UNIVERSITY OF CALIFORNIA, SAN DIEGO

S. aureus bacteria: A New Target of Serum Calcification Activity

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

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2009

The Thesis of Diane Jazmin Dy 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

2009

DEDICATION

This thesis is dedicated to my loving family and friends, who have always been there to support me and have pushed me to become a better person. I also dedicate this thesis to my deceased grandfather, Arturo Guevara, who is an exemplary example of how I want to live. His work ethic, honesty, perseverance, determination, his love for his family, and most importantly his love for life are just the few characteristics that define my grandfather.

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Figure 1 is a reprint of the material as it 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*, 4594-4604.

ABSTRACT OF THE THESIS

S. aureus bacteria: A New Target of Serum Calcification Activity

by

Diane Jazmin Dy

Master of Science in Biology

University of California, San Diego, 2009

Professor Paul Price, Chair

Staphylococcus aureus are gram- positive bacteria that cause skin and soft tissue infections. The continual incidence of infection is of great concern especially with the advent of methicillin resistant *S. aureus* (MRSA). Continued investigation on mechanisms our body uses to fight bacterial infection is vital. Our study suggests that the body takes advantage of a mechanism that mineralizes type-I collagen of bone and tendon to also mineralize bacteria. Serum driven bacterial mineralization may be a mechanism of innate immunity to control bacterial infection by affecting their viability or growth.

S. aureus was incubated in serum under various experimental conditions to determine the necessary factors in the mechanism of mineralization, as well as the extent and location of formed mineral. These experiments indicate that: 1) A large serum nucleator is required to generate mineral crystals 2) TNAP is essential for mineralization by activating the serum nucleator.

Inhibition of TNAP prevents bacterial mineralization and addition of pure TNAP restores the ability of serum to mineralize bacteria. 3) Fetuin is necessary for localizing mineralization within the interior of the bacteria. Without fetuin, mineral forms predominantly outside bacteria. These results demonstrate a similar mechanism of mineralization as type-I collagen 4)In addition, previous studies have demonstrated that bacteria have similar size exclusion properties as collagen[1,2,3]. Small molecules (< 14kDa) can penetrate the bacterial matrix while molecules (>50 kDa) are excluded [4,5,6]. Lastly, through TEM it is observed that the mineral is formed throughout bacteria and has a similar morphology to hydroxyapatite.

INTRODUCTION

Staphylococcus aureus bacteria commonly cause skin and soft tissue infections, and more serious infections such as bacterial endocarditis, abscesses, and osteomyelitis [7]. The increasing incidence of infection is of great concern especially with the advent of antibiotic resistant strains of *S. aureus*. Today there are many strains that are resistant to penicillin G. Although the introduction of methicillin largely contained the spread of penicillin resistant *S. aureus*, in 1961 methicillin resistant *Staphylococcus aureus* (MRSA) emerged. In 1997, the first case of vancomycin resistant *Staphylococcus aureus* bacteria arose. Currently, a strain of CA-MRSA, or community acquired MRSA is emerging that can cause pneumonia with a mortality rate of 30% in individuals that are co-infected with the influenza virus. There is a growing concern in fighting *Staphylococcus aureus* infection especially with their increasing resistance to antibiotics [8]. In this study, we examine one potential mechanism by which the body combats bacterial infection-bacterial mineralization by the previously discovered serum calcification factor. We believe that mineralization has the capacity to affect cell division, affect the mechanism of toxin transport, or affect the reaction of the immune system to bacteria.

A serum calcification activity, comprised of one or more proteins, was recently discovered while investigating the cause of normal bone calcification [9]. The serum calcification activity is 50 to 150 kDa in apparent size and has been discovered in all vertebrate species tested, which include human, rat, cow, cartilaginous fish, bony fish, and lamprey. The activity, however, was absent from invertebrates suggesting that its expression coincides with the evolution of a mineralized internal skeleton [1]. It has been demonstrated that this serum factor has the ability to calcify tendon, demineralized tibia, and calvaria, all of which are composed primarily of type I collagen [9].

By investigating the mechanism of serum-driven mineralization of type I collagen of bone, we have found that there are four requirements necessary for a matrix to become

mineralized in serum (refer to figure 1). First, a matrix must have size exclusion characteristics that allow small molecules (<6 kDa) such as calcium, phosphate or small mineral crystals, to access the matrix interior, while excluding large molecules (>40kDa) [2]. Second, a source of tissue-non-specific alkaline phosphatase (TNAP) is required to activate the serum calcification factor [3]. Third, the activated serum calcification factor must generate small crystals throughout serum, some of which diffuse into the aforementioned matrix [1,10]. Fourth, fetuin is required to ensure that mineral grows only within the matrix. Fetuin is the major calcification inhibitor in serum and is too large (48 kDa) to enter the matrix. Its presence in serum inhibits the growth of crystal nuclei that remain outside of the matrix [10]. This frees calcium and phosphate ions for the growth of crystals that have diffused into that matrix and therefore escaped fetuin's ability to inhibit their growth.

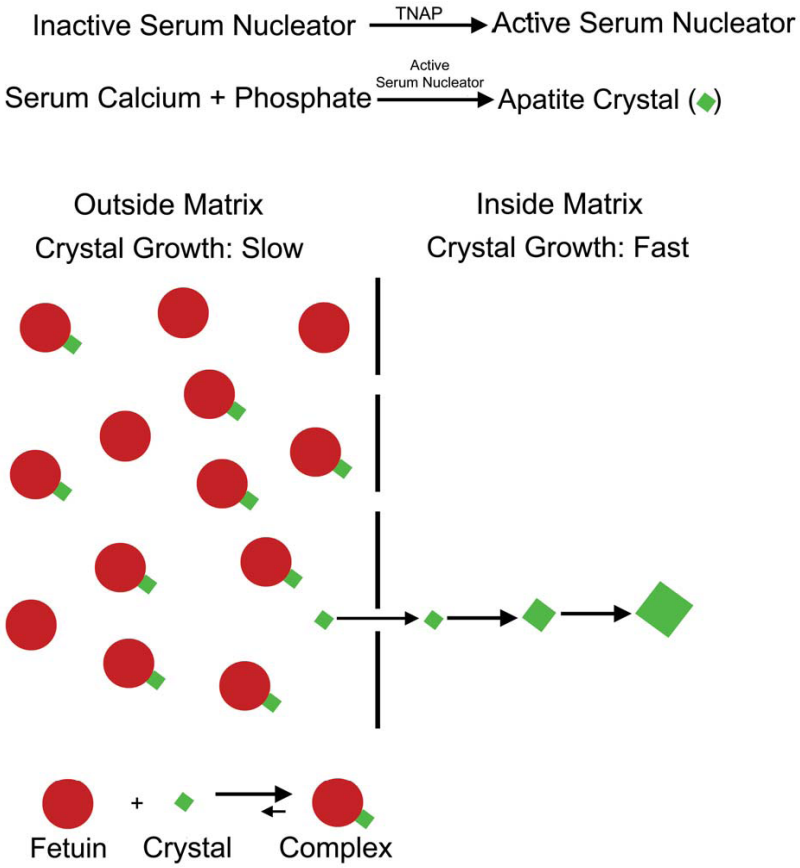

Figure 1: The shotgun mineralization mechanism. TNAP activates the high molecular weight nucleator of apatite formation, and the activated nucleator then forms small apatite crystals near the matrix. Most small apatite crystals (*green*) form complexes with serum fetuin (*red*) that inhibit their growth. A few of these crystals are free at any instant, however, and these can diffuse through pores in the matrix (represented by *gaps* in the *vertical black line*). Because fetuin is too large to pass through the matrix pores, the crystals inside the matrix are free of fetuin and grow rapidly [3].

The cell wall of S*taphylococcus aureus* bacteria has size exclusion properties that are similar to type 1 collagen (table 1) and our hypothesis is that *S .aureus* will become mineralized by the same serum driven process as collagen. The bacterial cell wall of S*taphylococci* and other gram-positive bacteria consists of peptidoglycan, which helps in providing structure, and a "secondary wall polymer" composed of teichoic acids, polysaccharides, and proteins [11]. Proteins smaller than 14 kDa can freely penetrate the *S. aureus* cell wall and proteins larger than 50 kDa are excluded from the cell wall [4,5,6]. Consequently, it stands to reason that it is possible for small ions such as calcium, phosphate, or small mineral nuclei to penetrate the cell wall and larger proteins, such as fetuin to be excluded. Therefore, the possibility of inducing calcification of a bacterial cell wall through the serum driven mechanism is feasible.

Table 1: Comparison of Size Exclusion and Matrix Calcification. Refer to references [2,4,5,6] for size exclusion characteristics of collagen and the bacterial cell wall. The similar size exclusion properties of the bacterial cell wall to collagen make it a strong candidate for serum driven calcification.

