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UNIVERSITY OF CALIFORNIA, SAN DIEGO

# The Calcification of *Staphylococcus aureus* Bacteria:
# A Potential Defense Mechanism Against Infections

A Thesis submitted in partial satisfaction of the requirements

for the degree of Master of Science

in

Biology

by

Catherine Krystle Arellano

Committee in charge:

Professor Paul A. Price, Chair
Professor Milton Saier
Professor Immo Scheffler

2010

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The Thesis of Catherine Krystle Arellano is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2010

## DEDICATION

This thesis is dedicated to my loving mom and dad, who have always been my inspirations. Without their continued guidance and encouragement, I would not be who I am today. I would like to thank them for allowing me the freedom and opportunity to pursue a higher education. I want nothing more than to make them proud. I would also like to dedicate this thesis to my best friend, Kevin Sendaydiego, who is the most caring and supportive person I know. He has been a continued source of motivation that has helped me achieve my goals to the best of my ability. Without his outstanding patience and compassion this thesis would not be possible.

## EPIGRAPH

*"If an experiment works, something has gone wrong"*
–Unknown

*"Science is always wrong.
It never solves a problem without creating ten more.*"
– George Bernard Shaw

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I would also like to acknowledge Ryan Anderson from the School of Engineering for helping me produce beautiful images with Scanning Electron Microscopy.

Lastly, I would like to thank my graduate counselor, Andrew Lukosus for all his administrative help and advice.

Figure 1 is adapted from an illustration that appears in "Tissue-Nonspecific Alkaline Phosphatase is Required for the Calcification of Collagen in Serum: A Possible Mechanism for Biomineralization". Price, P.A., Toroian, D., and Chan, W.S. (2009) *J. Biol. Chem. 284,* 4595-4604.

# ABSTRACT OF THE THESIS


The Calcification of *Staphylococcus aureus* Bacteria:

A Potential Defense Mechanism Against Infections

Master of Science in Biology

University of California-San Diego, 2010

Professor Paul A. Price, Chair

*Staphylococcus aureus* bacteria are gram-positive cocci that are notorious for causing various skin infections. Since the emergence of antibiotic resistant strains, the increasing incidence of bacterial infections has become worldwide concern. Therefore, continued investigation on mechanisms that may play a role in fighting bacterial infections is critical. It is our working hypothesis that the calcification of live *S. aureus* bacteria by the previously discovered serum calcification factor may be involved in a vertebrate's immunological response against bacterial infections.

Previous *in vitro* studies performed in our lab demonstrated that dead *S. aureus* bacteria are calcified in serum [1]. The goal of our study was to further understand the role of bacterial calcification in the defense against infections. To accomplish this, we developed a serum-free calcifying system where all parameters were defined. This system

allowed us to investigate the consequences of calcifying living bacteria, a phenomenon that was not previously explored. Without any serum factors, we are able to understand the repercussions solely due to the calcification of the bacteria. The results show that mineralization affects the viability and growth rate of the bacteria. Our studies also show that live *S. aureus* bacteria are likely to be calcified in blood by a mechanism that targets the size exclusion characteristics of the cell wall, a feature unique to bacteria. Future studies have been proposed to better understand the role of bacterial calcification *in vivo.*

# INTRODUCTION

*Staphylococcus aureus* bacteria are gram-positive cocci that are ubiquitous inhabitants of the skin and nasal passages of healthy individuals. Staphylococci are among the most robust pathogens to infect the human populations and are the likely culprits to cause various skin and suppurative infections. Furthermore, *S. aureus* infections can lead to serious life-threatening diseases such as pneumonia, meningitis, and urinary tract infections (UTIs) [2].

Since the emergence of antibiotic resistant bacteria, infections have become a global concern. Although the introduction of methicillin in 1959 was initially effective in treating *S. aureus* strains that were resistant to penicillin and other related antibiotics, the first incidence of Methicillin Resistant *S. aureus* (MRSA) emerged only a couple years later [3]. Outbreaks of MRSA became increasingly prevalent worldwide in the 1970's and have remained a problem ever since. More people die annually from invasive MRSA infections than from HIV, H1N1 flu, or infant mortality [3-9]. Therefore, continued investigation on mechanisms that may play a role in fighting bacterial infections is critical. It is our working hypothesis that the calcification of live *S. aureus* bacteria by a serum calcification factor may be involved in a vertebrate's immunological response against bacterial infections.

Previous *in vitro* studies in our lab demonstrated that dead *S. aureus* bacteria are calcified when incubated in serum [1]. In these past studies we accomplished two very important objectives. First, we verified that a bacterial matrix could be calcified in serum, and second, we determined the underlying mechanism of this serum-induced calcification. The evidence of this earlier study suggests that the mechanism that drives the mineralization of Type-I collagen of bone and tendon is also capable of mineralizing bacteria [1,10]. This "serum mechanism" was found to have four key requirements (Figure1): 1) A source of tissue non-specific alkaline phosphatase (TNAP) is required to activate a high molecular

weight serum nucleator of apatite formation [1,10]. The serum nucleator, which has yet to be identified, consists of one or more proteins that are 50-150 kDa in size [10]. It has been found in all vertebrate species tested including humans, rats, cows, cartilaginous fish, bony fish, and lamprey [11]. 2) The activated serum nucleator is necessary to generate small apatite crystals (<6 kDa in size) near the bacterial matrix [1,10] 3) The matrix must have size exclusion characteristics that allow small molecules (<6kDa) such as calcium, phosphate, and small apatite crystals to access the interior, while excluding large molecules (>40kDa) [1,13]. 4) A large protein such as fetuin (59 kDa), must selectively inhibit the growth of any crystals outside of the matrix [14]. Most small apatite crystals form complexes with serum fetuin that inhibit their growth. A few of the crystals are free and, and because of their small size, can diffuse through the pores of the bacterial cell wall. Because fetuin is too large to pass through the matrix pores, the crystals inside the matrix are free of fetuin, and grow rapidly [12].

## Four Key Requirements:

1. **Tissue Non-specific Alkaline Phosphatase**
   -activates serum nucleator
2. **Serum Nucleator**
   -generates crystals that are <6 kDa in size
3. **Matrix with appropriate size exclusion properties**
   <6 kDa can enter
   >40 kDa are excluded
4. **Fetuin**
   -a 59 kDa protein inhibits mineral growth outside of matrix

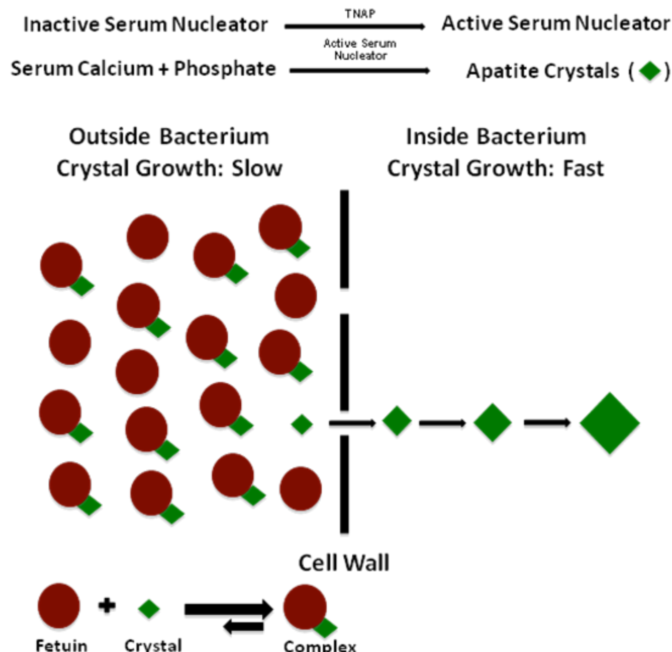



**Figure 1. Bacterial calcification by the serum mechanism.** Tissue non-specific alkaline phosphatase (TNAP) activates the high molecular weight nucleator of apatite formation, and the activated nucleator then forms small apatite crystals near the bacterial matrix. Most small apatite crystals *(green)* form complexes with serum fetuin *(red)* that inhibit their growth. A few of these crystals are free and, because of their small size, can diffuse through the pores of the bacterial cell wall (*represented by gaps in the vertical black lines*). Because fetuin is too large to pass through the matrix pores, the crystals inside the matrix are free of fetuin, and grow rapidly [1,10,12].

Since it has been established that dead *S. aureus* bacteria are capable of becoming mineralized in serum, the goal of our current study is to understand the repercussions of this mineralization. To accomplish this goal we have developed a novel serum-free mechanism for bacterial calcification based on our understanding of the serum-induced mechanism. This novel mechanism involves three key requirements (Figure 2): 1) A crystallization inhibitor, such as the protein fetuin (59 kDa), is required to selectively inhibits mineral growth outside of the matrix. 2) A solution that would, in the absence of the inhibitor, form a crystalline phase. 3) A semi-permeable barrier, such as the bacterial cell wall, that excludes the inhibitor but allows the solution containing the constituents of the crystalline phase to enter [1, 15, 16, 17].

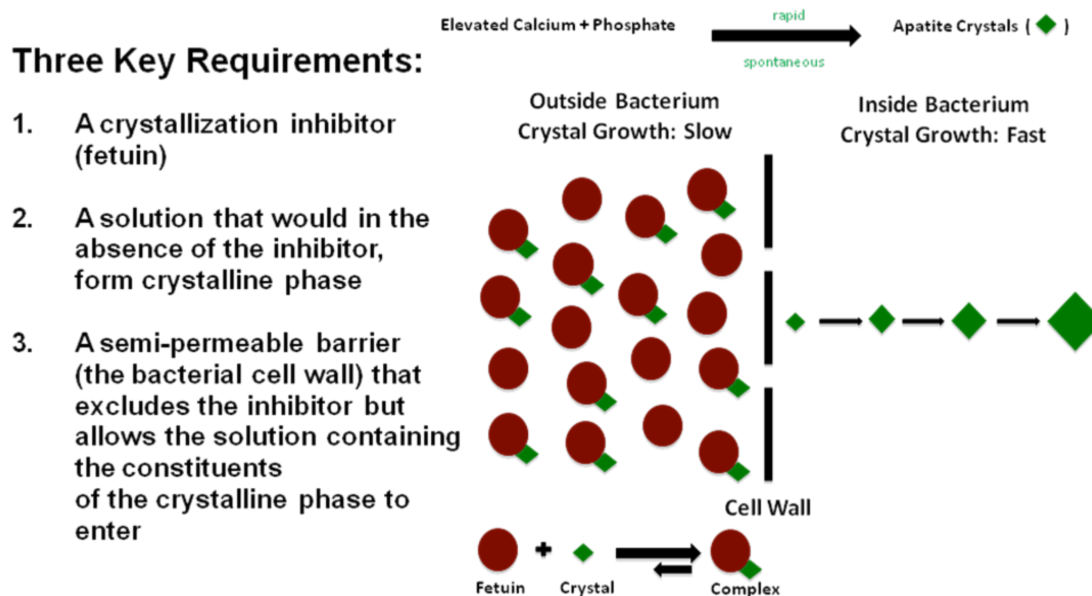



**Figure 2. Bacterial calcification by the serum-free mechanism.** Elevated concentrations of [Ca] and [$PO_4$] rapidly and spontaneously form apatite crystals throughout the solution. Apatite crystals (*green*) that are generated outside the bacteria form complexes with fetuin (*red*) that inhibit their growth. Because fetuin is too large to pass through the matrix pores (*represented by gaps in the vertical black lines*), the crystals that form inside the bacteria are free of fetuin, and are able to grow rapidly.

To test this mechanism, we have developed a simplified serum-free system where all parameters were defined. Though studies done with the serum mechanism have proven to be valuable, the composition of serum itself is too complex and not fully understood. Without the involvement of any confounding factors such as the ill-defined serum nucleator, this new system allowed us to investigate the affect on bacteria that is solely due to calcification. We have termed this novel mechanism "bacterial calcification by the serum-free mechanism".

It is worthy to note that both bacterial calcification in the serum system and bacterial calcification in the serum-free system involve mechanisms that are nearly identical to each other. When displayed as a side-by-side comparison (Figure 3), it is evident that the only differences in the two mechanisms are how and where the apatite crystals are generated. In serum mechanism, the presence of a serum nucleator is required to generate apatite crystals because the ion product of calcium and phosphorus are too low to form mineral spontaneously [12]. Because this entity is large in size, the nucleator cannot diffuse through the cell wall, and therefore its activity of forming crystals is limited to the outside of the bacterial matrix. The crystals that are not bound by fetuin are able to penetrate through the pores of the cell wall, and are free to grow rapidly. The serum-free system, on the other hand, has been designed to form crystals spontaneously and throughout the solution. This was achieved by elevating the concentrations of calcium and phosphate to a level that guarantees a high ion product. Apatite crystals rapidly form both inside and outside the bacterial matrix. The crystals that form inside the bacteria are free of fetuin and grow rapidly. All other aspects of the two systems related to bacterial calcification are mechanistically identical.

