Staphylococcus aureus: purification of RAP

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Project conducted under mentor Naomi Balaban, Ph.D., as part of the University of California at Davis Health System, Department of Pathology, Hugh Edmondson Fellowship Program of Summer 2000. In collaboration with Senbagam Virudachalan (senbagam@uclink4.berkeley.edu)

Abstract.

Staphylococcus auresus strain RN6911 is grown to the post-exponential phase. The supernatant is obtained and concentrated by lyophilization and Amicon which removes proteins smaller than 10 kilodaltons. The media sample is purified by gel filtration FPLC and become collected in different numbered tubes. These fractions are then subjected to in-vivo phosphorylation, Coomassie stainining, Northern blotting, and silver staining to test for the presence of a 38 kDa protein named RAP. A suspected fraction is targeted for sequencing. A sample of suspected fraction subjected to Coomassie staining reveals a distinct band between 30 and 46 kilodaltons that could possibly be RAP. The band is cut from the gel and sent to Sequencing laboratory. The results of the sequence will be compared to the current putative sequence of RAP.

Background.

Staphyloccoccus aureus is a Gram-positive bacteria that induces diseases such as pneumonia, endocarditis, meningitis, septicemia, and toxic shock syndrome (TSS). It is part of our natural flora. Currently in the United States, there are 2 billion people in hospitals. 750,000 of them are infected with *S. aureus* in a year, and 75,000 of them die. Although studies in bacterial infections have become less significant since the introduction of antibiotics, the recent increase in infections caused by methicillin-resistant *S. aureus* brings back attention to the pathology of bacterial infections. Such studies allow a thorough understanding of the process and provide means to develop blockade to the system in the forms of treatment or of vaccine.

When *S. aureus* first colonizes, it is relatively innocuous. Between the lag phase to mid-exponential phase, *S. aureus* produces stationary proteins that assist the bacteria in anchoring itself. Fro mid-exponential phase of growth, *S. aureus* begins producing secretory proteins, such as enterotoxins, hemolysins, and toxic shock syndrome protein. These proteins are proteases meant to digest away surroundings and provide the bacteria with living space.

The toxins thus produced are controlled by a global regulator named RNA III, which is transcribed from P3 transcription unit encoded by the *agr* locus. RNA III is regulated by a quorum-sensing mechanism. When a critical protein, RAP, accumulates to a certain threshold, which is reached at the mid-exponential phase of growth, RNA III is transcribed and toxins are produced.

RAP is a 38 kDa protein produced by *S. aureus*. RAP phosphorylates a 21 kDa protein named TRAP. TRAP-P, in turn, activates a signal transduction pathway, with the

result that RNA III is transcribed. If RAP could be purified, it could be made into vaccine by exposing animals to the antigen. In doing so, the animal can develop antibodies prior to contact with the bacteria. Subsequent, secondary exposure to the bacteria can then be combated quickly and efficiently.

Previously, RAP has been purified and its sequence determined to be IKKYKPITN, corresponding to a protein named L2 that lies within the *S. aureus* cell. The sequence, however, has not been verified to be, in fact, RAP. Moreover, attempts to repeat the finding have not been successful. Thus, the sequence of RAP remains in question.

Objectives.

The general objectives of this project is to determine 1) whether RAP is indeed L2, and 2) if it isn't, what the sequence of RAP is.

The specific objectives of this project are:

1) to purify RAP using *S. aureus* RN6911 strain by gel filtration FPLC and to sequence it.
2) to compare the new sequence of RAP with that of L2
3) to purify L2
4) to test L2 for RAP activity
5) to test anti-RAP antibodies against L2

In the duration of this Fellowship, I was responsible for most of the activities concerning RAP and also for a single test concerning L2. S. Virudachalam partook most of the activities concerning L2.

The methods and the results that I have obtained are described below.

Methods and Materials.

