputy and Costerton, 1982).
Unpublished data indicate that strain TG, as opposed to the other non-encapsulated strains, can produce a slime layer in a medium with increased carbohydrate concentration (Mortensen and Kapral, 1982). Certain encapsulated and non-encapsulated strains of _Staphylococcus aureus_ have been shown to produce slime layers _in vitro_ and _in vivo_ (Caputy and Costerton, 1982). Whether strain TG can produce slime _in vivo_ and if a slime layer can act to exclude lipids awaits further study.

More recently, a factor capable of inactivating bactericidal fatty acids has been described (Kapral et al., 1983). It is possible that this factor may influence resistance of some staphylococcal strains to bactericidal lipids.

It is also possible that differences in membrane structure or function can account for different sensitivity to free fatty acids. Such possibilities remain to be investigated.
Figure 1. Composite of electron micrographs. Three encapsulated strains of *S. aureus* (top three panels) and three nonencapsulated strains of *S. aureus* (bottom three panels) were mixed with India Ink, embedded in Poly/Bed 812 and thin sectioned for electron microscopy.
### Biochemical Characteristic of Strain M and Strain M-Al

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<tr>
<td>D - Dextrose</td>
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<td>+</td>
</tr>
<tr>
<td>D - Mannose</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
<td>D - Trehalose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
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<tr>
<td><strong>Agglutinations</strong></td>
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<tr>
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<tr>
<td>Protein A</td>
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*Other carbohydrates not fermented by either strain: D - Xylose, L - Arabinose, Rhamnose, Inositol, Dulcitol, Adonitol.*
<table>
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<th>303</th>
<th>TG</th>
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<td>3.2</td>
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<td>23.3</td>
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* 44% Palmitic, 43% oleic, 5% palmitoleic, 3% linoleic, 3% stearic, 1% myristic

² In terms of total lipid

ND = not done
CHAPTER TWO

Modification of Bactericidal Fatty Acids by an Enzyme of

*Staphylococcus aureus*

While studying the fate of *Staphylococcus aureus* in abscesses, Kapral and coworkers demonstrated a novel immune system responsible for the elimination of the cocci from these lesions (Kapral et al., 1983). Abscess homogenates were found to contain a staphylocidal substance that was distinct from any previously described lysosomal product. Work indicated that this substance was a free fatty acid with a number of unique characteristics that differentiated it from the more common saturated and unsaturated fatty acids that are also present in abscesses (Dye and Kapral, 1980, 1981a).

Subsequently, Shryock and Kapral found that certain strains of *S. aureus* produced a soluble factor capable of inactivating the bactericidal action of not only the staphylocidal fatty acid but also other bactericidal fatty acids (Shryock and Kapral, manuscript in preparation). When suspensions of palmitoleic, oleic or linoleic acids were reacted with culture filtrates and the resultant mixtures analyzed by gas-liquid chromatography (GLC), the appearance of modified lipids was noted. These lipids were not present in the culture filtrates and could not be attributed to the action of any known staphylococcal enzyme (Shryock and Kapral, manuscript in preparation).

20
It appeared likely that there was a correlation between the presence of unidentified lipids on GLC and the loss of bactericidal activity. Therefore, we planned further characterization of the factor.

This study describes the characteristics of a factor, subsequently found to be a fatty acid modifying enzyme (FAME), and examines its production by various strains of \textit{S. aureus}. 
MATERIALS AND METHODS

Preparation of FAME

*S. aureus* strain 18Z was incubated in Trypticase Soy Broth (TSB) (BBL Microbiological Systems, Cockeysville, MD, USA) at 37°C for 18 hours with continuous agitation. The culture was centrifuged at 10,000 x g for 20 minutes and the supernatant fluid sterilized with a 0.22 micron membrane filter. The filtered preparation was concentrated 10 fold on an Amicon PM30 membrane (Amicon Corporation, Danvers, Mass, USA) and the concentrate dialyzed overnight at 4°C against distilled water. After dialysis, the preparation was lyophilized, weighed and stored at 4°C until used.

Assay procedure for FAME

The lyophilized culture filtrate (LCF) was dissolved in 0.1 M phosphate buffer, pH 6.0, and serial 4-fold dilutions prepared in the same diluent. The fatty acid substrate [9,10-³H(N)]- oleic acid (New England Nuclear, Boston, MA, USA), was diluted with unlabeled oleic acid to provide 50,000 counts per minute (cpm) with a 200 µg aliquot of the fatty acid. This radiolabelled oleic acid was dissolved in hexane (Fisher Scientific Company, Pittsburgh, PA, USA) and stored at -20°C in lipid-free vials.