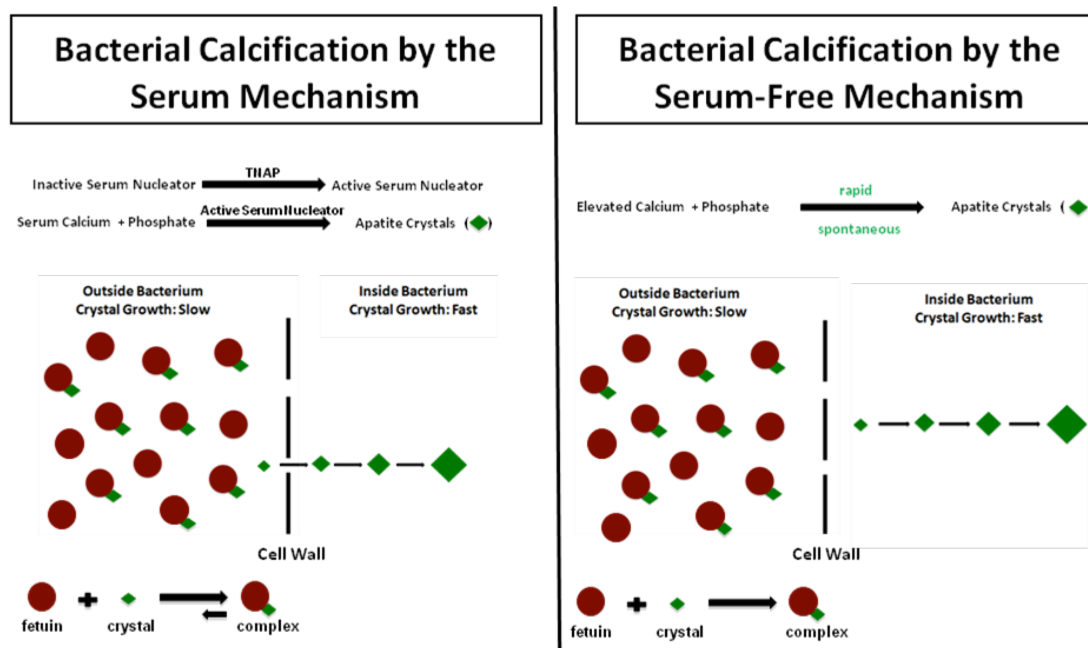



**Figure 3. Comparing bacterial calcification by the serum mechanism and bacterial calcification by the serum-free mechanism.** This figure highlights the mechanistic similarities between the serum mechanism and the serum-free mechanism of bacterial calcification. The only differences between these two mechanisms are where and how apatite crystals are formed (*see text above)*. All other aspects are mechanistically identical.

If our understanding of the serum mechanism and its requirements are correct, this newly designed serum-free mechanism should result in the successful calcification of a bacterial matrix. To test this, we performed an experiment to demonstrate that dead *S. aureus* bacteria with intact cell walls were effectively calcified in this new system.

However, the more remarkable, and probably the most important feature of this serum-free mechanism is the potential to investigate the consequences of calcifying living bacteria. This phenomenon was difficult to explore in the previous experiments due to the rapid bacterial division rate in serum. The magnitude of the bacteria's doubling rate in serum was overwhelmingly faster than the time required for robust calcification, thus making it nearly impossible to segregate the older mineralized bacteria from its newer non-mineralized

progeny. The serum-free system allows the use of any solution as long as the parameters of this mechanism's three requirements (Figure 2) are maintained.

It is interesting to note that several studies have revealed that factors required in the mechanism of bacterial calcification (mechanisms described above) are also involved in the various immunological responses (acute innate response). For example, hydroxyapatite particles have been shown to promote the release of various pro-inflammatory cytokines as well as recruit various polymorphonuclear cells, including neutrophils, basophils, and eosinophils. [18,19]. In addition, the protein fetuin and its human analogue $\alpha_2$ HS-glycoprotein, have been identified as potent opsonins that promote the bacterial phagocytosis by neutrophils and macrophages [20, 21]. With evidence of such a relationship with the immune response, it is reasonable to believe that bacterial calcification may play a role in the protection against infection.

Overall, our research is the first to provide a mechanism that permits the calcification of living *S. aureus* bacteria. This study suggests that the body may take advantage of a calcification mechanism that targets the size exclusion characteristics of the cell wall, a feature that is unique to bacteria. In order to investigate the consequence of bacterial calcification and analyze its defensive roles, our goal was to first, develop a chemically defined serum-free system that was suitable for the calcification of a bacterial matrix. To understand the implications associated with calcification and only calcification, we had to find a *S. aureus* medium that maintained bacterial viability but prevented its growth. We then modified this medium so that it could mineralize bacteria by our serum-free mechanism and determined whether it successfully calcified both dead and living bacteria. Lastly, we examined the impact of calcification on the bacteria's viability and growth rate. Permitting the study of live bacteria has much clinical relevance as it may provide the knowledge necessary to accomplish our long-term goal of understanding the consequences of bacterial calcification *in vivo.* We

are currently conducting preliminary experiments in a rat model to determine whether serum-induced calcification plays a role in fighting bacterial infections.

## MATERIALS AND METHODS

*Materials*---Male albino rats (Sprague-Dawley derived), with weights of 100g, were purchased from Harlan Labs (Indianapolis, IN). Dulbecco's Phosphate Buffered Saline (DPBS) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco (Grand Island, NY). Penicillin and Streptomycin were purchased from Invitrogen (Carlsbad, Ca). Sodium azide was purchased from EM Science (Gibbstown, NJ). DMEM was supplemented with penicillin and streptomycin and 10% azide. Didronel (etidronate disodium) tablets were purchased from Procter and Gamble Pharmaceuticals. The tablet was ground to a fine powder and added to a conical containing 50 ml of ultrapure water. The conical was rotated end-over-end for 30 minutes. The conical was spun down in a swinging bucket for 5 minutes at 4,000 rpm. The supernatant containing the drug was shell frozen and lyophilized. 10X MOPS Media was purchased from Teknova (Hollister, CA). 10% Buffered Formalin was purchased from Fisher Scientific (Pittsburg, PA). *Staphylococcus aureus* ATCC 25923 (lot # 485992, reference #085v) was purchased from MicroBioLogics Inc. (St. Cloud, MN).

*Methods*---*Preparation of Dead S. aureus bacteria*

A 5ml aliquot from a *S. aureus* glycerol stock was added to a flask containing 1L of Luria Broth (LB) and grown at 37°C with agitation for 8-19 hours. The culture was then spun down in a Sorvall superspeeds RC2-B centrifuge at 5,000 rpm (x4,068 g) in a GSA rotor for 10 minutes to pellet the bacteria. The supernatant was discarded. The pellet was resuspended in 40 mL of 0.5M EDTA, pH 7.5. The sample was rotated end-over-end at room temperature for 24 hours to kill the bacteria. The solution was spun down in an IEC Centra C12 Centrifuge at 8,500 rpm (x11,750 g) in a GSA rotor for 15 minutes. The pellet was resuspended in 40 mL of water and spun down in an IEC C12 centrifuge for 15 minutes. This

step was repeated four times. The final bacterial pellet was shell frozen and lyophilized. A 50 mg/ml solution of dead *S. aureus* in water was prepared for use in the experiments.

*Preparation of exponentially growing S. aureus*

The absorbance ($A_{600}$) of the culture was monitored with a spectrophotometer (Hitachi-2000) using a wavelength of 600nm and a one cm diameter cuvette. An aliquot from an overnight culture grown from a *S. aureus* glycerol stock was added to a flask containing 50 ml Luria Broth (LB) to achieve an initial $A_{600}$ of 0.04. This culture was grown at 37°C with agitation (150 rpm) for two hours until the bacteria reached its exponential growth phase ($A_{600}$~0.6-0.8). The culture was then spun down in a clinical centrifuge at 3,400 rpm for 10 minutes to pellet the bacteria. The supernatant was removed and the bacterial pellet was extensively washed with 1X MOPS media buffer, containing 0.2M HEPES buffer, pH 7.4, and 2% glucose.

*Preparation of the serum-free calcifying and control solutions*

The solution used for investigating the extent of the *S. aureus* bacterial matrix calcification was prepared at room temperature utilizing a procedure that was designed to achieve the near instantaneous mixing of calcium and phosphate. This would ensure that subsequent mineral formation would occur by homogenous nucleation in the resulting solution. One ml of 1X MOPS media buffer containing 0.2M HEPES, pH 7.4, 2% glucose, and 14mM $CaCl_2$ was placed into one 10x75mm test tube, and one ml of 1X MOPS media containing 0.2M HEPES pH 7.4, 2% glucose, and 14mM sodium phosphate buffer, pH 7.4, was placed into a second 10x75mm test tube. A Pasteur pipette was used to first, withdraw the 1 ml of $CaCl_2$ solution, then quickly and forcefully expel this solution into the second test tube containing 1ml of sodium phosphate buffer. This would achieve a final two ml volume

containing a 7mM calcium and phosphate solution. This procedure was repeated until a desired volume was achieved. A control 1X MOPS buffer solution with a final concentration of 1.32mM phosphate of was prepared by adding sodium phosphate buffer, pH 7.4 to 1X MOPS media buffer containing 0.2M HEPES, pH 7.4 and 2% glucose. For all fetuin-containing calcification solutions, 5mg of bovine fetuin per ml of 1X MOPS buffer was present. Unless otherwise noted, the bacteria that were tested using this procedure, were immediately added after mixing the solutions.

*Procedure for calcifying live S. aureus in the serum-free calcifying solution*

Experiments in 15 ml conicals were set up to determine the extent of calcification occurring within a bacterial matrix. Approximately $2.0 \times 10^8$ exponentially growing *S. aureus* bacteria were separately incubated in 15ml conicals at 37°C in a control solution or a calcifying solution containing added calcium and phosphate (see *Preparation of serum free calcifying solution* above for buffer description). The bacteria were treated under these conditions for a total of two sequential four hour incubations. After the first four hour incubation, the bacterial solutions were spun down and the supernatant was removed and saved. The bacterial pellets were re-suspended in a freshly mixed control or calcifying solution (see above for buffer description). The bacterial growth and extent of calcification were monitored by measuring the absorbance (see procedure below). After the second sequential four-hour incubation, the bacterial solutions were partitioned for further analysis. 500 µl of each solution was taken for $A_{600}$ measurements, 1 ml volume for biochemical analysis, 2 ml volume for Scanning electron microscopy (SEM), 30 µl for viable titer plating, and 100 µl for calcein staining analysis. Any remaining bacterial solutions were frozen in 10% glycerol for future use and analysis.

*Measuring the absorbance at a wavelength of 600 nm ($A_{600}$) to monitor the extent of calcification and bacterial growth.*

Approximately $2.0x10^8$ exponentially growing *S. aureus* bacteria were separately incubated at 37°C in 6ml of a control solution or a calcifying solution containing added calcium and phosphate (see above for buffer description). The bacterial growth and extent of calcification was monitored in the control and calcifying solution by measuring the $A_{600}$ using a Hitachi-2000 spectrophotometer. A 500 µl aliquot was removed for each $A_{600}$ measurement. A measurement was recorded before the start of incubation, after the first four hour incubation, and after the second four hour incubation to establish a baseline $A_{600}$ measurement. After each baseline measurement was taken, 25mM of EDTA, pH 7.5 was immediately added to the aliquot, and the $A_{600}$ was re-measured. The difference between the $A_{600}$ of the baseline measurement and after the treatment with EDTA is due to mineral. The same protocol was carried out to measure the extent of calcification for $7.8x10^7$ dead *S. aureus* bacteria.

*Quantification of bacterial calcification by calcein staining*

Calcein staining was utilized to detect mineral incorporation into the bacteria. After two sequential four hour incubations in a control or calcifying solution (see *calcification procedure* above), 30 µl of each bacterial sample was smeared onto an albumin-coated slide and air-dried. The slide containing the bacterial smear was then stained in a solution containing 20 µg/ml calcein in 1mM $NH_4OH$ for one minute to fluorescently label any mineralized bacteria. The smears were extensively washed by dipping each slide into a solution of 1mM $NH_4OH$ for three seconds. This wash was repeated three times in a fresh

solution of 1mM $NH_4OH$. Exposure to light was limited during the storage of the slide to maintain the quality of fluorescent labeling. The mineralized bacteria were quantified using light and fluorescent microscopy. The slide was viewed under a microscope with both fluorescent and visible light exposures. The percentage of mineralized bacteria was determined by the following equation:

% bacteria mineralized = (# fluorescent bacteria) / (total # of bacteria)

*Biochemical Analyses*

The calcium and phosphate incorporation into live *S. aureus* bacteria was determined by acid extraction. After two sequential four-hour incubations, 1 ml of each sample (see above) was centrifuged to pellet the bacteria. 0.5ml of 150 mM HCl was then added to each bacterial pellet to dissolve the mineral at room temperature overnight. The acid extracts were then quantitatively assayed for calcium and phosphate by a colorimetric assay [1,11].

*Scanning Electron Microscopy (SEM) an Energy Dispersive X-ray Spectrometry (EDX) Analysis of non-mineralized and mineralized S. aureus.*

Approximately $2.0x10^8$ exponentially growing *S. aureus* bacteria were separately incubated in either the control or calcifying solution (see *calcification procedure* above). 2ml of the bacteria at the specified time point were spun down in a Fischer Scientific Centra 228 Centrifuge at 3,400 rpm (x1,300 g) for 5 minutes and the supernatant was discarded. The bacterial pellets were then immediately resuspended in 3 ml of 10% buffered formalin phosphate and rotated end-over-end for at least 24 hours. 350 μl aliquots of the formalin fixed bacterial samples were transferred into separate microcentrifuge tubes and spun down in an Eppendorf Centrifuge 5415C for 5 minutes at 7,000 rpm. The supernatant was discarded. The bacterial pellets were then treated with sequential dehydration steps with 1ml of 20%, 50%,

70%, 90%, and 100% ethanol. A 10 μl of each sample was placed onto an ethanol-cleaned silicon wafer. Samples were analyzed at ~28,000 magnification with 7.50 kV by a Phillips XL30 ESEM FEG. Energy Dispersive X-ray Spectrometry (EDX) was performed using the Oxford EDX detector and INCA X-Ray Micro Analysis software to create an elemental map and elemental energy loss spectrum of the bacterial samples based on their chemical composition. Spot analysis allowed the analysis of a single bacterium.

*Effect of calcification on bacterial viability*

The affect of calcification on the bacteria's viability was determined by standard LB agar plate counts. Approximately $2.0x10^8$ exponentially growing *S. aureus* bacteria were incubated in either the control solution or calcifying solution (see above for *calcification procedure*) were plated on LB agar plates to determine the affect of calcification on the bacteria's viability. An aliquot was removed before the start of the incubation and several dilutions of the sample were made in 1X MOPS Minimal Media containing 0.2M HEPES buffer, and 2% glucose. 100ul of each dilution was immediately plated onto the LB agar. Each LB agar plate was incubated at 37° for 24 hours. This process was repeated after the first four hour incubation cycle and after the second sequential four hour incubation cycle. The colony forming units per milliliter was calculated for each sample.