Matrix	Can enter matrix if less than	Cannot enter matrix if greater than	Matrix calcified in serum?
Collagen	6 kDa	40 kDa	Yes
Bacterial cell wall	14 kDa	50 kDa	Yes (this study)

It is interesting to note that the two components of serum calcification, fetuin and alkaline phosphatase, have also been shown to play a role in the immune system. The human homologue of fetuin, α_2 HS glycoprotein has been shown to increase bacterial phagocytosis of *S. aureus* and *E.coli* by neutrophils through opsonization [12]. In addition, leukocyte alkaline phosphatase, whose function is unknown, increases in response to bacterial infection [13,14]. We propose to provide an explanation for the involvement of fetuin and alkaline phosphatase in the immune system by suggesting that serum driven bacterial mineralization may serve as a defense mechanism. The likelihood that bacteria fulfilling the four criteria described above become mineralized is not small especially since many previous experiments have shown that the mineralization of bacteria is not that uncommon.

Past experiments have demonstrated that microbial species have the ability to become calcified in vitro as seen in *Bacterionema matruchotii,* which is an oral microflora. Calcification was not limited to these species of oral microorganisms, but was extended to other typically non-calcifying microorganism variants of *Bacterionema* such as Gram-positive *Streptococcus.* These studies demonstrated the ability to form intracellular and extracellular mineral [15]. In these experiments, the nucleator of calcification was believed to be a proteolipid that is bound to the bacterial membrane. However, since these bacteria were grown in either a specifically defined medium or a metastable calcium and phosphate solution, it is difficult to assert whether this mechanism is involved in bacterial mineralization *in vivo*. Nevertheless, these studies demonstrate that calcification of bacteria is possible [16,17].

More recent studies have identified a controversial entity called a “nanobacteria”. Nanobacteria have been associated with causes of pathological calcification such as kidney stones, atherosclerosis, and cardiovascular diseases [18]. Some studies have suggested that these “nanobacteria” are self-replicating non-living particles that serve as a nucleus of calcification from which growth may ensue. Others suggest that nanobacteria are related to other mineral

forming bacteria and can induce calcification on structures such as epithelial cells. Based on EDX analysis, nanobacteria contain an apatite mineral of phosphate and calcium [19,20]. Despite the controversy of whether these “nanobacteria” are non-living or living, the mechanism by which they become mineralized *in vivo* has not been fully elucidated.

Our research will be the first to provide a general mechanism of bacterial mineralization that may occur in vivo. Understanding the mechanism of how our bacterial model, *S. aureus* becomes mineralized may provide a possible explanation for the calcification of nanobacteria and in other bacterial mineralization experiments. More importantly, we may provide a link between factors normally involved in vertebrate mineralization and their involvement in the immune system.

Therefore, our goals are first, to demonstrate that bacteria become mineralized in the physiological fluid of serum. Secondly, to investigate the mechanism of serum induced bacterial mineralization and determine factors that affect the amount of bacterial mineralization. And thirdly, determine the location of mineral incorporation in the bacteria.

METHODOLOGY

Materials

Rat serum was obtained by exsanguination from 44 day old rats. Adult Bovine serum and newborn calf serum were purchased from Invitrogen (Carlsbad, CA). Human serum was obtained by drawing blood from several volunteers at the Clinical Research Center at the University of California San Diego. Blood was clotted and spun down in a Fischer Scientific Centra 228 centrifuge at 3,400 rpm (x 1380 g) for 5 minutes to obtain pure human serum.

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (Grand Island, NY). Penicillin-Streptomycin was purchased from Invitrogen (Carlsbad, CA). Sodium azide was purchased from EM Science (Gibbstown, NJ). Unless otherwise stated, a 500 ml volume of DMEM was supplemented with 5 ml of penicillin/streptomycin, 1 ml of 10 % sodium azide, and 1.1 ml of 0.5 M sodium phosphate buffer, pH 7.4 (final medium Pi concentration = 2mM). Didronel (etidronate disodium) was purchased from Procter and Gamble Pharmaceuticals. Bovine kidney alkaline phosphatase (TNAP) was obtained from Calzyme Laboratory (San Luis Obispo, CA). Beryllium sulfate tetrahydrate was obtained from Fluka. (–) - tetramisole hydrochloride (levamisole), 1,10-phenanthroline monohydrate, calcein, and bovine fetuin were purchased from Sigma (St. Louis, MO).

Staphylococcus aureus ATCC 25923 (lot #485992, reference #0485v) was purchased from MicroBioLogics Inc. (St. Cloud, MN). 5 ml of an *S. aureus* glycerol stock was placed in a flask with 1 L of LB broth and grown in a shaker at 37° C for 8-10 hours. The broth was then spun down in a Sorvall superspeed RC2-B centrifuge at 5,000 rpm (x 4,068g) in a GSA rotor for 10 minutes. The supernatant was then removed and replaced with 40 mL of 0.5 M EDTA, pH 7.5 and rotated end over end at RT for 24 hours to kill the bacteria by osmotic shock. The solution was then spun down in a IEC Centra Cl2 centrifuge at 8,500 rpm (x 11,750g) in a GSA rotor for 15 minutes. The bacterial pellet was resuspended in 40 ml of water and spun down in a IEC

Cl2 centrifuge for 15 minutes. This step was repeated four times and the final bacterial pellet was shell frozen and lyophilized. A 50 mg /ml solution of dead *S. aureus* in water was then made.

Methods

Calcification procedures of S. aureus in neat serum

Experiments in 6 well plates were set up to determine the extent of calcification occurring within a bacterial matrix and the fraction of total bacteria that were calcified. Each well contained a 5ml volume of rat serum, human serum, or DMEM. Each 5 ml volume contained 0.02 % sodium azide and 1% penstrep to prevent bacterial growth. 40 ul of the 50 mg/ml dead *S. aureus* solution was then added to each well and the plate was placed in the incubator at 37° C and 5% CO_2 for 6 days. After the incubation, the bacteria in each well were transferred into 12 x 75 test tubes and spun down in a Fischer Scientific Centra 228 centrifuge at 3,400 rpm (x 1,380g). Bacterial pellets were resuspended in 3ml of 0.5% KOH. The 3ml volume was typically partitioned for various analyses: 300ul for Scanning Electron Microscopy (SEM), 1.2 ml for calcium and phosphate analysis, and 1.5 ml for calcein staining analysis.

Fluorescence Quantification

Calcein was utilized to detect mineral incorporation into the bacteria. 1.5 ml of calcified bacteria in 0.05 % KOH (see above) was centrifuged, and the pellet was then resuspended in 2 ml of 20 ug/ml calcein stain in 0.05 % KOH and rotated end over end for 1 hour. The bacteria were then spun down in a Fischer Scientific Centra 228 centrifuge at 3,400 rpm (x 1,380 g). Two washes with 4 mL of 0.05 % KOH were performed to remove any unbound calcein from the bacteria. Bacterial pellets were then resuspended in 1 mL of 0.05% KOH. 100 ul of this bacterial solution was then added to 900 ul of 0.05% KOH to make a 1:10 dilution. The 1:10 dilution of the calcein stained bacteria was then spun down in a Fischer Scientific Centra 228 centrifuge at 3,400 rpm (x 1,380 g) for 5 minutes. Pellets were then resupsended in 1 ml of 100 mM NH_4OH.

This was repeated 2 more times and from the final solution, 10 ul was then placed on a slide and dried under vacuum over night at room temperature, making sure to limit the amount of light exposure. The slide was viewed under a Leica DM microscope with both fluorescent and visible light capabilities. The bacteria were photographed and counted. The percentage of mineralized bacteria can then be determined as indicated:

$$\%\ calcified = \frac{(\#\ fluorescent)}{(total\#)}$$

Biochemical Analyses

Bacterial pellets for calcium and phosphate analyses were incubated with 0.5 ml of 150 mM HCl at room temperature for at least 2 hours to extract any calcium phosphate mineral. Acid extracts were then quantitatively assayed for calcium by a colorimetric assay (JAS Diagnostics, Miami, Fl). Phosphate concentrations were determined by a colorimetric assay, as previously described [1].

SEM analysis of mineralized and non-mineralized bacteria

For analysis by Scanning Electron Microscopy (SEM), the 300 µl of bacterial solution (see above) were spun down in a Fischer Scientific Centra 228 centrifuge at 3,400 rpm (x 1,380 g) for 5 minutes. Bacterial pellets were then brought up in 300 ul of 200 mM etidronate in 100 mM NH_4OH. The solution was then spun down in a Fischer Scientific Centra 228 centrifuge at 3,400 rpm (x 1,380 g) for 5 minutes and bacterial pellets were brought up in 600 ul of 1 uM etidronate in 100 mM NH_4OH. 10 ul of the solution were then placed on a slide and dried under vacuum over night at room temperature. Slides were then sputter coated with gold and palladium and then analyzed at 20 kV on a Phillips XL30 ESEM.