Aliquots of the labelled oleic acid stock solution containing 200 µg fatty acid were dispensed into lipid-free 2 dram glass vials.
and the hexane evaporated under a stream of nitrogen at 40°C. To each vial was added the alcohol substrate, the appropriate enzyme dilution and sufficient phosphate buffer to give a final volume of 1 ml. This mixture was incubated in a shaking water bath at 37°C for 20 minutes.

Following incubation, the lipids were removed from the aqueous phase by extracting the mixture twice with 2.5 ml diethyl ether-methanol (6:1 v/v) and once with 2.5 ml diethyl ether. The ether phases (upper phases) were combined and evaporated to dryness under nitrogen. The extracted lipids were dissolved in 70 µl of chloroform and the entire amount was spotted on 20 x 20 cm silica gel G coated glass plates (Fisher Scientific Company, Pittsburg, PA, USA). The vials were successively rinsed with 70 µl chloroform and 70 µl acetone and these rinses were added to the appropriate spots.

The fatty acids were separated from the ester by developing the plates in hexane-diethyl ether-acetic acid (80:20:1 v/v/v) and the lipid spots visualized with iodine vapors. The spots were scraped and collected in vials containing scintillation fluid cocktail and counted in a Beckman Liquid Scintillation Counter model LS 7000. The percent esterified was calculated by dividing the disintegrations per minute (dpm) of the ester spot minus the negative control by the total dpm in the sample-(fatty acid plus ester).

Characteristics of the FAME reaction

The effect of monovalent and divalent cations. The requirement for divalent ions in the FAME reaction was evaluated by dissolving
the lyophilized culture filtrate in 0.1 M sodium phosphate buffer (pH 6.0) containing either 5 mM, 10 mM or 20 mM EDTA.

The effect of sodium and potassium ions on the FAME reaction was examined by dissolving the preparation in 5 mega-ohm distilled water containing 10 mM N-2-hydroxy ethylpiperazine-N-2 ethane-sulfonic acid (HEPES, Calbiochem-Behring Corp., La Jolla, CA, USA) and the pH was adjusted with Tris-hydroxymethyl-amino methane (Tris base, Sigma Chemical Co., St. Louis, MO, USA).

**Substrates for esterification to oleic acid.** Initially, qualitative data was obtained to determine if alcohols other than ethanol might be suitable substrates for the esterification reaction. These included methanol, 1-propanol, 2-propanol, 1-butanol, and isoamyl alcohol. Certain staphylococcal metabolites, which might accumulate in abscesses were also studied. These included 2,3-butandiol; 2,3-butandione; β-hydroxybutric acid and 3-hydroxy-2-butanone. In addition, glycerol, cholesterol, and glucose were examined as host compounds that might be available to participate in the esterification reaction in abscesses. The concentration of the oleic acid was $1.4 \times 10^{-3}$ M and the concentration of the other compounds was $1.4 \times 10^{-1}$ M. Assay conditions were as when assaying FAME except that the incubation time was 4 hours. The molar ratio of alcohol to oleic acid for 30% esterification was determined with the same procedure was used as when assaying FAME except that due to low concentrations of some alcohols and the use of some insoluble alcohols such as cholesterol, the suspensions were made by combining phosphate buffer, fatty acid and alcohol and sonicating twice for
20 minutes in an 80 watt sonicating water bath at 25°C (Heat Systems, Inc., Plainview, NY, USA). Following sonication, 150 μg FAME was added to each vial and the mixtures were incubated at 37°C for 4 hours. The concentration of oleic acid was $7 \times 10^{-4}$ M and the concentration of the alcohols was varied from $2.6 \times 10^{-4}$ M to $8.6 \times 10^{-1}$ M.