*Effect of calcification on the growth of live S. aureus bacteria*

Approximately $2.3x10^9$ exponentially growing *S. aureus* bacteria were separately incubated in 6 ml of a control solution or a calcifying solution containing added calcium and phosphate (see above for buffer description) for two sequential 4 hr cycles at 37°C. The effects of calcification on the bacteria's division rate were determined. The samples were

spun down in a swinging bucket centrifuge at a speed of 3,400 rpm for 10 minutes. The bacterial pellets were re-suspended in a seeding buffer in a containing 2mM Calcium, 2mM Phosphate, and 1mg/mL fetuin in 1X MOPS medium. 0.05% yeast extract was added to induce the growth of the bacteria.

*In vitro experimental procedures*

Bacteria were separately incubated in either the calcifying solution or control solution. At each specified time point, aliquots were removed for each specific procedure (see materials and methods). There are three main time points described in these methods: The first is before the start of any incubation. The second time point is after the first four-hour incubation in either the calcifying or control solution. This specific time point is termed "the first four hour cycle or incubation". And the last time point is after the second sequential four-hour incubation in either the calcifying or control solution. This specific time point is referred to as "after the second sequential cycle or incubation".

Unless otherwise noted, the information from the "*Results*" section represents the data collected from one cumulative experiment containing several procedures. It is important to note that every procedure was repeated multiple times and have all produced similar results. The data presented in this thesis is therefore, a representation of these results.

*Animal Experiments---Injection of Etidronate and collection of serum*

Six 100g male albino rats (Sprague-Dawley-derived) were injected subcutaneously with etidronate at a dose of 25 mg / kg body weight/day for a total duration of 10 days. These rats were injected with etidronate two days prior to the *S. aureus* injections (*experimental group*). An additional six animals were inoculated with *S. aureus* only *(control group).* All twelve rats were killed by exsanguination while under isoflurane anesthetic 10 days after the

initial etidronate injection. The blood was clotted at room temperature for 30 minutes and spun down in a Fischer Scientific Centra 228 at 3,400 rpm for 10 minutes to obtain pure rat serum.

*Injection of S. aureus bacteria into the experimental group*

This procedure was based on the methods used by Hung, et al (Hung, Shyu et al. 2001) to inoculate animals in the experimental group with *S. aureus.* The peritoneal cavity of each rat was injected with 5ml Phosphate Buffered Saline (PBS) containing $1.5x10^9$ exponentially growing *S. aureus (*ATCC 25923) cells while under isoflurane anesthetic. The UCSD Animal Subjects Committee approved all animal procedures.

*Determining the effect of etidronate on serum-calcification activity*

The effect of the etidronate drug on the serum-calcification activity was determined by calcium assays in 24-well cell culture clusters (Costar 3524; Corning, Corning, NY) in a humidified incubator at 37°C and 5%$CO_2$. One ml aliquots from etidronate-treated serum (*see above)* or normal rat serum obtained by exsanguination from 44 day old rats were added to the wells. DMEM with added penicillin/streptomycin and sodium azide was added to the bordering wells to prevent evaporation and bacterial growth in the serum. A gel matrix was added into each well containing serum and incubated for 5 days. The procedures used to alizarin red stain the gel matrices have been previously described [22].

## RESULTS

*Developing a serum-free calcifying solution that maintains bacterial viability but prevents its growth*

The long-term goal of our study is to determine whether serum-induced bacterial calcification is a potential mechanism that the body uses to defend against infections. More specifically, we want to understand what kind of consequences calcification has on bacteria. However, this serum-induced mechanism is very complex and involves many ill-defined entities. In order to isolate the ramifications of calcification on the bacteria from any other confounding factors, we had to create a calcifying system that was free of serum. Additionally, the temporary disposal of serum allowed us to probe the effects of calcifying living bacteria, a phenomenon that was not possible because of its overwhelming growth in serum. Therefore, we had to first find a medium that allowed us to control and monitor the division rate of bacteria. We found that morpholinepropanesulfonic (MOPS) minimal medium, a buffer that was primarily designed to support the growth of the gram-negative bacteria *Escherichia coli*, actually maintained *S. aureus* bacterial viability while preventing its growth. Another beneficial feature of this buffer was that yeast extract could be added to induce bacterial division.

Next, we had to adapt this medium to create a serum-free calcifying solution that would mineralize bacteria by a mechanism (Figure 2) that is similar to what we understood occurred in the serum mechanism of calcification (Figure 1). This way, we can expect that any consequences observed on the bacteria that were calcified by the serum-free solution would also be the same consequences observed if bacteria were calcified in serum.

A serum-free calcifying and control solution was developed using 1X MOPS media as the buffer. The calcifying solution in 1X MOPS contained elevated concentrations of calcium and phosphate that guaranteed spontaneous and rapid apatite formation throughout the

solution. The high ion product of the calcifying solution was generated by rapidly mixing 14mM calcium and 14mM phosphate to achieve a homogenous mixture containing 7mM of each ionic component. Previous studies have shown that hydroxyapatite-like mineral forms throughout a high ion product solution within minutes of mixing [14]. However, because fetuin was added prior to mixing the constituents of the solution, there should be no evidence of mineral formation [14,23]. We hypothesized that mineral would only grow within the confines of a bacterial cell wall, where the large protein fetuin cannot reach.

The control solution in 1X MOPS, on the other hand, contained fetuin and concentrations of calcium and phosphate that were too low to form any apatite crystals spontaneously, and therefore would essentially remain free of mineral.

*Evidence that dead S. aureus bacteria are mineralized by incubation in the serum-free calcifying solution*

We first wanted to verify whether the incubation of dead *S. aurues* in the newly developed serum-free calcifying solution would reproduce similar results to that of the serum-induced mechanism. Approximately $7.8 \times 10^7$ dead *S. aureus* bacteria were separately incubated in 6ml of the control solution or the calcifying solution for at 37°C. Aliquots of each sample were removed during specified time points and used for different methods of analysis.

Calcein staining allowed the detection of mineral incorporation into the bacteria. Calcein is a fluorescent dye that binds to hydroxyapatite. With calcein staining, the percentage of the total bacteria mineralized could be calculated by simply viewing the same set of bacteria under visible and fluorescent light. Each bacterium within the viewable area of the objective lens will appear under visible light. However, the fluorescent calcein will only bind to hydroxyapatite, and thus only the mineralized bacteria will appear under fluorescent light. Figure 4 supplies evidence that dead *S. aureus* bacteria become robustly mineralized

after incubation in the serum-free calcifying solution for two sequential four-hour cycles. The top box in Figure 4 shows the total number of bacteria as seen under visual light, while the bottom box shows the same field of bacteria viewed under fluorescent microscopy. Only mineralized bacteria have taken up the calcein stain and are visible as bright green entities under fluorescent light. Both the total number of bacteria (top box) and the mineralized bacteria (bottom box) were quantified. The percent of mineralized bacteria was determined based on the fraction of these two numbers:

*% bacteria mineralized = (# fluorescent bacteria) / (total # of bacteria)*

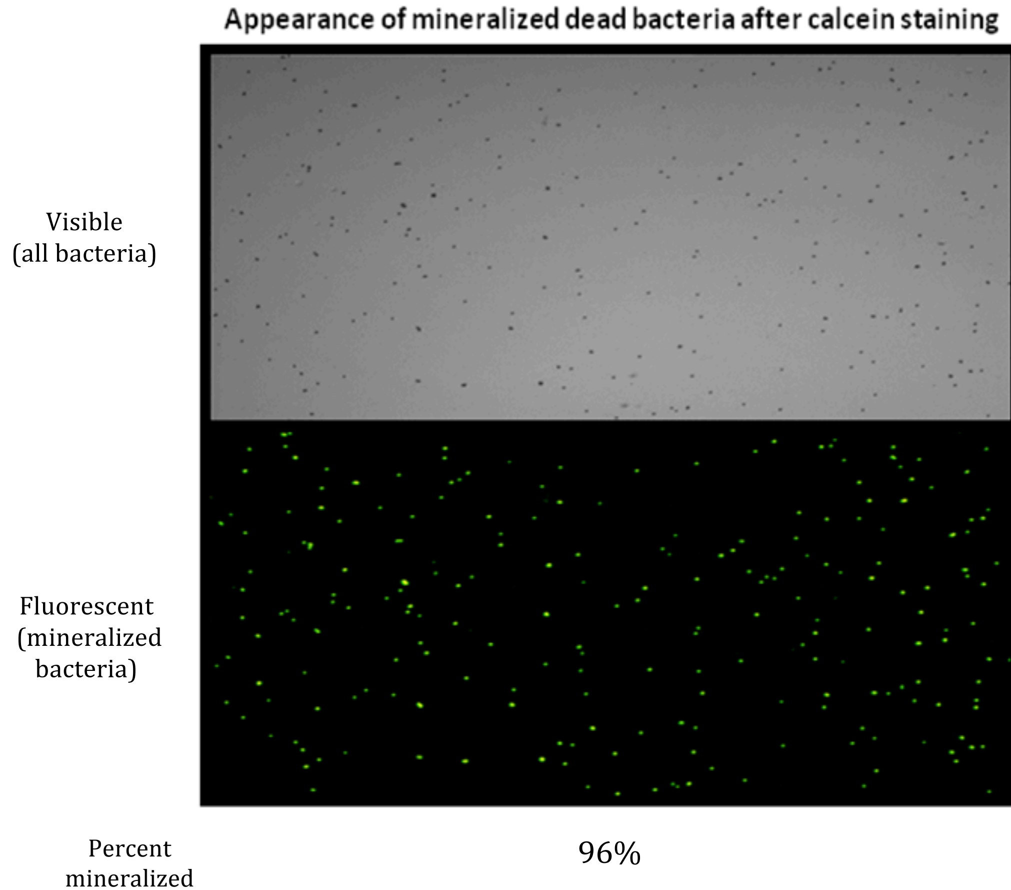



**Figure 4. Evidence that dead S. aureus bacteria are calcified by the serum-free mechanism after incubation in the calcifying solution.** Approximately 7.8x$10^8$ dead *S. aureus* bacteria were incubated in 6 ml of a calcifying solution containing added calcium and phosphate (see "Materials and Methods" for buffer description). After two sequential four hour incubations at 37°C, a 30 µl aliquot of each sample was smeared onto an albumin-coated slide and air-dried. The smear was then stained in 20 µg/ml calcein in 1mM $NH_4OH$ to fluorescently label any mineralized bacteria. The slides were extensively washed in 1mM $NH_4OH$. The mineralized bacteria were quantified using light and fluorescence microscopy. Top Box: Bacteria viewed under light microscopy at 20X magnification and 0.33s exposure. Bottom Box: The same bacteria viewed under fluorescence microscopy at 20X magnification and 1/250s exposure.
The percent mineralized = (# bacteria visible from Bottom Box/ # bacteria visible from Top Box) x 100.

The results of this experiment illustrated that 96% of the dead *S. aureus* bacteria were mineralized by incubation in the serum-free calcifying solution. With this data, we have proven that our new serum-free system has the capability of robustly calcifying a dead *S. aureus* bacterial matrix. It should be noted that the calcein staining method was also performed on bacteria that were incubated in the calcifying solution for only one four hour cycle (not shown). There was not a significant difference in the percent of total bacteria mineralized for either one or two calcification cycles. However, the bacteria that were incubated in the calcifying solution for two sequential four-hour cycles acquired a much stronger fluorescent signal than the bacteria that were only incubated for one four hour cycle. Further studies must be done to understand this phenomenon. The bacteria incubated in the control solution did not show any calcein staining (not shown) after the first or the second incubation cycle, and therefore were not calcified.

During the course of the experiment, we observed that the optical density of calcifying solution remarkably increased. Therefore, we invented a new method that utilized a spectrophotometric approach to also test the extent of bacterial mineralization. The absorbance at 600 nm wavelength ($A_{600}$) was measured before the start of any incubation, after the first four-hour incubation, and after the second four-hour incubation in either the calcifying or control solution to establish a baseline $A_{600}$ measurement. After each baseline measurement was taken ethylenediaminetetraacetic acid (EDTA), was immediately added to each aliquot and the $A_{600}$ was re-measured (Table 1). Because EDTA is a calcium chelator, EDTA will dissolve any mineral present in the solution. As a result, the measurement after the addition of EDTA is the absorbance due to the presence of bacteria. This experiment revealed that the absorbance due to bacteria remains relatively stable throughout the incubations in both the control solution and the calcifying solution.

It is of more interest to recognize the dramatic increase in absorbance that is due to mineral formation. The difference between the $A_{600}$ of the baseline measurement and the $A_{600}$ after the treatment with EDTA is due to mineral. The results observed in Table 1 show that the solution containing the calcified bacteria had an increase in $A_{600}$ due to mineral after the first four-hour incubation. Furthermore, there was an even a more dramatic increase in $A_{600}$ due to mineral after the second four hour incubation in the calcifying solution.