TEM analysis of mineralized and non-mineralized bacteria

For analysis by Transmission Electron Microscopy (TEM), 10% glycerol was added to the bacteria as a cryoprotectant. Bacteria were spun down in a Fischer Scientific Centra 228 centrifuge at 3,400 rpm (x 1,380 g) for 5 minutes. The pellet was then brought up in a small volume of 20 % BSA. The samples were then frozen in a specimen well using a Bal-tec HPM 010 high pressure-freezing machine. Samples were kept at -135 ºC in liquid nitrogen and freeze substituted on a Leica EM AFS2 containing 20% epon araldite resin in acetone as a fixative. The temperature was slowly raised to 0°C over 24 hours as follows: -90°C (8 hours), -60°C (8.5 hours), -30°C (8.5 hours) and 0°C (1 hour). The samples were thoroughly infiltrated with Epon araldite resin by incubating them in the following solutions at 0°C for the specified amount of time: 33% epon araldite in acetone (4 hours) and 66% epon araldite in acetone (4 hours). Samples were subsequently transferred to 100% epon araldite in a dessicator to remove residual acetone and several exchanges of 100 % epon araldite were performed over 24 hours. Samples were then flat embedded and placed in an oven for 2 days. 90-100 nm sections were then cut with a diamond knife and stained with 2% uranyl acetate for 30 minutes and alkaline lead citrate [21] for 5 minutes. Sections were washed with water and allowed to dry. They were visualized in a 1200 FX1 TEM at 80 kV with an exposure time of 2.8 seconds. Electron Energy Loss Spectrum (EELS) analysis was performed on unstained control and experimental sections using a JEOL JEM Z3100EF TEM at 300 kV and 40 K magnification. Images were taken with a Gatan Ultrascan 4-port 4Kx4K CCD camera.

Calcification procedures for Fetuin Depletion Experiment

10% fetuin depleted adult bovine serum was prepared as previously described by antibody affinity chromatography [10]. Briefly, antifetuin antibodies were purified from rabbit antisera by passing through a Sepaharose 4B column with covalently attached bovine fetuin. The

purified antibodies were then attached covalently to a Sepharose 4B column; bovine serum was then run over this column and freed of fetuin. The absorbance at 280 nm was determined and the fetuin content was determined by a radioimmunoassay. The serum was then diluted in DMEM until a concentration of 10 % serum was reached.

Experiments were carried out in a humidified incubator at 37˚C and 5% CO_2.. Each well contained a 5 ml volume of DMEM alone, DMEM containing 10% fetuin depleted adult bovine serum, or DMEM containing 10% fetuin depleted adult bovine serum supplemented with 130 μg/ml bovine fetuin. 40 μl of the 50 mg/ml dead *S. aureus* solution was added to each well and the plate was placed in the incubator for 12 days. After the incubation, the bacteria in the well was transferred into a 12 x 75 test tube and spun down in a Fischer Scientific Centra 228 centrifuge at 3,400 rpm (x 1,380g). Methods for SEM analysis were followed as described above.

Calcification procedures for TNAP deficient experiment

Serum deficient in alkaline phosphatase activity was prepared as previously described [3]. 150 μl of 1 M phenanthroline in methanol was added to 10 ml of newborn calf serum in a 15 ml conical. The conical was loosely capped and placed in a humidified incubator at 37˚C and 7.5% CO_2 for 72 h. The serum was then placed in dialysis tubing with a molecular weight cutoff of 6-8 kDa and dialyzed against four changes of 125 ml of DMEM (no phosphate boost) [3]. Experiments were carried out using 6 well plates in a humidified incubator at 37˚C and 5% CO_2. Each well contained 5 ml of DMEM with 2 mM P_i and 0.75 % by volume of one of the following: control newborn calf serum, alkaline phosphatase- depleted newborn calf serum, or alkaline phosphatase-depleted newborn calf serum + 0.2 unit/ml of purified TNAP (see table 2). 40 μl of the 50 mg/ml dead *S. aureus* solution was added to each well and the plate was placed in the incubator for 9 days. After the incubation, the bacteria in the well was transferred into a 12 x 75 test tubes and spun down in a Fischer Scientific Centra 228 centrifuge at

3,400 rpm (x 1,380g). Methods for biochemical analysis were followed as described above.

Table 2: The Alkaline Phosphatase activity of serum used in TNAP deficient experiment
The alkaline phosphatase activity was determined using a colorimetric assay for control newborn calf serum, alkaline phosphatase depleted newborn calf serum, and alkaline phosphatase depleted serum supplemented with 0.2 U/ml purified TNAP.

Sample	**Alkaline Phosphatase activity (*units/ml*)**
Control Serum	0.21
Alkaline Phosphatase-depleted serum	<0.002
Alkaline Phosphatase-depleted serum + 0.2 units/ml purified TNAP	0.18

RESULTS

Evidence that bacteria become mineralized in serum

In this study, we investigate the potential of *S. aureus* to become mineralized by a previously discovered serum calcification factor. These series of experiments establish that mineralization of *S. aureus* occurs through a mechanism that has previously been shown to mineralize type-I collagen of bone and tendon. We hope to better understand whether bacterial mineralization may be a possible mechanism to fight bacterial infection.

A calcein staining method that can detect mineral incorporation into bacteria was developed to evaluate whether incubation in serum can cause the mineralization of dead *S. aureus* bacteria. A calcein staining technique that has been previously shown to bind and detect hydroxyapatite mineral of newly synthesized bone was developed for use in this assay [22]. Utilizing calcein to stain bacteria not only allows visualization of mineralized bacteria under fluorescence, but also allowed determination of the fraction of all incubated bacteria that have been mineralized.

Initial studies to investigate serum driven mineralization of bacteria were performed by incubating 2mg (approximately 3.2×10^9) of bacteria in 5 ml of rat serum for 6 days at 37°C and 5 % CO_2. Figure 2 provides evidence that bacteria become mineralized in rat serum. The total number of bacteria can be visualized using light microscopy (fig. 2A). The same field of bacteria under fluorescence microscopy allows the detection of mineralized bacteria visualized by calcein labeling (fig. 2B). The number of visible bacteria seen in fig. 2A and the number of fluorescent bacteria seen in fig. 2B were quantified and the percent mineralized bacteria was determined based on the fraction of these two numbers:

$$\% \text{ mineralized} = (\# \text{ fluorescent bacteria} / \text{total } \# \text{ bacteria})$$

Results indicated that 91% of the bacteria became mineralized in rat serum. In contrast, bacteria incubated in DMEM alone did not mineralize (results not shown). This demonstrates that the

difference in mineralization capabilities is due to factors present in serum and not ionic composition since it has previously been demonstrated that the calcium phosphate ionic product of DMEM (3.6 $mM^{2)}$ and rat serum (3mM^2) are similar [9].

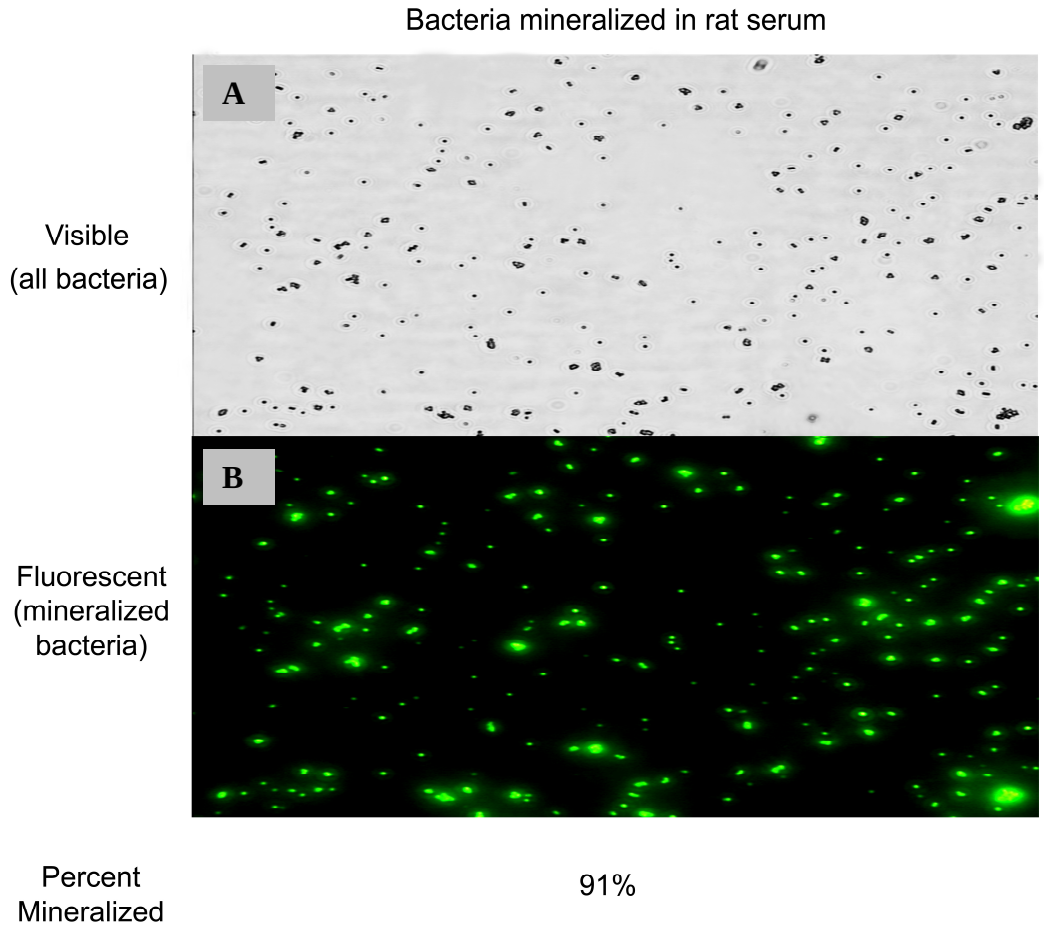

Figure 2: Evidence that Incubation in rat serum causes the mineralization of *Staphylococcus* aureus bacteria. 2mg (approximately 3.7 x 10^9) dead *S.aureus* bacteria were incubated in 5 ml of rat serum for 6 days at 37°C and 5% CO_2. Bacteria were stained in 20 μg/ml calcein in 0.05 % KOH to fluorescently label mineralized bacteria. All bacteria were then excessively washed with NH_4OH. An aliquot of the bacteria sample was placed on a slide and dried under vacuum. Quantification of mineralized bacteria was performed using light and fluorescence microscopy. (A) Bacteria visualized by light microcopy (B) The same bacteria visualized using fluorescence microscopy. Bottom: Percent mineralized= (# visible in B/ # visible in A) x 100. For details see "Materials and Methods"