**Production of FAME by staphylococcal strains**

The ability of various strains of *S. aureus* to produce FAME in either TSB or in a chemically defined amino acid medium (Nolte and Kapral, 1981) was assessed. Strains 18Z, P78, PG114, TG, 689, 303, 18Z-G, 18Z-H, P78-22 and PG114-1 have been previously described (Kapral and Miller, 1971; Kapral and Shayegani, 1959; Melish and Glasgow, 1970; Dye and Kapral, 1981b). All strains were soluble coagulase and clumping factor positive. Culture filtrates were prepared and assayed as described for FAME. The concentration of organisms in these cultures was determined by comparing optical density at 630 nm to a standard curve, and in some cases plate counts.

**Chemicals and glassware**

All glassware was cleaned with chloroform-methanol (2:1 v/v) before use.

Non-radiolabelled lipids and cholesterol were purchased from Sigma Chemical Company, St. Louis, Mo., USA.
RESULTS

Preliminary Investigations

We made the following observations to determine the nature of the factor responsible for the conversion of the free fatty acid to the modified lipid. Previous studies had shown that conversion activity of culture filtrate was destroyed by heating to 100°C for one hour (Kapral and Shryock, 1983) or after treatment with proteinase K (E. Merck, Darmstadt, Germany) at 1 mg/ml and 37°C for 1 hour. The activity of culture filtrates was maximal at 37°C to 40°C, but was less at 15°C and totally lacking at 0°C. The rate of the reaction was also dependent upon the concentration of the culture filtrate added. It was found that the modified lipid could be separated from the parent fatty acid by chromatography on Silica G plates developed with hexane: ethyl ether:acetic acid (80:20:1 v/v/v). This in turn, permitted the recovery of the modified lipid and its analysis by mass spectroscopy. When oleic acid was used as the starting material, the modified lipid proved to be ethyl oleate (Fig. 2). Considering the composition of the reaction mixture, it was obvious that the source of the ethyl group was the ethanol used in preparing the oleic acid suspension. These results suggested to us that the culture filtrate contained an enzyme capable of esterifying the free fatty acid with ethanol. Realizing that final
conformation of the nature of this factor was dependent upon its purification, it was nevertheless decided to characterize its activity by using crude preparations in the form of dialyzed and lyophilized culture filtrates (LCF).

Characteristics of the FAME reaction

The amount of oleic acid converted to the ester at various concentrations of LCF was examined over time (Fig. 3a). It was found that the rate and amount of esterification was directly proportional to LCF concentration. From these data, it was apparent that the incubation period could be shortened to 20 minutes and that by such standardization, an assay could be established which would relate esterification to enzyme concentration (Fig. 3b).

A single stock of LCF was prepared and used for all subsequent work. This preparation was found to have approximately 5 times the activity of the initial batch of LCF. The degree of oleic acid esterified was examined with different LCF concentrations (Fig. 4) and was found to be linear over the concentration range of 50-300 ug/ml. This data supported the earlier findings and allowed the standardization of the assay procedure. Standard assay conditions were established as 20 minute incubation at 37°C and a concentration of 200 ug oleic acid and 100 ul ethanol in a final volume of 1 ml. Under these conditions, a unit of activity of FAME was defined as that amount of enzyme needed to convert 10 nmoles of oleic acid to the ester per minute. Using the LCF working stock under standard conditions, the amount of LCF needed to convert 28.3% conversion of the oleic acid to the ethyl ester was equivalent to 1 unit of activity.
The effect of pH on the FAME reaction. LCF was suspended in either 0.1M sodium phosphate buffer (pH 5.0-8.0) or 0.1M citric acid/sodium citrate buffer (pH 3.5-6.0). The optimum activity was demonstrated at pH 6.0 with little activity below pH 4.5 or about pH 7.5 (Fig. 5).

The effect of temperature. Enzyme suspensions were incubated in a shaking water bath at 0°, 10°, 20°, 30°, 40°, 50°, or 60°C for 20 minutes. Ester conversion activity was linear, over the range of 0°C to 40°C. This linear range was followed by a plateau from 40°C to 50°C, and a decline in activity about 50°C.

The effect of monovalent and divalent ions on the FAME reaction. The FAME activity was not affected by the addition of 5 mM, 10mM or 20 mM EDTA to the reaction mixture or by the addition of 0.25%, 0.50% or 1.00% sodium or potassium ions.

Substrates esterified to oleic acid. Qualitative data indicated ethanol, methanol, 1-propanol, 2-propanol and 1-butanol were esterified to oleic acid by FAME. Isoamyl alcohol was not esterified under these conditions. Cholesterol was the only host related compound that could be esterified to oleic acid (Table III), and those staphylococcal metabolites tested were not esterified under these conditions.