**Table 1. The increase in absorbance due to the calcification of dead *S. aureus* bacteria by the serum-free mechanism.** Approximately $7.8 \times 10^8$ dead *S. aureus* bacteria were separately incubated at 37°C in 6ml of a control solution or a calcifying solution containing added calcium and phosphate (see *Materials and Methods* for buffer description). The extent of bacterial growth and calcification was monitored by measuring the absorbance at 600nm ($A_{600}$) using a spectrophotometer. The $A_{600}$ was recorded before the start of incubation, after the first four hour incubation, and after the second four hour incubation to establish a baseline $A_{600}$ measurement. After each baseline measurement was taken, EDTA, a calcium chelator, was immediately added to the aliquot, and the $A_{600}$ was re-measured. The difference between the $A_{600}$ of. the baseline measurement and the $A_{600}$ after treatment with EDTA is due to mineral

| | Incubation in the Control Solution | | | Incubation in the Calcifying Solution | | |
|---|---|---|---|---|---|---|
| **Sample** | **$A_{600}$** | **$A_{600}$ after EDTA added** | **$A_{600}$ due to mineral** | **$A_{600}$** | **$A_{600}$ after EDTA added** | **$A_{600}$ due to mineral** |
| **Initial** | 0.36 | 0.33 | 0.03 | 0.38 | 0.35 | 0.03 |
| **After 1st 4h incubation** | 0.29 | 0.27 | +0.02 | 2.08 | 0.26 | 1.82 |
| **After 2nd 4h incubation** | 0.24 | 0.23 | +0.01 | 5.25 | 0.25 | 5.00 |

It is important to remember that fetuin is present in the solutions and will bind to any apatite crystals to prevent its growth. Because fetuin is too large to pass through the pores of the bacterial cell walls, the increase in $A_{600}$ is presumably due to the mineral that has formed within the bacterial matrix, where fetuin is not present. The $A_{600}$ changes due to mineral in the control solution are negligible. Both the calcein staining procedure and the $A_{600}$ measurements are evidence suggesting that bacteria are robustly mineralized in the calcifying solution but not in the control solution.

*Evidence that living S. aureus bacteria are mineralized by incubation in the serum-free calcifying solution*

Next, we wanted to determine whether live *S. aureus* bacteria could also be mineralized by our newly developed serum-free mechanism. Approximately $2.0 \times 10^8$ exponentially growing *S. aureus* bacteria were separately incubated in 6ml of a control solution or a calcifying solution containing added calcium and phosphate (see *Materials and Methods* for buffer description). Aliquots were removed at specified time points and analyzed.

The same calcein staining method described above was performed to detect the mineral incorporation into the live bacteria after two sequential incubation cycles (Figure 5). We again quantified and compared the number of mineralized bacteria (bottom box) to the total number of bacteria (top box). We calculated that 99% of the bacteria were mineralized in the calcifying solution. This calcein staining procedure was repeated on bacteria that were incubated in the control solution for two sequential cycles. The results seen in Figure 6 show that 0% of the bacteria was calcified after incubation in the control solution. Calcein staining was also performed on bacterial samples that were incubated in the control or calcifying solution for only one four hour cycle. As observed with the dead *S. aureus*, the percent of

bacteria mineralized did not differ significantly when comparing one versus two four hour incubation cycles. However, the bacteria that were mineralized in two sequential calcifying cycles displayed a brighter color, and thus had a stronger fluorescent signal than the bacteria that were mineralized in only one cycle. There were no noticeable differences seen in the non-mineralized bacteria that were incubated in the control solution for one or two four-hour cycles.

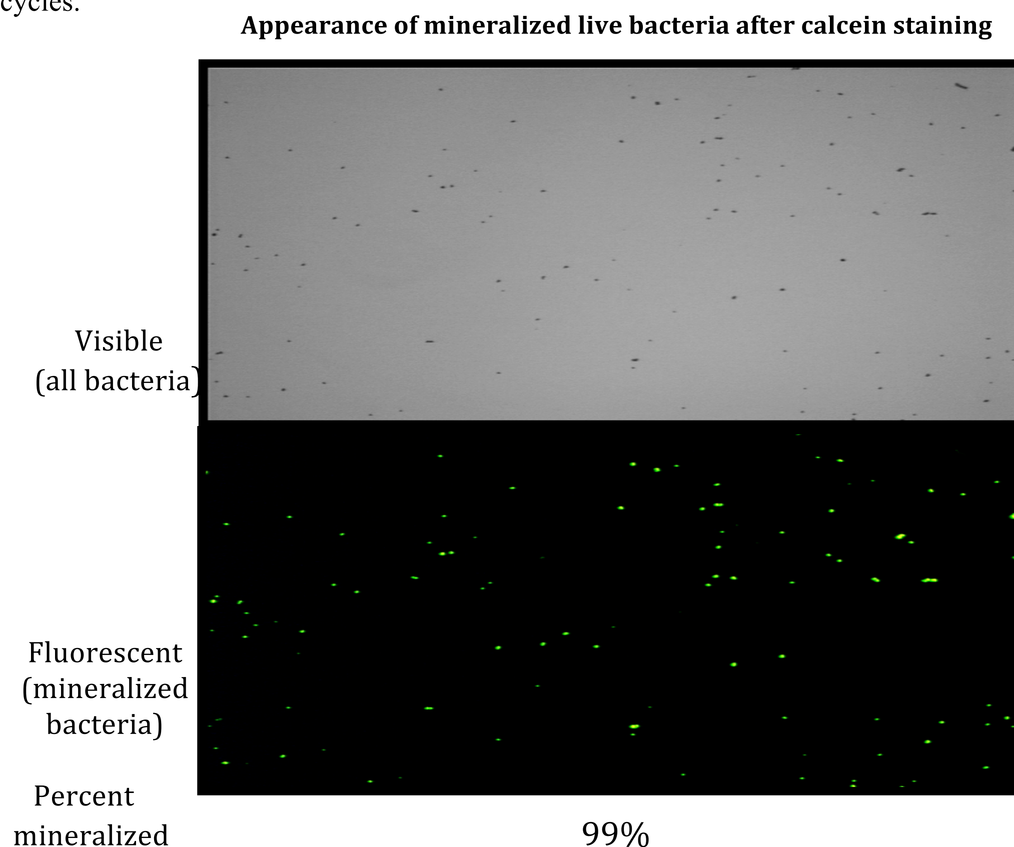



**Figure 5. Evidence that live *S. aureus* bacteria are calcified by the serum-free mechanism after incubation in the calcifying solution.** Approximately $2.0 \times 10^8$ exponentially growing *S. aureus* bacteria were incubated in 6 ml of a calcifying solution containing added calcium and phosphate (see *Materials and Methods* for buffer description). After two sequential four-hour incubations at 37°C, a 30 µl aliquot of each bacterial sample was smeared onto an albumin-coated slide and air-dried. The smear was then stained in 20 µg/ml calcein in 1mM $NH_4OH$ to fluorescently label any mineralized bacteria. The slides were extensively washed in 1mM $NH_4OH$. The mineralized bacteria were quantified using light and fluorescence microscopy.
Top box: Bacteria viewed under light microscopy at 20X magnification and 0.33s exposure. Bottom box: The same bacteria viewed under fluorescence microscopy at 20X magnification and 1/250s exposure.

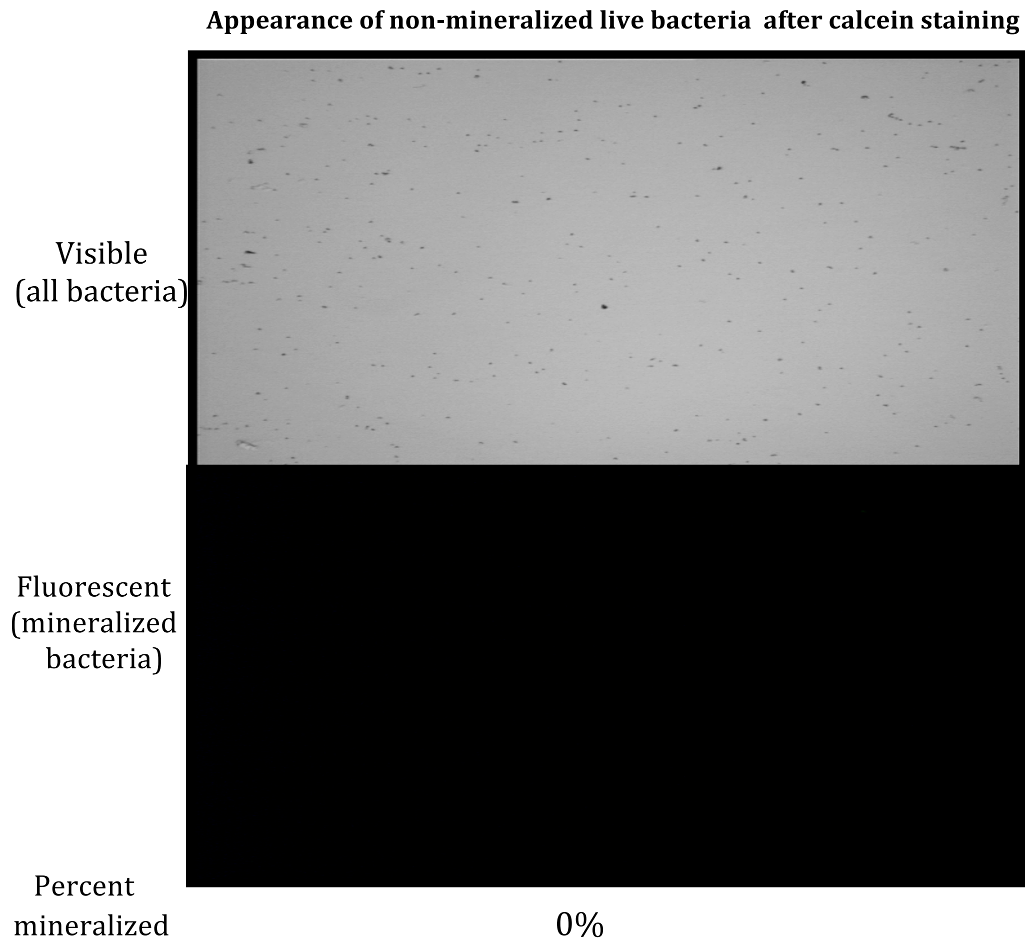



**Figure 6. Evidence that live *S. aureus* bacteria are not calcified after incubation in the control solution.** Approximately 2.0x$10^8$ exponentially growing *S. aureus* bacteria were incubated in 6 ml of a control solution containing no added calcium and phosphate (see *Materials and Methods* for buffer description). After two sequential four-hour incubations at 37°C, a 30 ul aliquot of each sample was smeared onto an albumin-coated slide and air-dried. The smear was then stained in 20 µg/ml calcein in 1mM $NH_4OH$ to fluorescently label any mineralized bacteria. The slides were extensively washed in 1mM $NH_4OH$. The mineralized bacteria were quantified using light and fluorescence microscopy. Top Box: Bacteria viewed under light microscopy at 20X magnification and 0.33s exposure. Bottom Box: The same bacteria viewed under fluorescence microscopy at 20X magnification and 1/250s exposure.

We also observed the $A_{600}$ changes in the calcifying solution containing the mineralized *S. aureus*. Because we were dealing with living bacteria, this method proved to be advantageous as it was utilized to detect both the extent of bacterial growth and calcification. The absorbance measurements discussed in this section will be referenced throughout the paper as it provides valuable information that can help interpret the other results. The $A_{600}$ was recorded before the start of incubation, after the first four-hour incubation, and after the second four-hour incubation to establish a baseline $A_{600}$ measurement. After each baseline measurement was taken, EDTA was immediately added and the $A_{600}$ was re-measured. We monitored the bacterial growth by comparing the $A_{600}$ measurements after the addition EDTA. Since EDTA dissolves mineral, the re-measured $A_{600}$ is attributed to the presence of bacteria. As seen in Table 2, the $A_{600}$ due to bacteria ($A_{600}$ after treatment with EDTA) stays relatively stable in both the control and calcifying solution. This data suggests that there was no significant bacterial growth or decline throughout the period of the experiment. Thus we successfully developed a serum-free system that prevented bacterial growth and allowed us to focus on the ramifications of calcification.

The difference between the baseline $A_{600}$ measurement and the $A_{600}$ measurement after EDTA is added is the absorbance due to mineral. It is evident that the bacteria incubated in the calcifying solution had a significant increase in absorbance due to mineral after the first four hour incubation with a value of 0.28, and an even more robust increase after the second four hour incubation with a value of 2.84 (Table 2). The bacteria incubated in the control solution, on the other hand, showed little to no absorbance changes due to mineral. Fetuin is present in both of the incubating solutions. Because fetuin is too large to pass through the pores of the bacterial cell walls [14], the mineral that has been formed in the solution, as implied by the increase in $A_{600}$, is presumably due to the mineral that has formed within the bacterial matrix, where fetuin is not present. On the other hand, the $A_{600}$ changes due to

mineral in the control solution are negligible. Both the calcein staining procedure and the $A_{600}$ measurements are evidence suggesting that live bacteria are robustly mineralized in the calcifying solution but not in the control solution.

**Table 2. The increase in absorbance due to the calcification of live *S. aureus* bacteria by the serum-free mechanism.** Approximately $2.0 \times 10^8$ exponentially growing *S. aureus* bacteria were incubated at 37°C in 6ml of a control solution or a calcifying solution containing added calcium and phosphate (see *Materials and Methods* for buffer description). The extent of bacterial growth and calcification was monitored by measuring the absorbance at 600nm ($A_{600}$) using a spectrophotometer. The $A_{600}$ was recorded before the start of incubation, after the first four hour incubation, and after the second four hour incubation to establish a baseline $A_{600}$ measurement. After each baseline measurement was taken, EDTA, a calcium chelator, was immediately added to the aliquot, and the $A_{600}$ was re-measured. The difference between the $A_{600}$ of the baseline measurement and the $A_{600}$ after treatment with EDTA is due to mineral.

| | Incubation in the Control Solution | | | Incubation in the Calcifying Solution | | |
|---|---|---|---|---|---|---|
| **Sample** | **$A_{600}$** | **$A_{600}$ after EDTA added** | **$A_{600}$ due to mineral** | **$A_{600}$** | **$A_{600}$ after EDTA added** | **$A_{600}$ due to mineral** |
| **Initial** | 0.50 | 0.50 | 0.00 | 0.55 | 0.55 | 0.00 |
| **After 1st 4h incubation** | 0.57 | 0.56 | +0.01 | 0.93 | 0.65 | 0.28 |
| **After 2nd 4h incubation** | 0.51 | 0.50 | +0.01 | 3.39 | 0.55 | 2.84 |

*Scanning Electron Microscopy (SEM): Morphological differences between mineralized bacteria and non-mineralized bacteria*

To determine any morphological differences between mineralized and non-mineralized bacteria, Scanning Electron Microscopy (SEM) was performed. Approximately $2.0 \times 10^8$ exponentially growing bacteria were separately incubated in the control or calcifying solution. After incubation at a specified time point, an aliquot of each sample was centrifuged to pellet the bacteria. The bacterial pellets were then resuspended in 10% buffered formalin to crosslink and preserve the bacterial morphology. After alcohol dehydration steps, the bacterial samples were placed onto silicon wafers and examined by SEM (see *Materials and Methods for detail)*. The images in Figure 7(A-C) are at ~28,500 magnification where the scale bar represents 1 μm. Figure 7A, 7B, and 7C represent the SEM of bacteria after incubation in the control solution, after the 1$^{st}$ four incubation in the calcifying solution, and after the 2$^{nd}$ four hour incubation in the calcifying solution, respectively. When comparing all three images, it is obvious that there are extreme morphological differences. The non-mineralized bacteria (as proven by calcein staining and absorbance readings above) in Figure 7A appear to have a smooth and spherical morphology. Each non-mineralized bacterium is approximately 1 μm in diameter. In contrast, the bacteria that were incubated in the calcifying solution for one four hour cycle (Figure 7B) have a rougher outer texture while maintaining a similar size to the control bacteria. It is our belief that this roughness in texture is due to the mineral that has formed within the bacterial cell wall by the serum-free mechanism. It is not clear whether the mineral extends beyond the interior of the cell wall. The bacteria that were incubated in the calcifying solution for two sequential cycles, on the other hand, have a much different morphological appearance (Figure 7C). The mineral incorporation is much more robust as

demonstrated by the extensive roughness in texture. Additionally, the bacteria have almost doubled in size.