To determine the amount of mineral that was incorporated in *S. aureus* incubated in rat serum, 150 mM HCl was used to dissolve the mineral inside bacteria and the extract was analyzed for calcium and phosphate content. There was 5.92 μmoles of calcium and 3.81 μmoles of phosphate incorporated into bacteria and the Ca/Pi ratio was 1.55. (fig.5) This ratio is comparable to the mineral formed in type-I collagen that had been incubated in serum [1,3,10]. In addition, the ratio is similar to the ratio of mineral found in bone [23,24]. This provides support that the mineral formed in bacteria is similar to the mineral formed in these earlier studies.

Additionally, to determine structural differences between mineralized and non-mineralized bacteria, scanning electron microscopy (SEM) was performed of unincubated *S. aureus* and *S. aureus* incubated in rat serum for 6 days at 37 °C and 5% CO_2. Bacteria were pelleted by centrifugation and samples were prepared for SEM analysis (see Materials and Methods). Figure 3 indicates that there are clear differences in the surface morphologies of mineralized and non-mineralized bacteria. Mineralized bacteria have a more spherical appearance in contrast to non-mineralized bacteria, which have a flatter appearance. Note that there are varying degrees of mineralization. It is possible that partially mineralized bacteria have a rougher texture in comparison to fully mineralized bacteria, which have a smoother appearance. Also note that there is no mineral detected outside mineralized bacteria, indicating that mineralization is specific within the bacterial matrix.

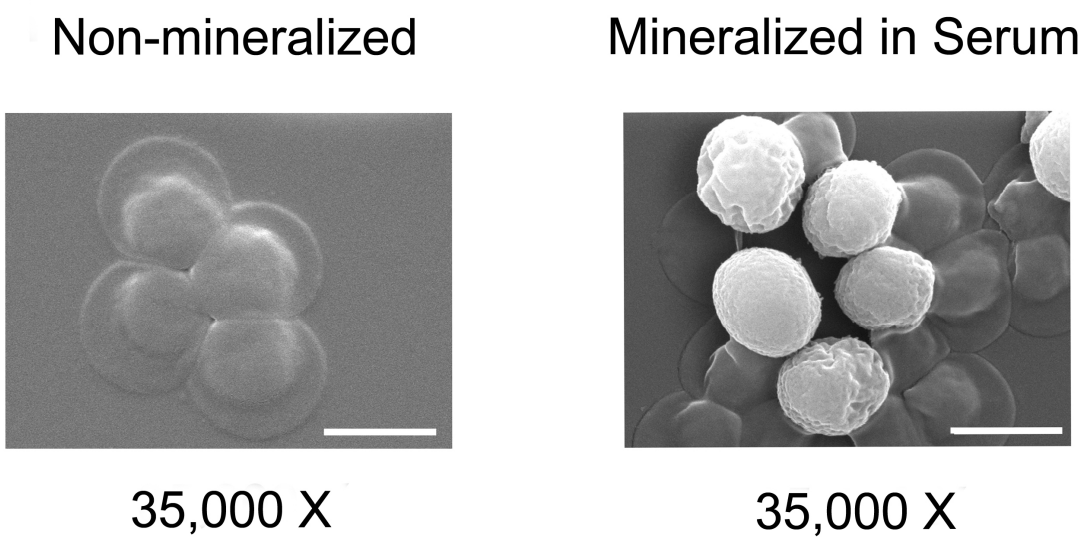

Figure 3: Scanning Electron Microscope (SEM) images of mineralized and non-mineralized *Staphylococcus aureus* bacteria.
2mg (approximately 3.2 x 10^9) of dead *S. aureus* bacteria were mineralized by incubation for 6 days in 5 ml of rat serum at 37°C and 5% CO_2. After incubation, samples were centrifuged to pellet bacteria, and bacteria were washed extensively in 100 mM NH_4OH containing etidronate. An aliquot of each bacterial sample was placed on a slide and dried under vacuum. Slides were sputter coated with gold/palladium and examined by SEM at 20 kV. An unincubated control sample was also prepared and visualized via the same methods. Scale bar = 500 μm.

The ability of bacteria to become mineralized in rat serum led to investigation of whether this activity could also be found in human serum. 2 mg of dead *S. aureus* were incubated in 5 ml of human serum for 6 days at 37 °C and 5% CO_2. Figure 4 demonstrates that bacteria incubated in human serum also become mineralized. The percentage of mineralized bacteria was determined to be 15 % via the same quantification method described above. These sets of experiments therefore provide evidence for the importance of serum in bacterial mineralization. It also suggests that bacterial mineralization is possible in human serum.

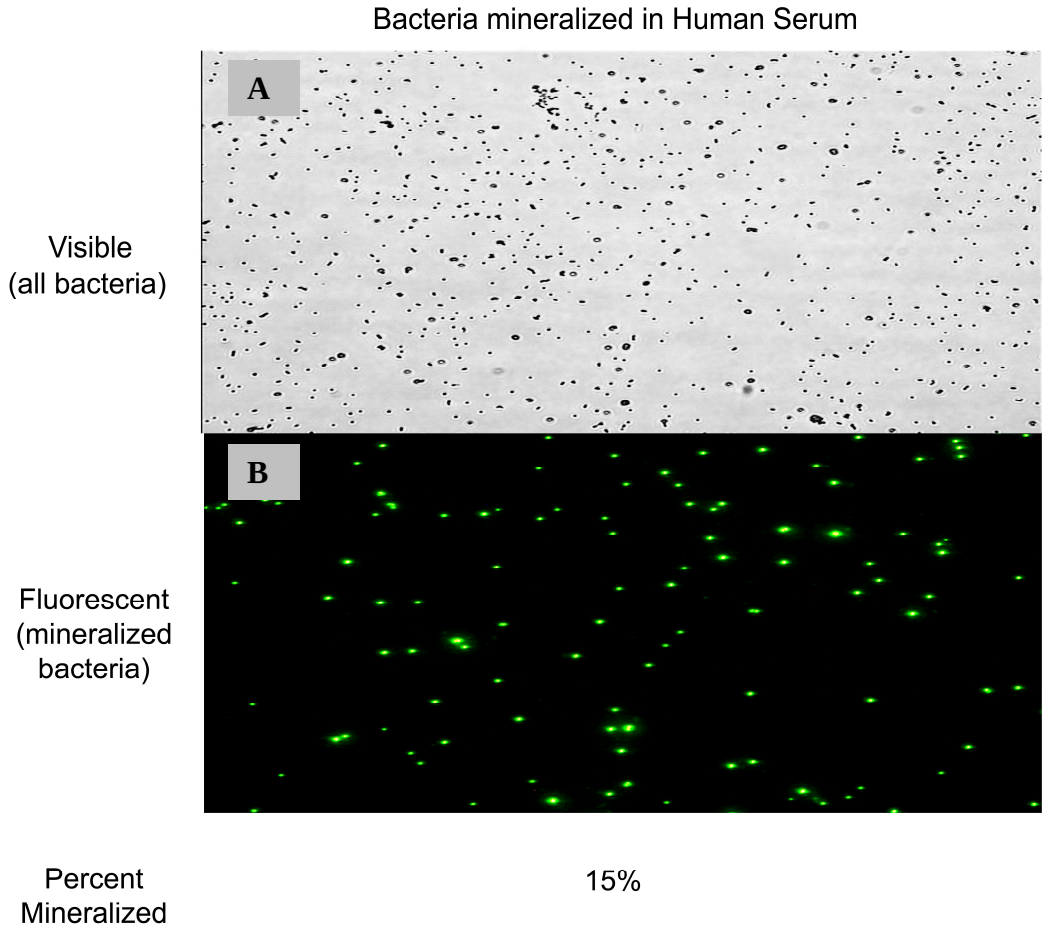

Figure 4: Evidence that incubation in human serum causes the mineralizaton of *Staphylococcus aureus* bacteria. 2mg (approximately 3.7 x 10^9) dead *S. aureus* bacteria were incubated in 5 ml of human serum for 6 days at 37°C and 5% CO_2. Subsequent procedures were followed as described in figure 2. (A) Bacteria visualized by light microscopy (B) The same bacteria visualized using fluorescence microscopy. Bottom: Percent mineralized= (# visible in B/ # visible in A) x 100. For details see "Materials and Methods".