Quantitative studies comparing the efficacy of various alcohols in the esterification process indicated that the chain length of the alcohol was important. As the chain length increased from one carbon (methanol) to four carbons (butanol), less alcohol was required per mole of oleic acid to give the same degree of esterification (30%)
with the same concentration of LCF (Table IV). However, cholesterol proved to be an even better substrate for this reaction than 1-butanol. A molar ratio (cholesterol:oleic acid) of approximately one was sufficient to convert 30% of the oleic acid to the ester.

Production of FAME by staphylococcal strains

When the production of FAME by various strains in both TSB and a defined amino acid medium was compared, two basic levels of FAME production were evident (Table III). Strains 18Z, PG114, TG and P78 produced significant amounts of the enzyme as compared to 18Z-G, 18Z-H, PG114-1, P78-22 and 303. Furthermore, the amount of FAME produced generally correlated with Dye and Kapral's findings concerning a strains' ability to survive within intraperitoneal abscesses (Dye and Kapral, 1981a) (Table V). Those strains which were rapidly eliminated from abscesses produce little or no FAME in these two media. Strains that either persisted in abscesses or were eliminated only after a delay were capable of producing significant amounts of FAME. The only exception was strain P78 (Table V). It was difficult to compare the production of FAME between the two media used because strains 303, P78-22, 689, 18Z-H, PG114-1 and 18Z-G produced levels of FAME near the sensitivity of the assay. However, the experimental error for strains PG114 and 18Z in defined medium was 7 and 20% respectively and in TSB was 20 and 50% respectively. These values indicate that these two strains produced significantly different levels of FAME in the two media tested (Table V).
DISCUSSION

Previous work described a model infection that involved the destruction of *S. aureus* in intraperitoneal abscesses by a unique bactericidal free fatty acid (Dye and Kapral, 1980; Dye and Kapral, 1981a). Recently, a factor capable of inactivating this staphylocidal fatty acid has been demonstrated in the culture supernatant of some strains of *S. aureus* (Shryock and Kapral, manuscript in preparation). This study describes the characteristics of this factor and examines its production by various strains.

Data indicates that this factor is an enzyme that esterifies free fatty acids to various short chain alcohols and cholesterol but, not to certain mono-, di- or polyhydric compounds. This fatty acid modifying enzyme (FAME) has optimal activity at pH 5.5 - 6.0. In 1974, Hays and Mandell reported that when abscesses were induced with turpentine and staphylococci, the pH of the abscess material was 6.2 to 7.0, while turpentine alone yielded abscesses at pH 7.1 to 7.2 (Hays and Mandell, 1974). Although the formation of these abscesses is unnatural and the effect of the turpentine on *S. aureus* was not examined, in support of this data we have found that homogenates of intraperitoneal staphylococcal abscesses have a pH of 5.9-6.1 (Mortensen, 1983 - unpublished data). This suggests that the conditions within staphylococcal abscesses may be close to the optimum for FAME activity.
Although we have elected to employ ethanol as a substrate when assaying for FAME activity because of its convenience in preparing fatty acid suspensions, the presence of ethanol in abscesses is unlikely. Cholesterol, however, is undoubtedly available in the form of membrane fragments contributed by the disintegration of the leukocytes at the core of the abscess. The fact that approximately equal molar concentrations of cholesterol to fatty acid suffice to esterify a significant proportion of the oleic acid whereas 700 fold greater amounts of ethanol are required to accomplish the same results, indicates that FAME has a marked preference for cholesterol in the esterification reaction.

FAME has yet to be purified and characterized. However, there are various types of enzymes that catalyze reactions involving esters of fatty acids (Vahouny and Treadwell, 1968). One possibility is that the FAME is an Acyl-transferase [EC 2.3.1]. Due to the requirements of this enzyme for ATP, magnesium ions and coenzyme A carrier (Vahouny and Treadwell, 1968), it seems unlikely that the FAME would fall into this category. The FAME reaction was found to be insensitive to EDTA and was unaffected by dialysis against distilled water.