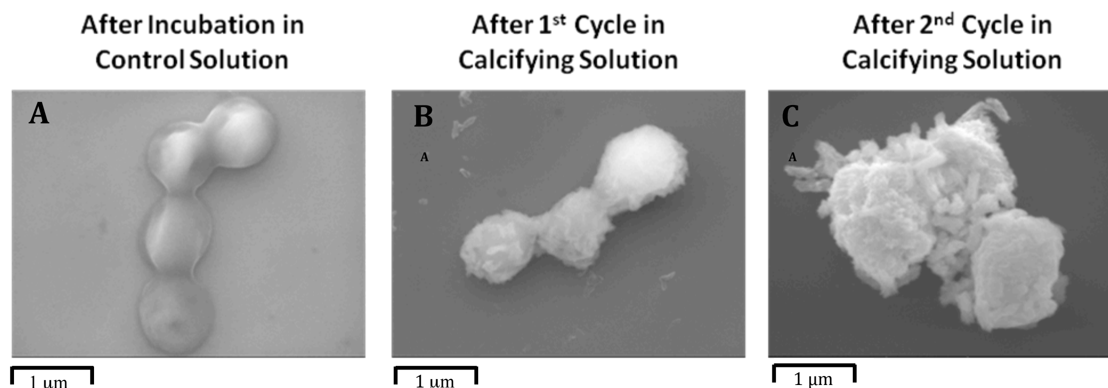



**Figure 7. Scanning Electron Micrograph (SEM) of mineralized and non-mineralized live *S. aureus* bacteria.** Approximately $2.0 \times 10^8$ exponentially growing *S. aureus* bacteria were separately incubated in 6 ml of a control solution or a calcifying solution containing added calcium and phosphate at 37°C (see *Materials and Methods* for buffer description). After incubation at the specified time point, an aliquot of each sample was centrifuged to pellet the bacteria. The bacterial pellet was re-suspended in 10% buffered formalin and rotated end-over-end for 24 h. The bacteria were then extensively washed in an ethanol gradient dehydration procedure (see *Materials and Methods*). A 10 µl aliquot of each sample was placed on a silicon wafer and examined by SEM at 7.50 kV at ~28,500 magnification.

The varying degrees of calcification can be associated with the different $A_{600}$ measurements due to mineral (Table 2). The non-mineralized bacteria in Figure 7A is associated with an $A_{600}$ measurement due to mineral of +0.01 while the mineralized bacteria in Figure 7B and 7C correlate to an $A_{600}$ measurement of 0.28 and 2.84, respectively (See Table 2). When viewing these image and its associated absorbance measurements due to mineral, it is difficult to question the fact that we have successfully calcified living bacteria.

*Analyzing the elemental composition of bacteria mineralized after incubation in the serum-free calcifying solution*

Now that it has been established that we successfully calcified live *S. aureus* bacteria in our newly developed serum-free mechanism, we wanted to determine the amount of mineral that was incorporated into the bacteria that were calcified for two sequential four hour cycles. An aliquot of each sample was spun down to pellet the bacteria, and 150 mM HCl was added to dissolve the mineral. The extract was analyzed for calcium and phosphate using the respective assays (see *Materials and Methods)*. The results seen in Figure 8 demonstrate that 4.11 μmol calcium and 3.77 μmol phosphate were incorporated into $4x10^8$ live bacteria that were incubated in the calcifying solution, while $<0.01$ μmol of calcium and phosphate were incorporated into bacteria incubated in the control solution. The incorporation of calcium and phosphorus into the bacteria incubated in the calcifying solution parallels the increase seen in absorbance (Table 2).

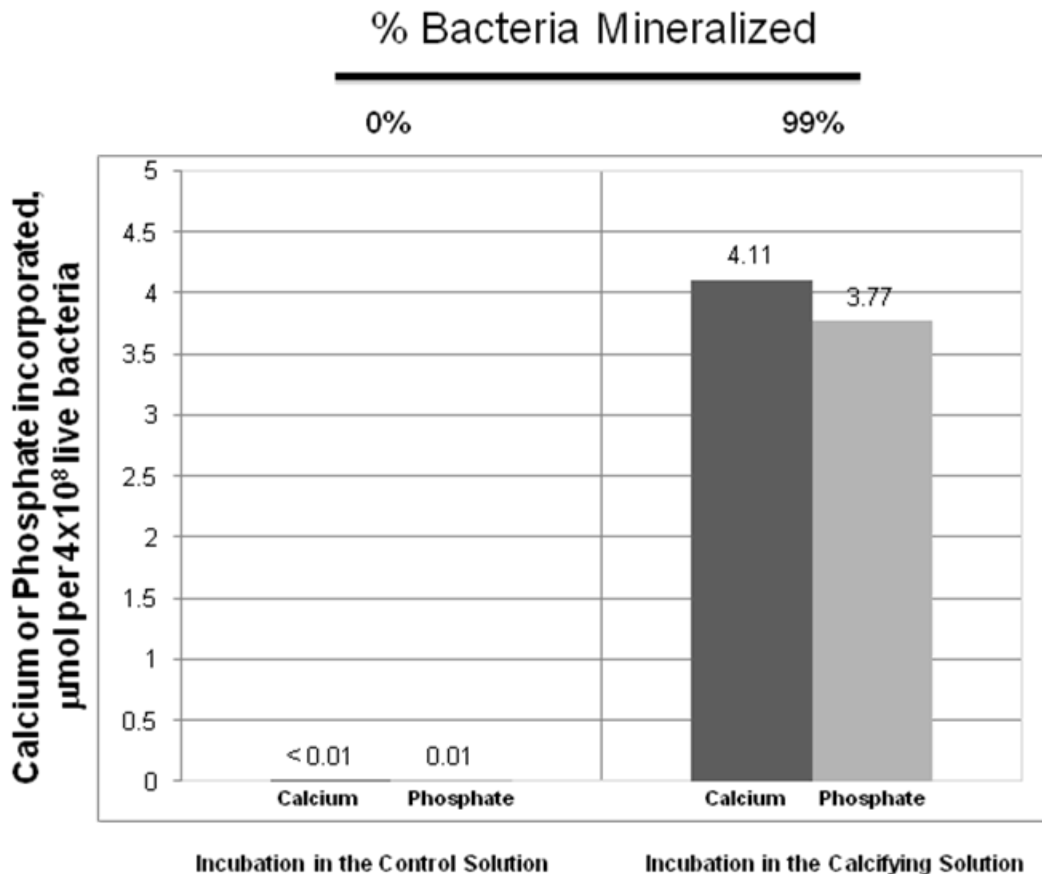



**Figure 8. Analysis of calcium and phosphate incorporation into live *S. aureus* bacteria after two sequential four-hour incubations in a control and calcifying solution.** Approximately $2.3x10^9$ exponentially growing *S. aureus* bacteria were separately incubated in 6 ml of a control solution or a calcifying solution containing added calcium and phosphate 37°C (see *Materials and Methods* for buffer description). After two sequential four-hour incubations, 1 ml of each sample was centrifuged to pellet the bacteria. 150mM HCl was then added to each bacterial pellet to dissolve mineral. The calcium and phosphate in each extract was analyzed as described under *Materials and Methods.*

Energy Dispersive X-ray Spectroscopy (EDS) was utilized to create elemental maps of the bacterial samples based on their composition. This system creates an image that demonstrates the spatial distribution of elements that are present in each sample. Figure 9 represents elemental maps that analyzed the presence of calcium and phosphorus in bacteria that were incubated in two sequential calcifying cycles versus bacteria that were incubated in the control solution. The left hand column represents the calcium maps while the right hand

column represents the phosphorus maps. The image located in the center is a SEM photo to allow the visual comparison of the elemental maps to the morphology of the bacteria. The SEM images have been adjusted to have the same size scale as the elemental maps. It is evident that calcium and phosphorus that are seen in the elemental maps (characterized by the dense red and orange color) are localized to the areas where the mineralized bacteria (bottom row) are present. Conversely, only background noise appears after analyzing the calcium and phosphorus content in the non-mineralized control bacteria.

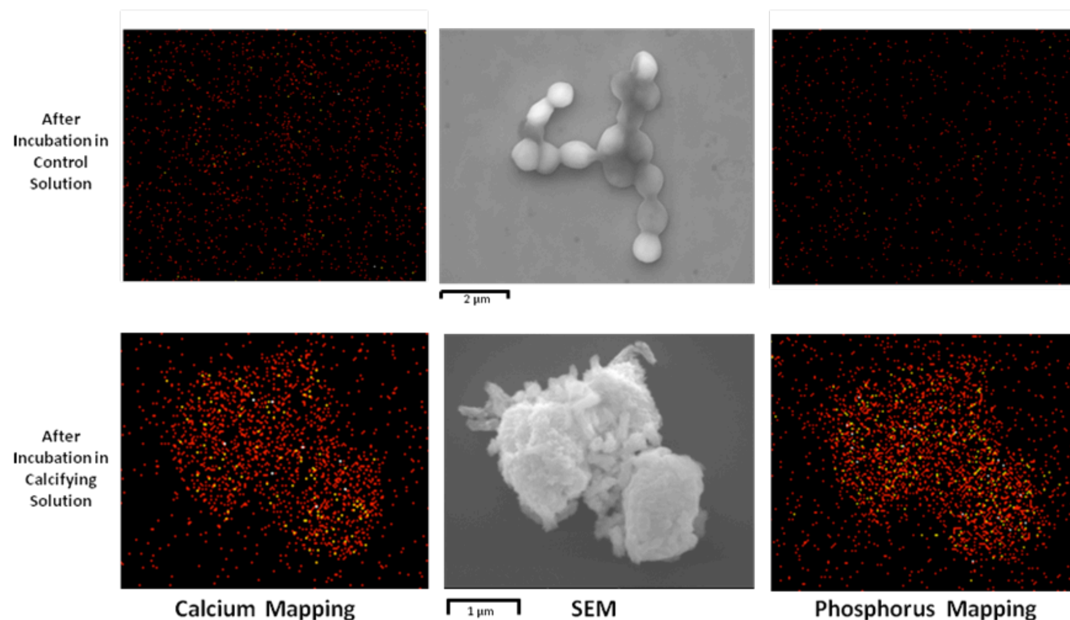



**Figure 9. Elemental Mapping of live *S. aureus* bacteria after incubation in the serum-free calcifying and control solutions.** Energy Dispersive X-ray Spectroscopy (EDS) was utilized to create elemental maps of the bacterial samples based on their composition. This system creates images showing the spatial distribution of elements in each of the samples. The left hand column represents the elemental calcium maps while the right hand column represents the elemental phosphorus maps. The image located in the center is a scanning electron micrograph to allow visual comparison of the elemental maps to the morphology of the bacteria. The scale bars have been adjusted to 2 µm and
1 µm for the bacteria incubated in the control solution or the calcifying solution, respectively.

Another way to examine the elemental composition of our bacterial samples is to produce an electron energy loss spectrum (EELS) using EDS. By doing a spot analysis using this system, we were able to identify the elemental composition of a single bacterium. Elemental analysis revealed distinct peaks for calcium and phosphorus in the bacterium that was incubated in the calcifying solution but not in the bacterium incubated in the control solution (data not shown). Additionally, EELS analyzed the weight ratios of the different elements present in the sample. By normalizing the values of the elements' weight ratios to carbon, we were able to establish relative weights of calcium and phosphorus. The data shown (Table 3) implies that the there is an increase and calcium and phosphorus in a single bacterium after each sequential incubation in the serum-free calcifying solution. Moreover, we were able to calculate the molar ratio of Ca/P incorporated into a mineralized bacterium as 1.44 after the 1$^{st}$ four-hour incubation in the calcifying solution and 1.49 after the 2$^{nd}$ for hour incubation in the calcifying solution (Table 3). These numbers are similar to the ratio of Ca/P found in biological apatite, [10,11,24,25].