Further tests examined the amount of calcium and phosphate that were incorporated into *S. aureus* bacteria incubated in human serum. Results from bacteria incubated in human serum indicated that 1.71 μmoles of calcium and 0.84 μmoles of phosphate were incorporated into 3.2 x 10 ^ 9 bacteria (fig. 5). There is an increase in the amount of calcium and phosphate incorporation into the same amount of bacteria incubated in rat serum, which corresponds to values of 5.92

μmoles of calcium and 3.81 μmoles of phosphate (fig. 5). It is expected that the larger percentage of bacteria mineralized in rat serum would demonstrate an increase in calcium and phosphate incorporation. An explanation for the differences in percent mineralized and amount of calcium and phosphate incorporation in bacteria incubated in rat or human serum may be due to differences in the components of these sera, such as ionic composition.

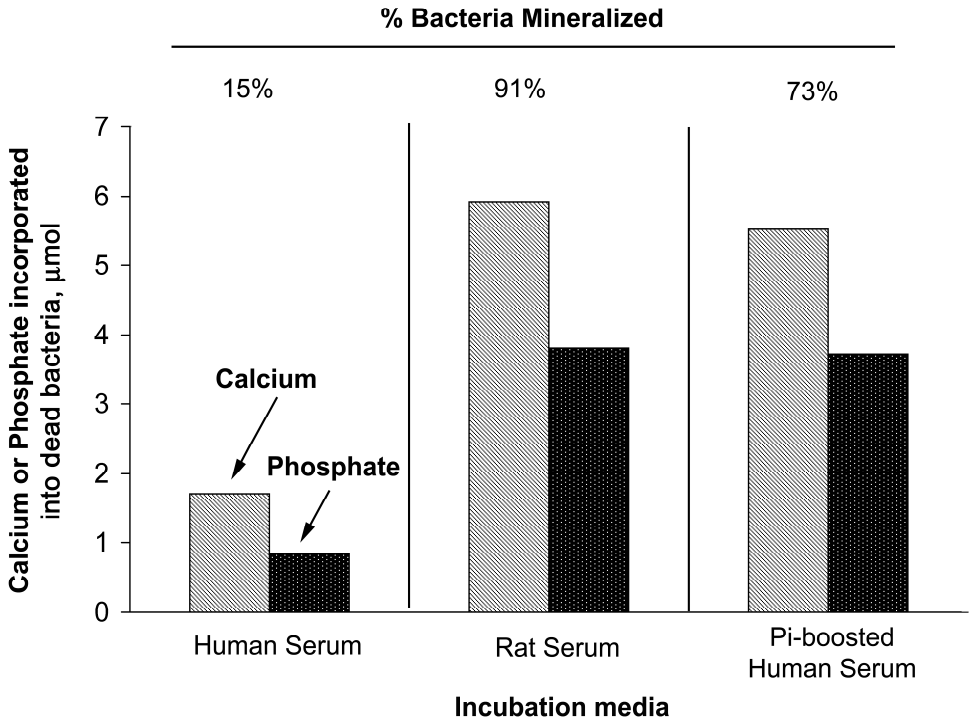

Figure 5: Analysis of calcium and phosphate incorporation into *Staphylococcus aureus* bacteria after separate incubation for 6 days in human serum, rat serum, or phosphate boosted human serum. 2mg (approximately 3.7 x 10^9) dead *S. aureus* bacteria were separately incubated in 5 ml of human serum, rat serum, and human serum + 2mM Pi 6 days at 37°C and 5% CO_2. After incubation, samples were centrifuged to pellet bacteria. Bacterial pellets were then acid extracted using 150 mM HCl, and the extracts were analyzed for calcium and phosphate as described under "Materials and Methods".

Evidence that Phosphate concentration in serum is an important component in bacterial mineralization.

It has previously been shown that devitalized arteries incubated in rat serum with an ion product of 1.2 mM [Ca] x 3.2 mM [Pi] become calcified after 6 days. It was demonstrated that devitalized arteries also become calcified in human serum if the phosphate level is increased by 2mM from 1.2 mM to 3.2 mM; the same level of phosphate found in rat serum. Arteries incubated in human serum alone did not calcify [25]. Since the level of serum phosphate plays a role in the extent of calcification seen in arteries, we wanted to test whether phosphate levels also play a role in serum driven bacterial mineralization.

In order to determine if the serum phosphate concentration is limiting the amount of mineralization based on percentage of bacteria mineralized and total mineral incorporation, the phosphate concentration of human serum was increased by 2 mM. Results indicated that there is a connection between the initial phosphate concentration of serum and the amount of calcium and phosphate incorporated into bacteria. As seen in figure 5, increasing the phosphate concentration of human serum resulted in an increase of calcium incorporation from 1.71 μmoles to 5.5 μmoles. This is comparable to the 5.92 μmoles of calcium incorporated into bacteria incubated in rat serum. Additionally, there was an increase in phosphate incorporation from 0.84 μmoles to 3.73 μmoles of phosphate, which is again comparable to the 3.81 μmoles of phosphate incorporated into bacteria incubated in rat serum. The difference in phosphate concentrations of human and rat serum, thus provide a possible explanation for the differences seen in both the amount of mineral incorporation and the differences in the percent mineralized bacteria.

Evidence that Alkaline Phosphatase (TNAP) is required for the serum driven mineralization of bacteria

Additional experiments were performed to determine the mechanism and components necessary for bacterial mineralization in serum. Previous studies have indicated that tissue non-specific alkaline phosphatase (TNAP) is important mechanistically for the calcification of Type I collagen [3]. These studies led to experiments investigating whether alkaline phosphatase may also play an important role in the mineralization of bacteria in serum. In this study, human serum was separately treated with three TNAP inhibitors: 30 mM levamisole, 30 μM beryllium sulfate, or 3mM phenanthroline. 2mg of *S. aureus* bacteria were added to each sera and incubated at 37ºC and 5 % CO_2 for 6 days. As indicated in figure 6, it is evident that inhibition of TNAP largely affects calcium and phosphate incorporation into bacteria in comparison to bacteria incubated in neat human serum.

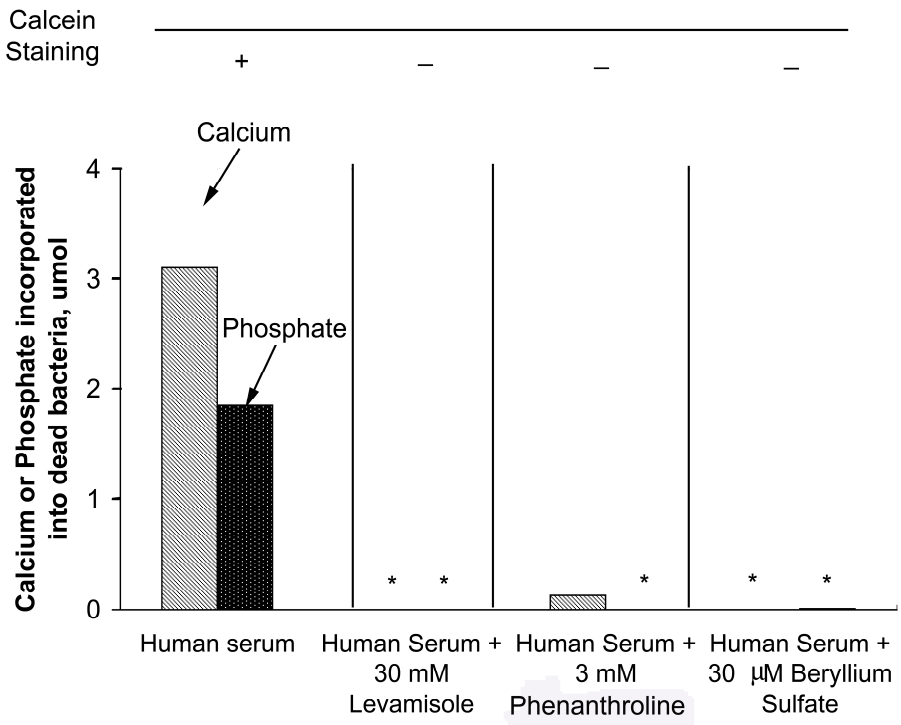

Figure 6: Evidence that tissue nonspecific alkaline phosphate (TNAP) activity is necessary for bacterial mineralization: calcium and phosphate analysis. 2mg (approximately 3.2 x 10^9) dead *S. aureus* bacteria were separately incubated in 5 ml of human serum containing: 30 mM Levamisole, 3mM Phenanthroline, 30 µM Beryllium Sulfate, or no additives for 6 days at 37°C and 5% CO_2,. After incubation, samples were centrifuged to pellet bacteria. Bacterial pellets were then acid extracted using 150 mM HCl, and the extracts were analyzed for calcium and phosphate as described under "Materials and Methods". *, less than 0.06 µM.