A second possibilities is that FAME is a representative of the sterol-ester hydrolases [EC 3.1.1.13], a group of enzymes which catalyze the hydrolysis of sterol-fatty acid esters. These hydrolases have been described in a number of mammalian tissues, including liver and pancreas. A representative enzyme of this group isolated from liver tissue catalyzes the synthesis and hydrolysis of cholesterol esters of 14-18 carbon fatty acids. The pH optimum, for
hydrolysis is pH 6.6. This enzyme is dialysis stable and inactivated by heating at 65°C for 15 minutes (Byron, Wood and Treadwell, 1953). Studying a pancreatic form of this group of enzymes, Hernandez and Chaikoff found that esterification required a sterol substrate with the beta configuration of the hydroxyl group on the number 3 carbon. When the esterification of cholesterol to various fatty acids was studied, unsaturated and diunsaturated fatty acids, 14-18 carbons in length, were esterified preferentially at pH 6.2 (Hernandez and Chaikoff, 1957). Additional results demonstrated that the pH optimum of the pancreatic form for esterification was pH 6.1 to 6.2 whereas for hydrolysis the optimum was pH 6.6 to 7.0. At this pH's, both the reactions go to 80% completion (Bahouny and Treadwell, 1981).

Cholesterol ester hydrolases have also been described in Saccharomyces cerevisiae (Taketani, Osumi and Katsuki, 1978), Pseudomonas fluorescens (Uwajima, and Terada, 1975) and S. aureus (Harvie, 1977). In addition, there has been a report that S. epidermidis and at least two propionibacteria are capable of esterifying cholesterol to fatty acids in the skin (Puhvel, 1975). These enzymes have not been studied extensively; however, some information is available. For the P. fluorescens enzyme, the optimum pH for hydrolysis of cholesterol esters to cholesterol and fatty acids is pH 7.3 (Uwajima and Terada, 1975).

Although the production of FAME within abscesses has not yet been measured, with most strains examined, there is a high correlation between the strain's ability to produce FAME in vitro and its
ability to survive in abscesses. Strain P78 is the exception in
that it produces significant amounts of the FAME in vitro and yet
is rapidly eliminated from abscesses. This suggests either FAME
production alone does not assure survival of S. aureus in abscesses
or that the demonstration of in vitro production of the enzyme does
not necessarily guarantee its production in vivo.

The ability of strains 18Z, P78, TG, 689 and PG114 to produce
certain toxins also appears to correlate with production of FAME.
Strain 18Z and P78 produce both alpha and delta toxins and strain
TG produces the alpha, beta and delta toxin. Strain 689 produces
only alpha toxin and strain PG 114 only delta toxin. The alpha
toxin negative variants, 18Z-G, 18Z-H, P78-22, and the delta toxin
negative variant PG114-1, were derived from their respective parents
after ultraviolet irradiation (Kapral and Miller, 1971; Kapral and
Shayegani, 1959). In this study, the non-hemolytic mutants produce
less FAME than the parent strains. This suggests a possible genetic
linkage between the loci for toxin production and FAME production
such that the derived mutants are, in fact, pleiotropic mutants
lacking multiple factors.

The data indicate that there is a difference in the amount of
FAME produced when certain S. aureus strains are cultivated in TS8
or in chemically defined medium. The difference in the amount of
FAME produced in the two media may indicate a limiting nutritional
requirement for enzyme production or the presence of substances
that regulate enzyme production or secretion.
The role of the fatty acid modifying enzyme in the interaction between the parasite and the host remains to be delineated; but, this work contributes to our understanding of the complex interaction in abscesses between the staphylococci and the host. A major factor in the ability of some strains of *S. aureus* to survive in abscesses and cause disease, may be their ability to inactivate bactericidal lipids produced by the host specifically in response to the infection.
9-Octadecenoic acid (Z)-, ethyl ester

C_{20}H_{38}O_2

111-62-6

Me\, (CH_2)\, CH\, CH(CH_2)\, COOEt
Figure 2. Mass spectra of the modified lipid. Oleic acid (200 μg) was mixed with 100 μl ethanol in the presence of culture filtrates of *Staphylococcus aureus* strain 18Z.

A. Analysis of the modified fatty acid with a Hewlett-Packard 5985 Quadropole Mass Spectrometer utilizing electron impact at 70 eV.

B. EPA/NIH mass spectral data base standard for the ethyl ester of oleic acid (Heller and Milne, 1963).
Figure 3. The amount of oleic acid converted to ethyl oleate.