**Table 3. Increase in calcium and phosphorus in a single bacterium after incubation in the serum-free calcifying solution.** Energy Dispersive X-ray Spectroscopy (EDS) system was used to identify the different elements incorporated into the specified bacterial samples to produce an energy loss spectrum. By doing a spot analysis using this system, we were able to identify the elemental composition of a single bacterium. Elemental analysis revealed distinct peaks for Calcium and Phosphorus in the bacterium that was incubated in a calcifying solution but not in bacterium incubated in the control solution. By normalizing the mass weights to Carbon, we were able to establish a molar ratio of Ca/P in each sample.

| | After 1st 4h Incubation in the Calcifying Solution | After 2nd 4h Incubation in the Calcifying Solution |
|---|---|---|
| Weight Calcium | 9.2 | 41.5 |
| Weight Phosphorus | 5 | 21.7 |
| Molar Ratio of Calcium to Phosphorus | 1.44 | 1.49 |
| Weight Ratio of Mineral to Carbon | 0.25 | 1.08 |

*The effect of calcification on the growth rate of live S. aureus bacteria*

The data that has been presented thus far show that live *S. aureus* is mineralized by the serum-free mechanism. We hypothesize that this robust mineralization may affect bacterial growth and generation time. Approximately $2.3 \times 10^9$ exponentially growing *S. aureus* bacteria were separately incubated at 37°C in either a control or calcifying solution for two sequential four-hour cycles at (see *Materials and Methods* for buffer information). The bacteria incubated in the calcifying solution were extensively mineralized while the bacteria incubated in the control solution were not mineralized. Aliquots of the mineralized and non-mineralized bacteria were spun down and their corresponding incubation solutions were removed. The mineralized and non-mineralized bacteria were then resuspended and incubated in seeding buffers containing identical constituents. The seeding buffer was composed of 1X MOPS media containing 1mg/ml fetuin to selectively inhibit apatite growth outside of the matrix and 2mM concentrations of calcium and phosphate. The concentrations of calcium and phosphate in this seeding buffer were versatile for the purpose of this experiment, as it proved to be low enough to prevent spontaneous apatite formation but high enough to maintain any mineral that was previously formed. Moreover, the seeding buffer was a source that allowed any crystals that were already incorporated within the bacterial matrix to grow further. Because this seeding buffer was still composed of MOPS media, a bacteriostatic medium for *S. aureus*, equal amounts of yeast extract had was added to each sample induce bacterial growth. This way, we were able to monitor the division rate of bacteria that remained calcified throughout the growth curve. We compared this division rate to the rate of the non-mineralized control bacteria. We utilized spectrophotometery to monitor the growth of the bacteria by frequently measuring the absorbance ($A_{600}$) changes at specified intervals. The $A_{600}$ of the samples at t=0, measured immediately after the bacteria were added to the seeding buffer containing yeast extract denoted the start of the growth experiment. The growth curve (Figure 10) depicts $A_{600}$

as a function of time on a semi-logarithmic graph. The trend of red diamonds represent the growth rate of bacteria that were previously incubated in the control solution (non-mineralized bacteria), while the trend of green squares represent the growth rate of the bacteria that were previously incubated in the calcifying solution (mineralized bacteria).

The non-mineralized control bacteria (*red*) show signs of growth immediately after being incubated in the seeding buffer. In contrast, the mineralized bacteria (*green*) have a prolonged lag period and do not show any signs of growth for at least two hours. This data suggests that bacterial calcification may lead to suppression in growth and consequently a delayed lag time.

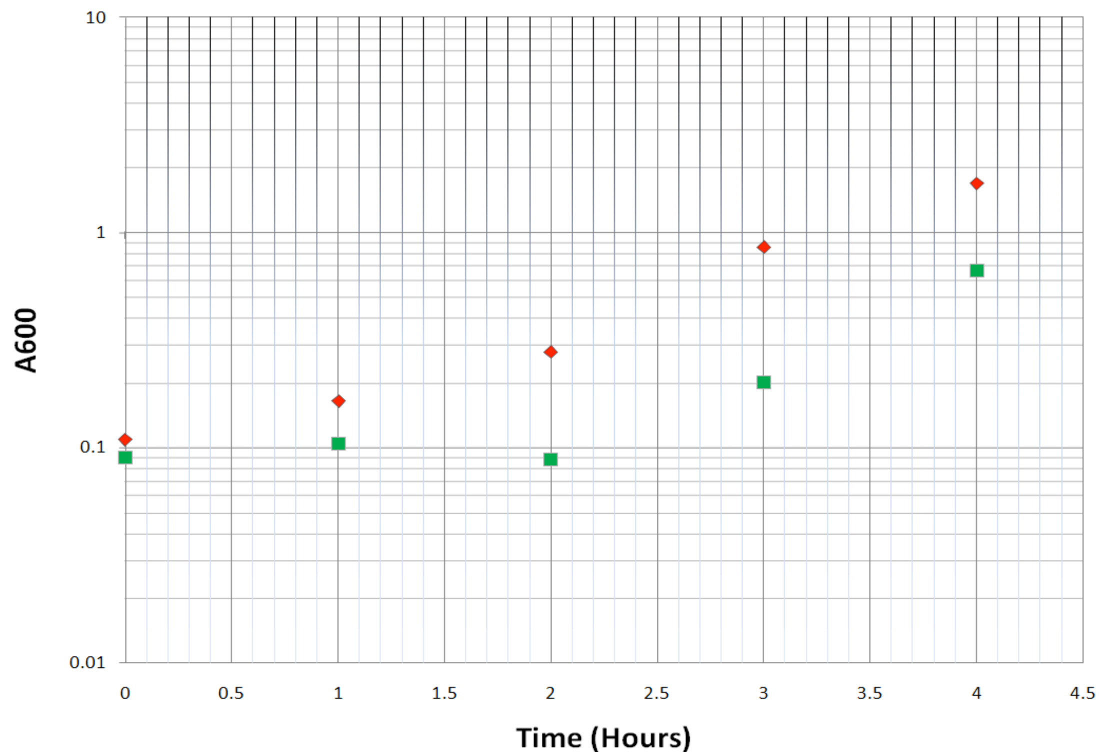



**Figure 10. Growth Curve of Live S. aureus after two sequential four-hour cycles in the calcifying and control solution** Approximately $2.3x10^9$ exponentially growing *S. aureus* bacteria were separately incubated in 6 ml of a control solution or a calcifying solution containing added calcium and phosphate at 37°C for two sequential four-hour cycles (see *Materials and Methods* for buffer description). The affects of calcification on the bacteria's division rate were determined. The samples were spun down in a swinging bucket centrifuge at a speed of 3.,4000 rpm for 10 minutes. The bacterial pellets were re-suspended in a seeding buffer containing 2mM Calcium, 2mM Phosphate, 1mg/mL fetuin. 0.05% yeast extract was added to induce the growth of the bacteria. The trend of the red diamonds and the green squares represent the growth rate of the bacteria incubated in the control solution and the bacteria incubated in the calcifying solution, respectively.

*The impact of calcification on the viability of S. aureus bacteria*

Next, we wanted to determine whether the extensive calcification impacted the viability of *S. aureus* bacteria. To test this, the number of viable bacteria was determined using LB agar plates. Approximately $2.0 \times 10^8$ exponentially growing *S. aureus* bacteria were incubated in either a calcifying solution or a control solution. Before the start of any incubation, an aliquot of each bacterial sample was removed and several dilutions were made. 100 μl of each dilution was immediately inoculated onto the LB agar plate and incubated at 37°C for 24 hours. This procedure was repeated after the 1$^{st}$ four-hour incubation and again after the 2$^{nd}$ four-hour incubation. Both bacterial samples had the same initial colony count. After each specified time point, the impact of bacterial calcification on viability was assessed by comparing the CFU/ml grown from the mineralized bacteria that were incubated in the calcifying solution, to the CFU/ml grown from the bacteria incubated in the control solution. Table 4 reveals that the mineralized bacteria showed little decrease in viability (<5%) after the 1$^{st}$ cycle in the calcifying solution. However, after the 2$^{nd}$ cycle in the calcifying solution, a significant decrease in viability (69%) was observed. The $A_{600}$ due to mineral (Table 2) is shown to highlight the fact that the increasing bacterial calcification parallels the decrease in bacterial viability.

**Table 4. The impact of calcification on the viability of live S. aureus bacteria.** The impact of calcification on the bacteria's viability was determined by standard LB agar plate counts. Live *S. aureus* bacteria were calcified as described in the Figure 4 legend. Before the start of any incubation, an aliquot of each bacterial sample was removed and several dilutions of each sample were made. 100 µl of each dilution was immediately plated onto the LB agar and incubated at 37° for 24 hours. This procedure was repeated after the first 4h cycle in the calcifying solution and again after the 2$^{nd}$ cycle in the calcifying solution. At each time point, the CFU/ml present in the bacteria incubated the calcifying solution was determined (see *Materials and Methods* for equation) and compared to the CFU/ml calculated in the bacteria incubated in the control solution. The table below shows that there is a <5% decrease in viability after the first calcifying cycle but a 69% decrease in viability after the 2$^{nd}$ calcifying cycle. The $A_{600}$ due to mineral (see Table 2 for method) is shown to highlight the fact that an increase in bacterial calcification parallels the decrease in bacterial viability.

| Sample | Decrease in Viable Bacteria | Increase in Bacterial Calcification ($A_{600}$) |
|---|---|---|
| **After 1$^{st}$ Cycle in the Calcifying Solution** | **<5%** | **0.28** |
| **After 2$^{nd}$ Cycle in the Calcifying Solution** | **69%** | **2.84** |

*Evidence that etidronate administration inhibits serum-calcification activity in rats*

Our studies done with live bacteria have inspired us to explore the roles of bacterial calcification *in vivo*. We are currently conducting preliminary experiments in a rat model to determine whether serum-induced calcification plays a role in fighting bacterial infections.

Previous studies in our lab have shown that first generation bisphosphonate drugs inhibit normal bone mineralization at doses comparable to those that inhibit bone resorption [26,27]. The initial animal experiments were carried out to determine the effects of etidronate, a bisphosphonate drug, on the serum-calcification activity in rats.

Rats were injected subcutaneously with etidronate at a dose of 25 mg/kg body weight/day. A preliminary experiment was done to test whether three sequential days of injections would inhibit serum calcification activity, since this was the time at which *S. aureus* bacteria would be injected. Blood of the etidronate-treated rats were obtained by exsanguination, and the serum was collected (See *Materials and Methods*). Acrylamide gel matrices, which have the correct size exclusion properties, have previously been shown to calcify in normal rat serum when incubated under physiological conditions. Acrylamide gels were incubated in the serum from etidronate-treated rats to see if the drug had successfully inhibited calcification activity. After several days of incubation, the gels were stained with Alizarin [10] and no distinct foci throughout the gel matrix were detected. In contrast, stained gels that were incubated in control rat serum under identical incubation conditions, exhibited numerous calcification foci throughout the gel matrix. The results of this experiment imply that we have successfully determined a dose of etidronate that effectively prevents the serum calcification activity in blood. This suggests that rats treated with etidronate will lose the ability to calcify bacteria in serum.

To further support that this dose of etidronate was sufficient enough to knock out serum calcification activity, the serum from etidronate-treated rats was diluted to 5% in

DMEM. Again, Alizarin stained gel matrices did not show any signs of foci even after two weeks of being incubated in this diluted serum. This suggests that the injected dose is above and beyond what is needed for the interests of our experiments, guaranteeing that *S. aureus* will not become calcified by the serum-calcification mechanism. This allows us to compare the response against bacterial infections of normal rats that could calcify bacteria to etidronate-treated rats that are not able to calcify bacteria.

*The effects of injecting etidronate on the clearance of S. aureus bacteria from the peritoneal cavity of rats*

Successfully establishing a dose of etidronate that inhibits all normal calcification in rats enables us to answer our key question: What role does bacterial calcification have on a vertebrate's defense against infections? The method we utilized to answer this question is illustrated in Figure 11 below. First, we subcutaneously administered the drug, etidronate, for several days at a dose that inhibited all serum calcification activity. Next, we injected a specified number of exponentially growing *S. aureus* bacteria into the peritoneal cavities of the rats. Then, we tried to determine whether inhibiting the serum calcification activity in these etidronate-treated rats had any affect on their ability to fight bacterial infections.

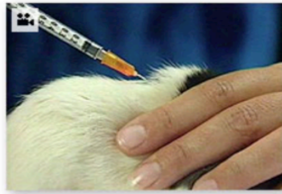


**Figure 11. Preliminary experiment to determine if serum-induced calcification plays a role in fighting bacterial infections in the peritoneal cavity**

Since, it is our working hypothesis that bacterial calcification *in vivo* may play a role in the defense against infections, and it was presumed that the etidronate-treated rats have lost the ability of to calcify bacteria by the serum-induced mechanism, we believed that these rats would have a more difficult time clearing the bacteria than the control rats. What we did observe were nodules that were distributed throughout the peritoneal cavities of both the control rats and the etidronate-treated rats. Additionally, we performed a peritoneal lavage on the rats ten days after the bacteria were inoculated. We attempted to analyze the number of viable bacteria present in the control and etidronate-treated rats by plating the lavage fluid on LB agar. We observed more growth on the plates that contained lavage fluid from the etidronate-treated rats. While this finding was promising, there were too many inconsistencies in acquiring the lavage fluid, such as the total volume re-collected from the rats or the different potential cells present in the fluid that could have grow on LB, for proper analysis to be conclusive. Therefore, we could not properly analyze our hypothesis since we did not work out a procedure well enough to determine the ability of a rat to fight bacterial infection.

Though these results did not provide the information we needed in order to answer whether bacterial calcification had an affect in the defense against infections, it did provide constructive information on how we could improve this experiment. These improvements will be described in the discussion.

## DISCUSSION

It is our working hypothesis that the calcification of live *S. aureus* bacteria in serum may be involved in a vertebrate's immunological response against bacterial infections. Previous studies in our lab have demonstrated that dead *S. aureus* bacteria are calcified in serum [1]. In these previous studies we accomplished two important objectives. First, we verified that a bacterial matrix could be robustly calcified in serum, and second, we determined the fundamental mechanism of this serum-induced calcification. The results suggest that the mechanism that drives the mineralization of Type I collagen based on a size exclusion principle is likely to mineralize bacteria for the same reasons [13]. The four key features [1,10] that are necessary to cause bacterial calcification in serum are as follows: 1) Tissue non-specific alkaline phosphatase (TNAP) is required to activate a high molecular weight serum nucleator of apatite formation. This unidentified serum nucleator consists of one more proteins that are 50-150kDa in size. 2) The now activated serum nucleator is necessary to generate small apatite crystals (<6kDa in size) near the bacterial matrix. The normal calcium and phosphate concentrations in both rat and human serum, are such that mineral cannot form spontaneously. 3) A matrix must have the appropriate size exclusion characteristics. It has been shown that the cell wall of *S. aureus* bacteria allows molecules that are smaller than 14kDa to enter the interior of the matrix, while excluding molecules that are larger than 50kDa [15,16,17]. 4) A large inhibitor of growth, such as the protein fetuin (59kDa), which selectively inhibits the growth of any crystals outside of the matrix. Most crystals form complexes with serum fetuin that inhibit their growth. A few of the crystals are free, and because of their small size, are able to diffuse through the pores of the bacterial cell wall. Fetuin is too large to enter, and therefore, the crystals inside the matrix are free of fetuin and grow rapidly (Figure 1) [14].