In order to further explore the importance of alkaline phosphatase in bacterial mineralization, an experiment was performed using a previous study as a model. In this study it was demonstrated that type-I collagen incubated in serum with TNAP, irreversibly inactivated by phenanthroline, did not calcify. Activity was only restored when purified TNAP was added to TNAP deficient serum [3]. For this study, endogenous alkaline phosphatase was irreversibly inhibited with phenanthroline (see Materials and Methods). To demonstrate the importance of TNAP in bacterial mineralization, 0.2 U/ml of purified TNAP was added to phenanthroline

treated serum to bring it to the level found in untreated newborn calf serum (see table 2). Results indicated that TNAP-deficient serum prevented mineralization of bacteria while addition of purified TNAP restored mineralization activity to a level comparable to that seen in control newborn calf serum (fig.7). This study provides further evidence that TNAP is a necessary component for the mechanism of bacterial mineralization.

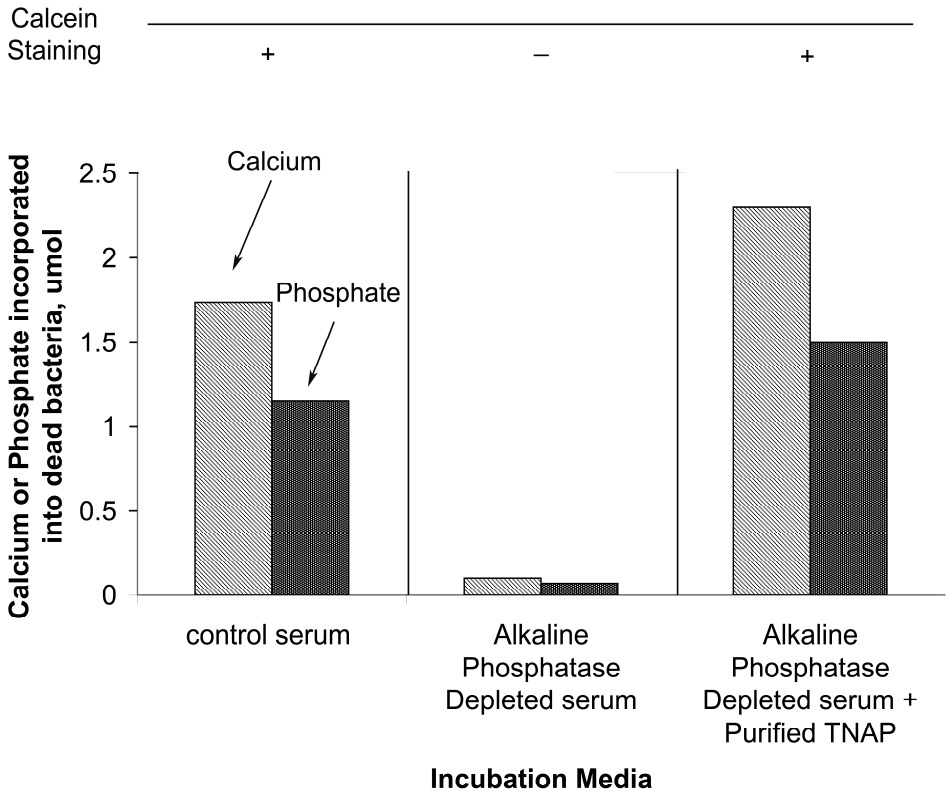

Figure 7: Evidence that mineralization of *S. aureus* bacteria in serum is inhibited by TNAP depletion and restored by repletion of purified TNAP. 2mg (approximately 3.2 x 10^9) dead *S. aureus* bacteria were separately incubated for 6 days at 37°C and 5% CO_2, in 5 ml of DMEM containing 2mM phosphate and 0.75 % by volume of newborn calf serum, TNAP depleted serum, or TNAP depleted serum + 0.02 U/ml purified TNAP (see table 2 for the alkaline phosphatase activity in each serum). After incubation, samples were centrifuged to pellet bacteria. Bacterial pellets were then acid extracted using 150 mM HCl, and the extracts were analyzed for calcium and phosphate as described under "Materials and Methods".

Evidence that Fetuin is an important component for serum induced mineralization of bacteria

Previous studies have also indicated that fetuin is necessary for the calcification of type-I collagen. Specifically, fetuin, the major calcification inhibitor, prevents mineral nuclei from growing outside a matrix. Since fetuin cannot access the matrix interior, those mineral nuclei that have escaped fetuin's inhibition can enter the matrix and freely grow. The mineral nuclei inside the matrix grow more rapidly than mineral outside the matrix. Therefore fetuin paradoxically plays a role in the selective calcification of a matrix [10].

The impact of fetuin in serum driven mineralization of bacteria was explored by separately incubating 2mg of bacteria for 6 days at 37°C and 5% CO_2 in 10% fetuin depleted bovine serum or 10 % fetuin repleted bovine serum. Results indicated that removal of fetuin from serum led to the predominant formation of a mineral phase outside the bacteria (fig. 8). Repletion of 10 % fetuin depleted bovine serum with 130 μg/ml of purified fetuin led to mineralization of bacteria and prevention of a mineral phase outside the matrix. This study consequently provides evidence that fetuin is also essential for bacterial mineralization.

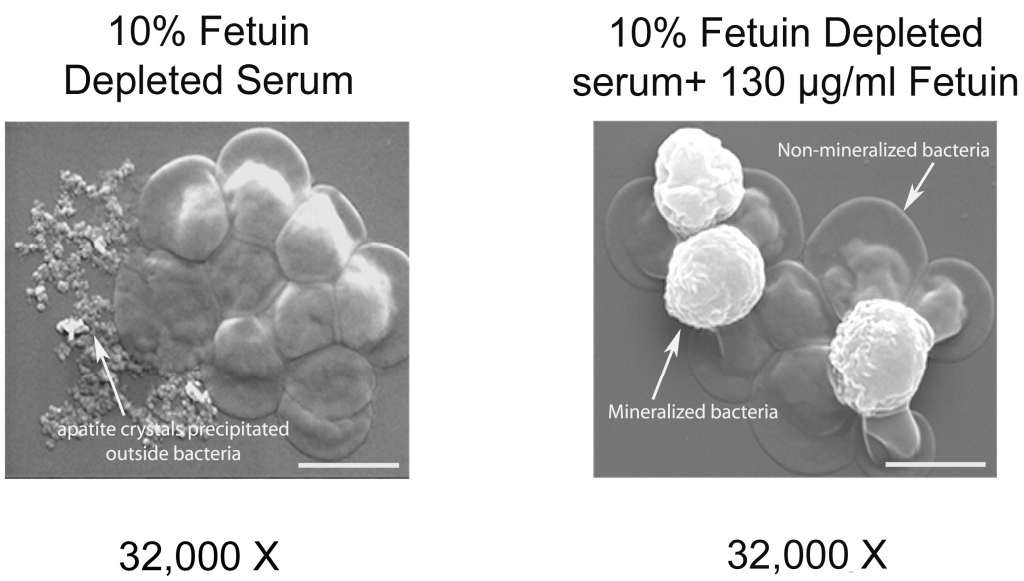

Figure 8: Evidence that Fetuin is required for serum driven mineralization of dead *Staphylococcus aureus*. 2mg of dead *S. aureus* were separately incubated in 10% fetuin-depleted bovine serum and 10% fetuin depleted bovine serum +130 µg/ml fetuin for 12 days at 37 ºC and 5% CO_2. After incubation, samples were prepared for SEM analysis as described in figure 3. Scale bar = 1 µm.

Trasmission Electron Microscopy: Studies on the Location of Mineral inside S. aureus bacteria

Of interest in this study is not only to determine the mechanism of bacterial mineralization in serum, but to also determine where mineral is localized in the bacteria. Transmission electron microscopy (TEM) and electron energy loss spectrum analysis (EELS) were performed on *S. aureus* incubated in rat serum for 6 days at 37 ºC and 5% CO_2 and on control unincubated *S. aureus*. As evident in figure 9, mineralized bacteria have a darker interior due to mineral than control non-mineralized bacteria. There is even some evidence of needlelike and plate like structures that are absent in non-mineralized bacteria.