A. The conversion of oleic acid to ethyl oleate by (●) 0.5, (■) 1.0, (▲) 2.0, (○) 4.0 and (□) 9.0 mg/ml lyophilized culture filtrate (LCF).

B. The data was replotted with the percent of oleic acid esterified at (●) 20, (■) 40, and (▲) 60 minutes over a range of LCF concentrations.

Percent Esterified = \[
\frac{\text{dpm ethyl ester}}{\text{dpm ethyl ester + dpm fatty acid}}
\]
Figure 4. Esterification of oleic acid to ethyl oleate. LCF was mixed with 200 μg oleic acid and 0.1 ml ethanol in 0.1M phosphate buffer (pH 6.0). Vertical bars represent the standard deviation of the mean of 3-8 experiments.
Figure 5. The effect of pH on the FAME reaction. LCF was suspended in either 0.1M sodium phosphate buffer (pH 5.0-8.0) or 0.1M citric acid/sodium citrate buffer (pH 3.5-6.0) with 200 μg oleic acid and 100 μl ethanol at 37°C and 20 minutes incubation.
Figure 6. The effect of temperature on the FAME reaction. A mixture of 150 μg LCF, 200 μg oleic acid and 100 μl ethanol was incubated in a shaking water bath, at various temperatures, for 20 minutes. The coefficient of correlation of percent esterification vs temperature over the range of 0°C-30°C equals 0.996.
<table>
<thead>
<tr>
<th>Substrates Esterified to Oleic Acid by FAME&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol + Cholesterol +</td>
</tr>
<tr>
<td>Ethanol + Glucose -</td>
</tr>
<tr>
<td>N-Propanol + Glycerol -</td>
</tr>
<tr>
<td>2-Propanol + β-Hydroxybutyric acid -</td>
</tr>
<tr>
<td>N-Butanol + 2,3-Butanediol -</td>
</tr>
<tr>
<td>2,3-Butanedione -</td>
</tr>
<tr>
<td>Isoamyl Alcohol - 3-Hydroxyl-02-Butanone -</td>
</tr>
</tbody>
</table>

<sup>1</sup> The concentration of oleic acid was $1.4 \times 10^{-3}$ M and the concentration of the other substrates was $1.7 \times 10^{-1}$ M.
### TABLE IV

**Molar Ratio for 30 Percent Conversion**

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Alcohol:Oleic Acid Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>848</td>
</tr>
<tr>
<td>Ethanol</td>
<td>732</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>141</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>36.6</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.89</td>
</tr>
</tbody>
</table>

7.1 x 10^{-4} M oleic acid, 37°C/4 hours, 150 µg enzyme preparation
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>DEFINED (^1)</th>
<th>TSB</th>
<th>ELIMINATION (^2) IN ABSCESSES</th>
</tr>
</thead>
<tbody>
<tr>
<td>303</td>
<td>&lt;0.01 (^3)</td>
<td>&lt;0.01</td>
<td>IMMEDIATE</td>
</tr>
<tr>
<td>P78-22</td>
<td>0.03</td>
<td>0.01</td>
<td>IMMEDIATE</td>
</tr>
<tr>
<td>689</td>
<td>0.03</td>
<td>0.02</td>
<td>IMMEDIATE</td>
</tr>
<tr>
<td>18Z-H</td>
<td>0.04</td>
<td>0.01</td>
<td>IMMEDIATE</td>
</tr>
<tr>
<td>PG114-1</td>
<td>ND</td>
<td>&lt;0.01</td>
<td>IMMEDIATE</td>
</tr>
<tr>
<td>18Z-G</td>
<td>ND</td>
<td>&lt;0.01</td>
<td>IMMEDIATE</td>
</tr>
<tr>
<td>P78</td>
<td>0.47</td>
<td>0.20</td>
<td>IMMEDIATE</td>
</tr>
<tr>
<td>PG114</td>
<td>0.24</td>
<td>0.65</td>
<td>DELAYED</td>
</tr>
<tr>
<td>18Z</td>
<td>0.76</td>
<td>0.10</td>
<td>DELAYED</td>
</tr>
<tr>
<td>TG</td>
<td>0.24</td>
<td>0.10</td>
<td>NONE AT 35 DAYS</td>
</tr>
</tbody>
</table>

\(^1\) Amino acids - salts - vitamins
\(^3\) Units/10\(^9\) staphylococci
ND = Not done
BIBLIOGRAPHY