The objective of our present studies was to continue the investigation of bacterial calcification and determine the impact calcification has on live bacteria. Though research involving serum-induced calcification has proven to be valuable, the composition of serum is very complex and not fully understood. Therefore, in order to understand the ramifications of calcification, and only calcification, we had to create a system that was free of serum. To accomplish this, we developed a chemically defined calcifying system that included elevated concentrations of calcium and phosphate that guaranteed spontaneous crystal formation, and purified serum fetuin to selectively inhibit crystal growth outside of the bacterial matrix.

From our current understanding of the serum mechanism, we have adapted and identified three key requirements that are necessary to mineralize a bacterial matrix in a serum-free calcifying system (Figure 2). These requirements are: 1) A crystallization inhibitor such as the protein fetuin (59 kDa). 2) A solution that would, in the absence of the inhibitor, form a crystalline phase. This was achieved by elevating the concentrations of calcium and phosphate to an ion product that guaranteed the spontaneous and rapid formation of mineral 3) A semi-permeable barrier, such as the bacterial cell wall, that excludes the inhibitor but allows the solution containing the constituents of the crystalline phase to enter.

If we have correctly identified the key features from the serum mechanism, then our serum-free system should also have the capability of mineralizing a bacterial matrix. The key differences between the two mechanisms are how and where the apatite crystals are formed. In serum, the unidentified serum nucleator is required to generate apatite crystals [12,14]. Because the nucleator is large in size, the crystals are only formed on the outside. Most crystals are bound by fetuin and therefore, not able to grow. The small crystals that are free of fetuin are able to penetrate through the pores of the bacterial cell wall, escaping fetuin's inhibition, and grow rapidly. The serum-free mechanism, on the other hand, has elevated concentrations of calcium and phosphate to spontaneously form crystals throughout the

solution. The crystals that grow within the interior of the matrix, are free of fetuin, and able to grow. The other aspects of the two systems are mechanistically identical.

*Dead S. aureus are mineralized by the serum-free mechanism*

Since it has been established that dead *S. aureus* are robustly calcified when incubated in serum, we first wanted to verify that the serum-free calcifying solution was also capable of mineralizing dead *S. aureus* bacteria. Calcein stain, a fluorescent dye that binds to mineral, was used to determine the extent of mineralization. The results of our calcein staining experiment illustrates that 96% of dead *S. aureus* are mineralized after incubation in the serum-free calcifying solution (Figure 4) and 0% of dead *S. aureus* are mineralized after incubation in the serum-free control solution. It is important to note that we developed a calcein assay that was much simpler and faster than the one previously used in the lab [1,28]. The older method required the bacteria to be submerged in a calcein staining solution for several hours and extensively washed before being able to place the bacterial samples onto a slide for viewing. The method we developed bypassed this arduous work and save hours of time.

After incubation in either the serum-free calcifying or control solution, the samples of bacteria were aliquotted and smeared onto an albumin-coated slide. This smear resulted in an evenly distributed population of bacteria, making it easier to view and quantify under a microscope. The dried smears containing the bacteria only required a one-minute submersion in a calcein solution in order to stain. The method of washing was to simply dip the slides into a mild basic wash for a couple of seconds.

In the course of this study, we developed another breakthrough method of monitoring the extent of mineralization. Because we observed a drastic increase in the optical density of the calcifying solution, we decided to validate the robust mineralization by a utilizing a

spectrophotometric approach. The absorbance ($A_{600}$) readings were measured throughout the duration of the experiment. We observed a slight increase in opacity in the calcifying solution after the first four-hour incubation and a dramatic increase after the second four hour incubation. A baseline measurement was taken before the start of any incubations, after the first four hour incubation, and after the second four hour incubation in the serum-free calcifying solution. By utilizing EDTA, a calcium chelator, we were able to monitor the extent of mineralization at each specified time point. Consequently, the $A_{600}$ measurements that were examined immediately after the addition of EDTA represented the absorbance that was due to the presence of bacteria. As expected, this number remained relatively constant throughout both incubation cycles suggesting that there was no gain or loss in bacteria. Therefore, the difference between the baseline $A_{600}$ measurement and the $A_{600}$ measurement after the treatment of EDTA must be due to mineral. Because fetuin, an inhibitor of apatite growth, is too large to enter the pores of the cell wall, mineral will only grow inside the bacterial matrix where it is free of fetuin. For that reason, the mineral responsible for the dramatic increase in opacity and $A_{600}$ measurements is presumably incorporated within the bacteria and not in the outside solution. No mineral was detected in the non-mineralized bacterial controls.

*Live S. aureus bacteria are mineralized by the serum-free mechanism*

The success of calcifying dead *S. aureus* lead to the interest of calcifying live *S. aureus* by the serum-free mechanism. The noteworthy advantage of using this serum-free calcifying solution was the potential to understand the consequences of mineralizing living bacteria. In the past, the attempts to calcifying live bacteria in serum and understand the consequences of calcification were not successful. The overwhelming division rate of bacteria in serum proved to be extremely difficult to separate the older mineralized bacteria from the newer non-mineralized progeny that may have become mineralized at a later time. The

resolution was to develop this serum-free system using MOPS minimal medium, a buffer that is known to support the growth of *Escherichia coli.* We found that MOPS media actually maintained *S. aureus* viability while preventing its growth, allowing us to probe the consequences of calcifying the same set of living bacteria throughout the duration of the experiments. This way, we can monitor the various growth rate and viability of specific bacterial samples as a function of time.

The calcein staining procedure done to detect mineral incorporation in the dead bacteria was also performed on live *S. aureus*. The results indicate that 99% of bacteria were mineralized after incubation in the calcifying cycle for two sequential four-hour cycles (Figure 5). We also observed an astounding increase in the optical density of the calcifying solution as the experiment was carried forward. We used absorbance measurements before and after the addition of EDTA to show that the increase in optical density is due to bacterial calcification (Table 2). This method proved to be more versatile and beneficial for the examination of living bacteria as it allowed us to monitor both bacterial growth and the extent of calcification. After a baseline measurement was recorded, EDTA was added to dissolve any mineral in the solution. The $A_{600}$ readings after the treatment of EDTA, representing the $A_{600}$ due to the bacteria, did not change much throughout the duration of the experiment, suggesting that the MOPS buffer successfully prevented bacterial growth. A more robust mineral incorporation, as signified by the $A_{600}$ due to mineral, was observed in the bacteria that were incubated in the calcifying solution for two sequential four-hour cycles than those bacteria that were only incubated for one four-hour cycle. The reason for this trend still needs to be examined further, but it can be deduced that the amount of time that the bacteria are incubated in the calcifying solution determines the extent of calcification. The bacteria incubated in the control solution did not show any signs of mineralization in either the calcein staining assay or the spectrophotometric measurements.

Calcifying live *S. aureus* bacteria in the serum-free system illustrated that we understood serum-induced calcification, and correctly isolated the key requirements that we hypothesized were necessary to mineralize a bacterial matrix.

*Dead S. aureus bacteria may be more extensively calcified than live S. aureus*

Though the results from the calcein staining assays implied that the majority of both dead and live bacteria, dead *S. aureus* seemed to be more easily and more robustly mineralized. This presumption can be supported by the more dramatic increase in $A_{600}$ measurements due to mineral that was observed in the dead bacterial samples incubated in the calcifying solution (Table 1). The $A_{600}$ measurements of the dead bacteria after the first and second four-hour incubation in the calcifying solution were 1.82 and 5.00, respectively. In contrast, $A_{600}$ measurements correlating to the live *S. aureus* bacteria were only 0.28 and 2.84. One may argue that the measurements observed in the dead *S. aureus* samples are higher because these numbers represent the mineral that is incorporated in every bacterium, and their may have been a larger number of dead bacteria that were initially incubated in the serum than the live. However, only $7.8 \times 10^7$ dead bacterial cells were separately incubated in an identical calcifying solution as $2.0 \times 10^8$ living bacterial cells. Therefore you would expect that the more numerous living bacteria would accumulate more total mineral in the solution since there are more cells available for mineral to grow in. However, the $A_{600}$ measurements suggest that $7.8 \times 10^7$ dead bacterial cells had more mineral formed in its solution than $2.0 \times 10^8$ living bacterial cells. This suggests that each individual dead bacterium is able to incorporate more apatite within its matrix. This may be because non-living bacteria have a much more hollow interior than living bacteria, and thus will have more space to accommodate mineral. Additionally, researchers that have done studies on *Staphylococcus* isolated from dental calculus have found that the vitality of some organisms may actually retard calcification [29]

Therefore, it is reasonable to believe that living bacteria might have a mechanism that resists the process of becoming mineralized. This presumption must be researched further.

*The morphological differences between mineralized bacteria and non-mineralized bacteria*

SEM was utilized to determine any morphological differences between the mineralized and non-mineralized bacteria. With these SEM images to provide further evidence, it is hard to question that we have successfully calcified living bacteria. Figure 7 demonstrates the extreme morphological differences between the bacterial samples. The non-mineralized control has maintained a round spherical shape while the two other samples, consisting of bacteria with varying degrees of calcification, have much rougher outward appearances. It is also evident that there is a progressive increase in calcification from bacteria that were incubated in the calcifying solution for one four-hour cycle to the bacteria that have been incubated in the same solution for two four-hour cycles. The bacteria incubated in one four-hour cycle have a rough outer appearance but maintained a comparable shape and size to the control bacteria. Although the bacteria that have been incubated in two four-hour calcifying cycles maintain a somewhat rounded shape, they have almost doubled in size. It is possible that this increase in size was caused by extensive mineral that have formed both inside and outside of the cell wall. It is believed that the mineral began to grow inside the bacterial cell wall. Once the interior of the cell wall became fully occupied with mineral, it is possible that the apatite crystals began to extend beyond the interior of the cell wall, making the bacteria appear larger. Another possibility is that the mineral growing within the bacterial matrix pushed against the inner walls, and consequently increased the volume within the bacteria. Moreover, the idea of calcification resulting in the death of the bacteria cannot be neglected. If this was the case, then it is reasonable to assume that the very robustly mineralized bacteria (Figure 7C) were probably dead or dying during the time it was being

mineralized by the calcifying solution. This is a practical theory since it is believed that dead bacteria are more easily calcified than live bacteria and other studies have illustrated that changes in morphology may indicate an altered bacterial function [30,31]. Further examination of this theory will be discussed later.

*The elemental analysis of live S. aureus that were mineralized by the serum-free calcifying solution*

We wanted to provide evidence to support the obvious physical differences between non-mineralized and mineralized bacteria by quantifying the mineral that was incorporated into the bacteria. Both acid extraction (Figure 8) and EDS (Figure 9) confirmed the presence of calcium and phosphorus in the bacteria that were incubated in the serum-free calcifying solution for one and two four hour incubation cycles, but not in the bacteria incubated in the control solution. Furthermore, EDS was utilized to create elemental maps of calcium and phosphorus (Figure 9). When viewing a side-by-side comparison to an SEM image, the elemental maps of the mineralized bacteria clearly suggest that the calcium and phosphorus are localized to the confines of the bacterial structure. However, it was EELS spot analysis that provided strong evidence to demonstrate that mineralization in the bacteria progressively increased from the first four-hour incubation in the calcifying solution to the second sequential incubation in the calcifying solution (Table 3). Since EELS determined the relative elemental composition in a bacterium, we were able to establish the relative weights of calcium and phosphorus when normalizing the weights to carbon. It was clear that both the calcium and phosphorus incorporation significantly increase the longer the bacterium is incubated in the calcifying solution. Interestingly, the molar ratios of Ca/P remained relatively constant between the two incubation periods. Since these numbers were comparable to the molar ratio of biological apatite, it is likely that the entity being formed by these elements within the

bacteria is actually hydroxyapatite [10,11,24,25]. A much more accurate analysis must be performed to confirm this speculation. What is more fascinating is the fact that weight of mineral in the bacteria calcified for two cycles is slightly higher than the weight of carbon. This suggests that the mineral that has incorporated into the bacterium is equal to weight of carbon, the very element that makes up the bacteria.

*Bacterial calcification slows down bacterial growth*

Once it was firmly established that living *S. aureus* bacteria were successfully calcified, we were interested to know what effects the robust mineral incorporation had on the growth rate and generation time of the bacteria. To test the impact of calcification on the bacterial growth, we took bacteria that were previously mineralized from two sequential four-hour incubations in the calcifying solution and compared its division rate to that of non-mineralized bacteria that were incubated in the control solution for the same duration. These two bacterial samples were separately incubated in an identical seeding buffer with concentrations of calcium and phosphate that maintained the mineral already present in the solution while preventing spontaneous formation of new crystals (see *Materials and Methods* for detail). This was to prevent the mineral that was incorporated within the calcified bacteria from dissolving while preventing any new mineral from forming within the non-mineralize bacteria. Because MOPS, a bacteriostatic medium for *S. aureus*, was still the buffer being used in the seeding solution, equal amounts of yeast extract were added to induce the bacterial growth. When graphed on a semi-logarithmic curve (Figure 10), it is apparent that the mineralized bacteria had a prolonged lag period and did not show signs of growth until two hours after the start of the incubation. In contrast, the non-mineralized bacteria control began to grow almost immediately after the start of the experiment.