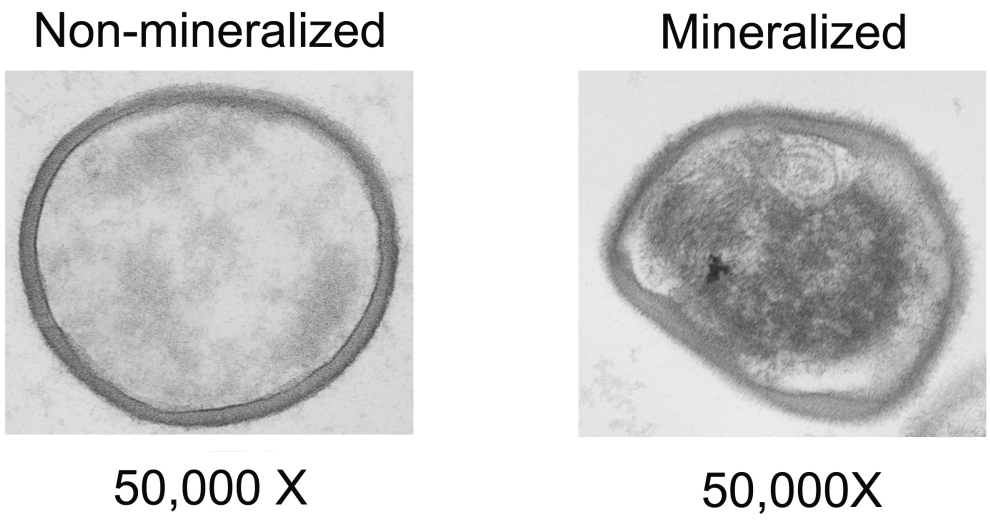

Figure 9: Transmission Electron Microscope Analysis of Non-mineralized and Mineralized *S. aureus*

Experimental and control samples were high pressure frozen and fixed in epon aralidite. 90-100 nm sections were cut and stained with 2 % uranyl acetate and Sato lead citrate. Sections were visualized by TEM at 50,000 magnification.

With higher resolution, it is confirmed that mineralized bacteria form needlelike and plate like crystals throughout the matrix interior, which is comparable to the morphology of hydroxyapatite mineral, depending on orientation (10A). Additionally, using EELS it is evident that calcium and phosphate are present inside the mineralized bacteria and map to the same location as the needlelike structures (10B-10F), while there is no evidence of calcium and phosphate mineral in control bacteria (10G-10H). This study provides further evidence that bacteria mineralize in serum and the mineral formed in bacteria is localized within the matrix. It is also evident that mineral formation does not extent beyond the cell wall of the bacteria.

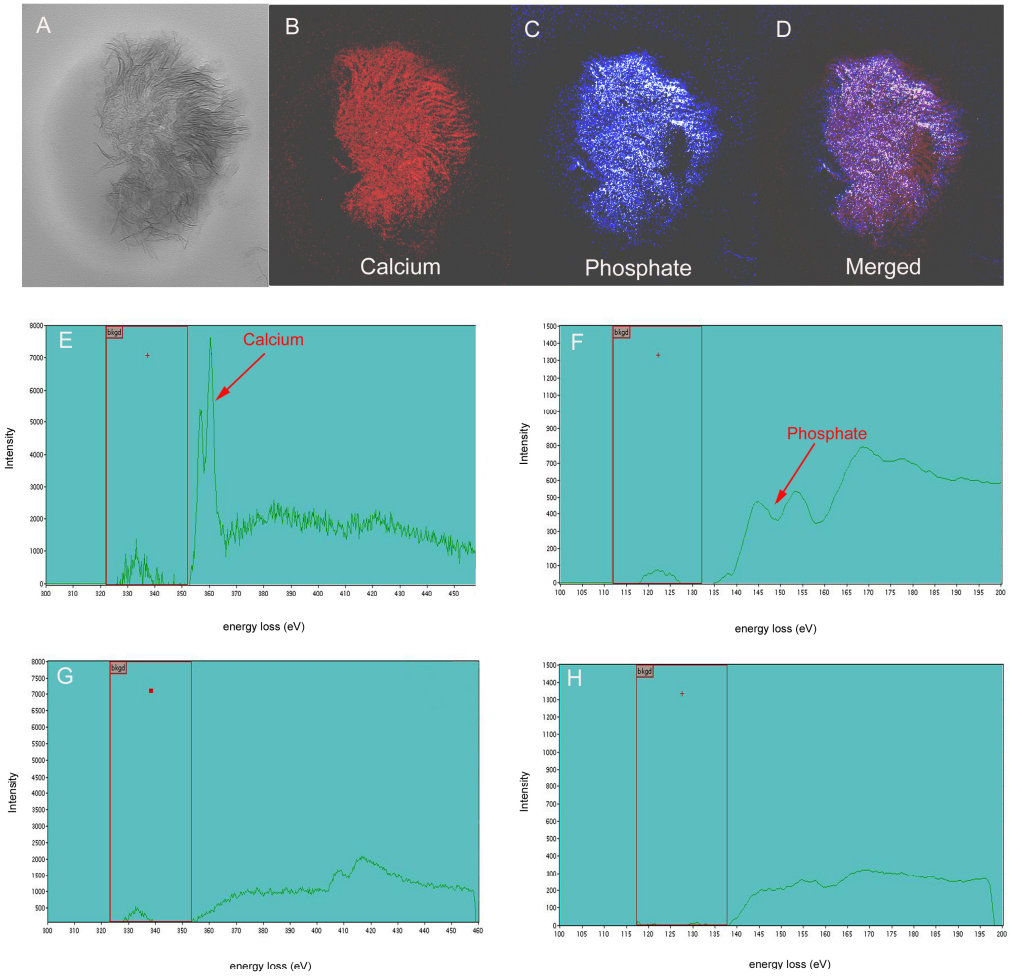

Figure 10: Transmission Electron Microscope (TEM) and Electron Energy Loss Spectra (EELS) analysis of mineralized and non-mineralized *Staphylococcus aureus* bacteria.

A) 2mg of dead *S. aureus* were mineralized by incubation for 6 days in 5 ml of rat serum at 37 ºC and 5 % CO_2. Sample was high pressure frozen and fixed in epon aralidite. Unstained 90-100 nm sections were then visualized via TEM. (see materials and methods for details) EELS was then performed to determine location of calcium and phosphate.

B) Calcium map C) Phosphate map D) Merged image of B) & C). Calcium and phosphate spectra (with background subtracted) obtained for experimental sample shown in E) & F). Calcium and phosphate spectra of control sample with background subtracted shown in G) & H).

DISCUSSION

Evidence that serum is required for the mineralization of bacteria under physiological conditions

The goal of our studies was to investigate whether the serum calcification factor previously shown to be able to mineralize type I collagen fibrils, tendon, and bone is able to drive the mineralization of bacteria [9]. As mentioned in the Introduction, our hypothesis was that the serum calcification factor would be able to mineralize dead *S. aureus* bacteria because the cell wall of these bacteria have similar size exclusion properties to those of collagen [2,4,5,6] Based on the results seen for bacteria incubated in rat serum and human serum, it was evident that bacteria incubated in serum become mineralized (fig.2-5). Specifically, it is evident that mineralized bacteria form both needlelike and plate like crystals, throughout the matrix, which have a morphology that is similar to hydroxyapatite [26]. EELS analysis provided strong evidence that bacteria mineralize in serum; calcium and phosphate peaks are present in mineralized bacteria, but absent in non-mineralized bacteria (fig.10A-H). However, confirmation that the mineral inside bacteria is hydroxyapatite will be necessary through powder X-ray diffraction analysis. Nevertheless, these results suggest the importance of serum for the mineralization of bacteria and supports our hypothesis that a factor found in serum can cause the mineralization of bacteria. It also suggests that bacterial mineralization can be accomplished under more physiological conditions than past studies.

Previous studies on the calcification of bacteria such as *Bacterionoma matruchotii* demonstrated via electron microscopy that calcium and phosphate mineral forms intracellularly. These past studies have utilized bicarbonate buffered metastable calcium and phosphate at pH 7.2 [15-17, 27, 28]. Our studies build on the ability of microorganisms to become mineralized in vitro by suggesting that this calcification can occur not only in a metastable solution, but in the physiological fluid of serum. Our studies provide physiological relevance to matrix specific

mineralization since the media utilized, rat serum and human serum, contain levels of calcium and phosphate that in a buffer system are too low to cause calcification. It is known that under high concentrations of calcium and phosphate, mineral will form spontaneously [25, supplemental data, 29, 30]. It suggests that factors found in serum are necessary to cause the mineralization of bacteria.

Factors that influence bacterial mineralization

Although the results of the experiments support our hypothesis that serum or a serum factor can induce mineralization of bacteria, they also suggested a difference in the mineralization capabilities of human and rat serum. There was both an increase in the fraction of mineralized bacteria and increase in the amount of calcium and phosphate that was incorporated into bacteria that were incubated in rat serum. An investigation into several potential reasons for these differences led to three discoveries regarding bacterial mineralization. First, the phosphate levels of serum influence the amount of mineralization that can occur. Second, alkaline phosphatase is a requirement for mineralization. Third, fetuin is necessary for mineralization.

Phosphate concentration as a factor in mineralization.