These results suggest that bacterial calcification may lead to a suppression of growth, and consequently a delayed lag time. However, this hindrance in growth can be explained by several theories. If bacteria that are calcified even have the ability to divide, it is possible that the mineralization of bacteria, or the process of becoming mineralized, has affected the function or functions of the cell that are required for proper growth and generation. If this were true, then the calcified bacteria that were initially injured were able to recover and start dividing two hours after the non-mineralized controls. Nevertheless, such a delay can prove to have much potential for future medical application. If bacteria experience a prolonged lag time because of calcification, then potential defense mechanisms, both inborn and synthetic, will be given more time to become activated and work more efficiently. If bacterial calcification does occur in the body as we hypothesize, then this lag may allow more time for cells involved in the immunological response to recognize the presence of bacteria and even clear the infection. This delay also gives a better opportunity for pharmacological products, such as antibiotics, to target and kill the bacteria. Yet, even if no defenses were to act on the bacteria, the delay proves to cause such a substantial impact in itself, that, at any given time point, the number of bacteria in the mineralized population will always be magnitudes less than the number of bacteria in the population of non-mineralized controls. Another question that follows is whether the progeny of the now growing calcified bacteria acquire the characteristic of being mineralized.

Another theory to explain the delay in bacterial growth is the possibility of calcification actually killing the bacteria. Because absorbance readings do not discriminate between viable and non-viable cells, and our measurement of the number of bacteria that were initially incubated in the seeding buffer was based on the $A_{600}$, some of the bacteria that have contributed to the absorbance reading could have already been dead. Therefore, if a majority

of the mineralized bacteria were already dead at the time our growth curve experiment began, then only the small number of surviving bacteria was able to divide.

Whether this delay in growth was caused by cell injury, bacterial death, or even a combination of the two, the results of this experiment has shown that bacterial calcification may have potential for many clinical applications.

*Bacterial calcification has an impact on bacterial viability*

The results of the different experiments described above suggest that the bacteria may have been killed as a consequence of becoming mineralized. Therefore, it was beneficial to test this hypothesis in order to interpret the role of bacterial calcification on viability. To determine the effects on viability due to calcification, we determined the cfu/ml that grew on standard LB plates at specified time points. Because the food source to support *S. aureus* bacteria in MOPS may be limited, it was possible that the bacteria may have experienced cell death because of prolonged incubation periods. In order to account for this, the cfu/ml grown from the mineralized samples were compared to the cfu/ml grown from non-mineralized bacteria that contained the same initial number of exponentially grown bacteria and were incubated in the control solution for identical time periods. We found that there was little decrease in bacterial viability (<5%) after the first four-hour incubation in the calcifying solution. However, there was a staggering decrease in viability (69%) that was observed after the second four-hour incubation in the calcifying solution. This correlation demonstrates the calcification may have a major impact on bacterial viability. It is important to remember that the remarkable increase in mineral incorporation into the bacteria during the second four-hour incubation in the calcifying solution, as supported by the $A_{600}$, calcein, and SEM experiments, parallels this noteworthy decrease in viability. In other words, the more robust mineral incorporation is likely to be explained by the fact that the calcification may have killed the

bacteria, thus allowing more mineral incorporation into the more hollow interior of the dead bacteria that lack possible resistance to becoming mineralized. Additionally, this finding supports the theory, that the delay in bacterial growth observed in the mineralized bacteria, but not the non-mineralized bacteria, is in large part due to the fact that the majority of the bacteria were already dead, and therefore, only the few surviving bacteria were adequate to divide and contribute to the growth curve.

These optimistic discoveries induce the strong interest in knowing whether performing a third sequential incubation cycle in the calcifying solution would result in even more dramatic effects than those already seen. It is likely that the longer a bacteria sample is incubated in the calcifying solution the more robustly calcified it becomes. Therefore the question that comes to mind is, whether a third calcifying cycle would conclude in total eradication of living bacteria by calcifying the entire bacterial population.

However, more accurate and sensitive tests must be performed in order to verify these assertions since there are many confounding factors that may have contributed to these observations. One flaw that is inherent in the viable plate counting method is the assumption that one colony forming unit (cfu) directly correlates to one bacteria [31]. However, this is not likely to be the case, especially with bacteria such as *S. aureus*, which are frequently found in clusters [2,32] . Therefore, it cannot be known whether one cfu consists of one or more bacteria. This could mean that the cfu/ml counts in both the non-mineralized and mineralized bacteria were underestimated. It would be helpful to know whether one treatment, either incubation in a calcifying solution or control solution, is more likely to cause clusters than the other before solid conclusions are made. Additionally, we also assume that the lack of growth on the LB media implies that the bacteria are dead. In reality, calcification could have injured the cell where its functions that are required for growth were compromised, and thus, could not grow on the agar. Also, our viability counts were setup in such a way that mineral

incorporated in the calcified bacteria was likely to dissolve in the agar during the incubation period, since the media lacked adequate concentrations of calcium and phosphate to maintain the mineral. Therefore, it is not known whether the cfu that have grown on the plates represent the bacteria that were still calcified, or if the cfu represented de-mineralized bacteria whose mineral was already dissolved into the agar. Thus, there is still the probability that mineralized bacteria are actually incapable of growing on a nutrient rich medium such as LB.

Lastly, if we have successfully killed bacteria because of calcification by our *in vitro* serum-free mechanism, then we have introduced an opportunity to develop and optimize potential topical treatments for infections in the future.

*Factors involved in the bacterial calcification mechanism may play role in the immunological response against infections*

The goal of our research was to examine the potential roles that serum-induced calcification may play in fighting against bacterial infections. The results of our current serum-free experiments have shown that calcification has potential to affect the growth rate and viability of the bacteria. It should be noted that our serum-free calcification mechanism was meant to emulate what we hypothesize would happen in serum. Therefore, if the serum-induced calcification mechanism really is similar to the serum-free calcifying mechanism that we have developed in our studies, then we can presume that the calcification that occurs in serum would enforce similar consequences on the bacteria.

With this in mind, it is interesting that several studies have shown that factors required in the mechanism of bacterial calcification are also important factors that are involved in the immune response.

For example, the protein fetuin and its human analogue $\alpha_2$-HS-glycoprotein, are identified as potent opsonins that are released by the liver during an acute innate response to

bacterial infections. Fetuin has been identified to promote the phagocytosis of bacteria by neutrophils and macrophages [20,21]. Therefore it is possible that fetuin may play a dual role during bacterial infections. We believe that fetuin, an inhibitor of crystal growth, first promotes bacterial calcification by localizing mineral formation within the interior of the cell walls by the serum-induced mechanism. Then, fetuin may serve as an opsonin to promote the phagocytosis of the calcified bacteria.

Another factor that has been shown to affect the immune response is the presence of hydroxyapatite. Both the results of the previous study involving the calcification of dead *S. aureus* in serum [1] and the elemental composition analysis of our live bacterial studies suggest that the mineral that was formed within the bacterial matrix was indeed hydroxyapatite. Numerous studies have revealed that hydroxyapatite particles promote the release of various pro-inflammatory cytokines and help recruit various polymorphonuclear cells such as neutrophils, basophils, and eosinophils [18,19]. Since hydroxyapatite can be found within the interior of the bacterial cells, it is reasonable to believe that these immune cells are recruited to destroy the calcified bacteria.

The association of these calcification factors with the immune response, coupled to our results that suggest calcification decreases growth rate and viability, provides evidence to support that bacterial calcification can indeed play an important function in the defense against infections. More research must be performed to confirm these assertions.

*Conclusions of bacterial calcification in vitro*

Our studies were the first to demonstrate that living bacteria can be calcified by a potential mechanism our body may utilize to defend against infections. Further research must be performed to determine whether bacterial calcification leads to altered functions, prolonged changes in the ultrastructure, and greater susceptibility to phagocytes and antibiotics. Our

success in calcifying living *S. aureus* bacteria by the newly developed serum-free system illustrated that we understood serum-induced calcification and have correctly isolated the key requirements that we hypothesized were necessary to mineralize a bacterial matrix. Therefore, if we are correct in our presumption that bacterial calcification occurs in serum by a similar mechanism, then, the consequences of mineralization exemplified by the results of these studies may demonstrate what may potentially occur in serum.

In the future, it would be interesting to test whether serum-induced calcification would be effective on other potential pathogens such as other bacteria, viruses, and fungi.

*A Future study: Investigating the role of bacterial calcification on the clearance of S. aureus bacteria from blood*

Our present experiments have provided us valuable information that help lead us to the conclusion that living *S. aureus* bacteria are likely to be calcified in the blood by a mechanism that is based on the size exclusion characteristics of the cell wall, a feature that is unique to bacteria. Furthermore, this calcification has shown to have a substantial impact on both bacterial growth and bacterial viability. These results of our *in vitro* studies have encouraged us to explore the effects of calcification that would occur in a living rat model.

Our preliminary animal experiments were set out to test the effects of the bisphosphonate drug, etidronate, on serum-calcification activity. Previous studies have shown that the administration of etidronate in a rat inhibits normal bone mineralization [26,27,33] Because the mineralization of Type I collagen in bone is driven by the same serum mechanism that we hypothesize would also calcify bacteria, it was believed that etidronate would also prevent the mineralization of *S. aureus* bacteria when injected in the rats.

We subcutaneously administered the rats with 25mg/kg body weight/day etidronate to determine the effects of the drug on serum calcification activity. From this study, we

successfully determined an effective dose of etidronate that would prevent normal serum calcification. This dose of etidronate was effective for a duration that guaranteed that the bacteria would not become mineralized throughout the course of the experiment. Establishing an effective dose of etidronate can have noteworthy consequences. With this information, we now had the necessary tools to properly design an experiment that would target our long-term objective of the bacterial calcification studies: To explore the roles of serum-induced bacterial calcification *in vivo* on the defense against infections.

To test this objective, we carried out the experiment described in Figure 11. We first subcutaneously administered etidronate at a dose that inhibited bacterial calcification. Next, we injected a specified number of exponentially growing *S. aureus* bacteria into the peritoneal cavity of the rats. Then, we attempted to determine what kind of effects etidronate, and thus the inhibition of serum calcification activity, had on the rat's capacity to clear the infection. What we found at the conclusion of this experiment, was that the etidronate-treated rats that have lost serum calcification activity, and the control rats that maintained serum calcification activity, both formed nodules throughout their peritoneal and body cavities. However, we could not make any proper conclusions based on this observation since we did not work out a procedure well enough to even analyze the effects on bacterial clearance. Though these results did not provide information to tackle our main objective, it did provide constructive information on how we could optimize this experiment.

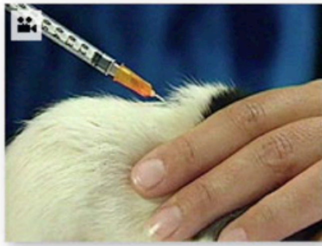
The procedure that we have developed to knock out serum calcification activity by the administration of etidronate can still be used as a valuable tool in our future experiments. However, we have realized that the fault in our preliminary experiment was the area chosen to inoculate the bacteria. Knowing that the very mechanism that drives bacterial calcification is in fact present in serum, it is only sensible to inject the *S. aureus* directly into the bloodstream. However, the limitations that were given by the animal studies committee, only allowed us to

inject bacteria into the peritoneal cavity. Since it was our interest to determine the effects of bacterial calcification, and the mechanism to drive bacterial calcification was present in serum, injecting *S. aureus* into the peritoneal cavity prevents us from really gaining the information that we need to answer our questions. This is because inherent defenses present in other parts of the body may inhibit the bacteria from ever reaching the blood, and thus, also preventing the possibility of the bacteria even becoming calcified by the serum mechanism. The nodules that were observed in the etidronate-treated rats and the normal control rats are probably examples of such an obstruction. More importantly, studies involving the clearance of bacteria from the blood are vital, since it is the bacterial infections that have entered the bloodstream that have the most fatal consequences. Understanding a potential mechanism that our body utilizes to clear infections in the blood is critical.

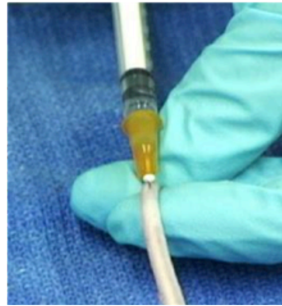
With this in mind, we are optimistic in our quest to understand the repercussions of serum-induced bacterial calcification *in vivo.* Figure 12 illustrates the future experiment we plan on carry out in order to explore this phenomenon further. First, we need to subcutaneously administer etidronate into the experimental groups rats, at the dose that we discovered would inhibit all serum calcification activity. Then we need to determine a sublethal dose of *S. aureus* bacteria, and identify the time required for a control rat to clear the infection. This dose of exponentially growing bacteria should be injected directly into the bloodstream of both the experimental etidronate-treated rats and control rats for the reasons stated above. Next, we need to compare the rate of bacterial clearance from the blood of calcification-inhibitor-treated rats to the rate from control rats. In theory, the etidronate treated rats will have lost the ability to calcify bacteria by the serum mechanism. Without this mechanism working, the *S. aureus* will not be calcified, and bacterial growth and viability are unlikely to be adversely affected in the same ways we saw in our *in vitro* studies. Therefore,

we hypothesize that etidronate-treated rats will have a more difficult time clearing the bacteria from the blood than the control rats.

1) Inject a drug that inhibits all serum calcification activity



2) Inject a sublethal dose of *S. aureus* directly into the bloodstream



3) Determine the rate of bacterial clearance from the blood of calcification-inhibitor- treated rats and control rats

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**Figure 12. Future experiment to determine if serum-induced calcification plays a role in fighting in fighting bacterial infections in blood**

If successful, this future experiment can prove to be a milestone for microbiologists and biochemists alike. More importantly, it can provide the information that is necessary for future clinical applications that can help save the thousands of people that die from bacterial infections everyday. Understanding and enhancing potential for the calcification of bacteria by the serum-mechanism as a defense mechanism, can pave the way for the development of new and more effective treatments for bacterial diseases.

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