Human serum phosphate levels are around 1mM and those of rat are around 3mM [25]. This is a difference that we hypothesized influenced bacterial mineralization in serum. Increasing the phosphate concentration of human serum to the level found in rat increased the calcium and phosphate incorporated into bacteria. This amount was comparable to the amount of calcium and phosphate that was incorporated into bacteria incubated in rat serum. There was also an increase in the fraction of bacteria that became mineralized in phosphate-boosted human serum (fig. 5). A requirement for an increase in phosphate is logical because inorganic phosphate is one of the components necessary for the formation of hydroxyapatite mineral. In addition, it has been

demonstrated that there is a dependence on phosphate concentration on the amount of calcification seen in arteries and that the threshold of phosphate for calcification of arteries is between 1.5 mM and 2 mM [25]. Given this, it is reasonable to assert that there is also a phosphate concentration dependence on bacterial mineralization. Perhaps the reduced phosphate level is limiting the amount of mineral formation in human serum and therefore limiting the amount of bacterial mineralization.

Evidence of the Importance of Alkaline Phosphatase (TNAP) in bacterial mineralization

Although the exact function of alkaline phosphatase is not known, previous studies have suggested the importance of alkaline phosphatase for normal bone mineralization. The importance is illustrated in studies with hypophosphatasia, caused by genetic defects in alkaline phosphatase. Patients with this defect have various bone developmental problems [31]. In addition, it has been shown that inhibiting alkaline phosphatase activity also inhibits mineralization [32]. Studies with alkaline phosphatase knockout mice demonstrate that the absence of alkaline phosphatase causes skeletal disease [33]. Additionally, a recent study has shown that a source of tissue-non-specific alkaline phosphatase (TNAP) is required to activate a serum calcification factor that has been shown to be important in the calcification of type-I collagen. It is hypothesized that the calcification factor circulates in serum in an inactive form and becomes activated upon dephosphorylation by TNAP [3].

Our studies therefore investigated whether inhibiting alkaline phosphatase activity in serum would also affect bacterial mineralization. Results shown in figure 6 indicate that the three alkaline phosphatase inhibitors used, levamisole, phenanthroline, and beryllium sulfate prevented mineral incorporation into bacteria. To further investigate the importance of TNAP, serum was treated with phenanthroline, an irreversible inhibitor of alkaline phosphatase. As shown in figure 7 bacteria are unable to mineralize in this serum. However, upon addition of purified TNAP to

TNAP deficient serum, bacteria are once again able to mineralize to levels to those seen in normal serum. These sets of experiments therefore provide evidence that alkaline phosphatase partakes in an important role in mineralization and that the same components responsible for normal bone mineralization are also important for bacterial mineralization in serum. Further discussion on a role of TNAP for bacterial mineralization will be discussed later.

Evidence that Fetuin is necessary for serum-induced bacterial mineralization

In order to further establish that the mechanism of serum driven bacterial mineralization is similar to the mechanism that causes the mineralization of type I collagen (figure 1), an experiment was performed to determine the importance of fetuin in bacterial mineralization. Results shown in figure 8 provided evidence that fetuin directs mineral formation inside bacteria. With the depletion of fetuin, bacteria were not mineralized and the apatite crystals that formed grew outside the bacteria. Upon addition of purified fetuin to fetuin depleted serum, mineral growth was restricted to the interior of the bacteria. This experiment further supports our hypothesis that bacterial mineralization occurs through the same mechanism of mineralization as type I collagen. However, additional tests need to be performed to confirm the activity of fetuin in bacterial mineralization. Techniques need to be devised to separate the mineral formed during incubation in 10% fetuin depleted serum from the bacteria. The amount of mineral that formed outside the bacteria could then be quantified and compared to the amount of mineral that formed inside the bacteria. It is expected that the same amount of mineral that forms outside the bacteria, in the absence of fetuin, would be incorporated into the bacteria, in the presence of fetuin.

The Molecular Mechanism of bacterial mineralization

One of the main goals in this study was to determine if serum driven bacterial mineralization is similar to the mechanism of serum driven calcification of type-I collagen. As

outlined previously, there are four key requirements in this mechanism. 1) A matrix must have size exclusion properties that allow calcium, phosphate, and small mineral nuclei (<6 kDa) and must exclude larger proteins (>40 kDa) to cause mineralization of a matrix [2]. 2) A serum calcification factor that generates apatite crystals outside a matrix is necessary for mineralization [2,10]. 3) TNAP is necessary to activate the serum calcification factor [3]. 4) Fetuin is required to promote mineralization inside the collagen matrix by specifically inhibiting the growth of mineral nuclei outside the matrix.

With this study, we propose that the same four key features are necessary in the mechanism of serum driven bacterial mineralization. 1) A matrix with appropriate size exclusion properties is necessary for mineralization. Previous studies have shown that *S. aureus* bacteria meet this requirement. Molecules smaller than 14 kDa can access the bacteria matrix interior, but molecules larger than 50 kDa are excluded [4,5,6].

2) Serum or a serum calcification factor is essential for bacterial mineralization. Human, bovine, and rat serum are able to cause mineral incorporation into bacteria as evident through calcein staining, SEM, calcium and phosphate analyses, and TEM (fig.2-5, 9, 10A-H). Since the levels of calcium and phosphate are too low to cause spontaneous mineral formation, this provides evidence that serum contains a calcification factor required for the formation of mineral. 3) A source of TNAP is required. Our study has demonstrated that bacterial mineralization is inhibited with the use of three TNAP inhibitors, but addition of purified TNAP to TNAP deficient serum is sufficient to restore mineralization of bacteria (fig. 6, 7). 4) Fetuin is required to promote mineralization inside a bacterial matrix by specifically inhibiting growth of mineral nuclei outside the bacteria. Depletion of fetuin causes there to be predominant mineral growth outside the bacterial matrix. Repletion of fetuin restores the ability of bacteria to become mineralized in serum (fig.8). Our results not only support our hypothesis that a similar and possibly identical mechanism as type-I collagen mineralization are involved in bacterial mineralization, but they

also provide evidence that bacteria can become mineralized in a physiological system. We postulate that serum driven bacterial mineralization is not a random process, and we further discuss reasons as to why bacteria may be a target for mineralization *in vivo*.

The relationship between factors necessary for serum driven bacterial mineralization and factors involved in the immune system

Our studies have established a connection between serum driven bacterial mineralization and serum driven mineralization of type I collagen. This connection may also provide an explanation for why components necessary for collagen mineralization are also important factors in the immune system. First, there have been studies that suggest that leukocyte alkaline phosphatase activity increases in response to bacterial infection [13, 34, 35, 36]. Additionally, it has been shown that a patient with repeated infections had a deficiency in leukocyte alkaline phosphatase [14]. Although, the precise function of leukocyte alkaline phosphatase has not been elucidated, we draw from these studies that alkaline phosphatase may be an important factor in the immune system. It seems that alkaline phosphatase is upregulated during infection to participate in a mechanism to combat infection. This is further supported by the observation that in the absence of alkaline phosphatase there is a greater prevalence of bacterial infection. Since alkaline phosphatase typically plays a role in mineralization, we theorize that the secretion of alkaline phosphatase by leukocytes may play a role in combating bacterial infection by causing mineralization of bacteria. Bacterial mineralization may affect their ability to divide and their ability to import or export molecules; all of which may affect their viability or normal function.

Second, fetuin promotes bacterial phagocytosis by neutrophils through opsonization. It is thus an important regulator of the innate immune response [12]. Our study provides further insight into the potential role of fetuin in innate immunity by demonstrating that fetuin is

necessary for bacterial mineralization. If bacterial mineralization is an important mechanism of innate immunity then this is another reason why fetuin is significant in the immune system.

Finally, previous studies have demonstrated that hydroxyapatite particles can mediate the release of inflammatory cytokines and promote the recruitment of various immune cell types, such as polymorphonuclear neutrophils and leukocytes [37]. It can be imagined that these same cell types can be recruited to sites of bacterial mineralization. Our studies have shown that bacteria become mineralized in serum with the requirement of factors involved in the immune system and the most likely mineral formed inside the bacteria is hydroxyapatite based on morphology and Ca/Pi ratio. Therefore, bacterial mineralization may well be a viable mechanism of innate immunity.

A possible mechanism of combating bacteria through innate immunity can be illustrated as follows. Bacteria circulating through blood come in contact with macrophages or leukocytes. Leukocytes locally release TNAP and activate a serum calcification factor. Bacteria that have the appropriate size exclusion properties become mineralized through a mechanism that requires fetuin. Mineralized bacteria promote the secretion of various cytokines, which in turn promote the recruitment of more macrophages or neutrophils to the site of infection and the viability of these bacteria may also be compromised by mineralization. In this way, the components necessary for type I collagen mineralization are also necessary for innate immunity.

Further research will be necessary to confirm the involvement of bacterial mineralization in the immune system. First, it will be necessary to demonstrate that live bacteria can become mineralized in serum. Appropriate conditions still need to be developed in order to have bacteriostatic growth with the proper environment for mineralization to still occur. If mineralization of live bacteria is possible, different assays will be developed to determine the purpose of bacterial mineralization such as affecting viability or growth. Additionally, the impact of mineralization of live bacteria as a leukocyte response can be investigated. Assays can be

developed to determine if mineralization of live bacteria causes an increase in recruitment of macrophages or neutrophils. Lastly, an *in vivo* model can be developed to determine what affect live mineralized bacteria may have on the immune system of a rat.

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