<u>Purifying RAP: overall procedure.</u>

RN 6911 is grown to post-exponental phase. The supernatant is then concentrated and applied to FPLC gel filtration column, which separates the proteins in the cells according to size. The substances in the column become collected in different tubes (called fractions) due to the varying time at which the substances come out. The densities of the substances collected are also recorded continuously via a UV detector that detects absorbance readings. Substances in tubes that are visualized to contain proteins (by way of the UV detector) are then subjected to testing by in-vivo phosphorylation, Comassie stain, Northern blotting, and silver stain.

During in-vivo phosphorylation, the fractions are each applied to 6390B early exponential cells. These cells contain TRAP but have not produced RAP in any significant amount. If a particular fraction contains RAP, the sample will phophorylate TRAP. Such phosphorylation can be visualized using a radioactive phosphate probe, and thus the existence of RAP in a particular fraction can be confirmed.

The gel which have been used for in-vivo phosphorylation are further stained by Comassie. Comassie staining reveals the amount of cells that are present in the gel. This

ensures that a sample will not be suspected of being phosphorylated more than others simply because there are more cells that were applied.

The sample fractions are also tested by Northern blotting. In this procedure, the fractions are combined with 6390B early exponential cells and incubated. The presence of RAP in the fractions will induce the transcription of RNA, which can be visualized by combining the sample and cells with radiolabeled RNA specific DNA.

Silver staining is performed by running the sample fractions on a gel which separates proteins according to size. The presence of the protein is then visualized by staining the gel with silver. If RAP is present in a particular fraction, it should reveal a unique, distinct band at roughly 38 kDa. This test is used as a supplement to the verification process since verification is determined mainly on activity, not on physiology.

After the tests are run, the suspected fraction are then subjected to SDS PAGE followed by Commassie staining. A band correlating to RAP are then cut from the gel and sent for sequencing. Results of the sequencing would be back in a few weeks.

<u>Testing anti-RAP antibodies against L2:</u>
Western blot

Sample putatively containing L2 (as eluted from nickel column) is run on a gel and transferred to a nitrocellulose membrane. The membrane is cut into strips, and each strip is exposed to serum from different mice, some of which have been injected with RAP collected from FPLC and would thus develop anti-RAP antibody. The strips are then tagged with anti-mouse antibody, which binds to H2O2 to give off fluorescence.

For in-depth description of methods, please see protocol section at the end of the document.

Results.

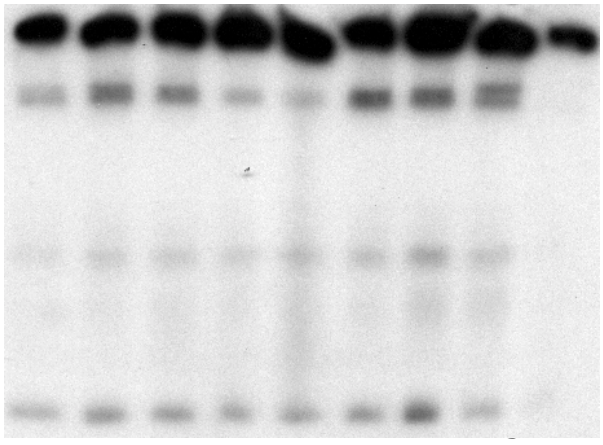
14+ FPLC run throughs were performed and two sets of fractions were obtained.

The two sets of fractions are subjected to the tests described above, although not all tests were conclusive.

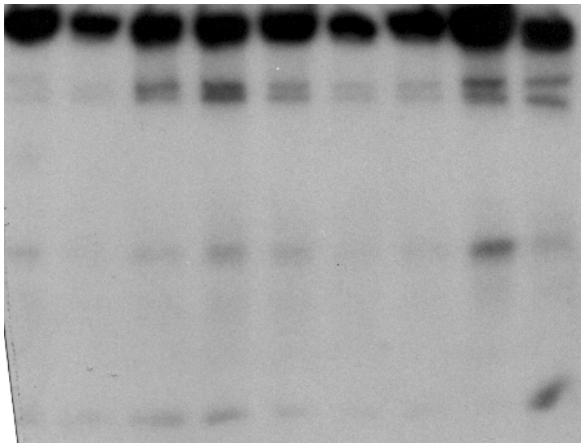
Some samples of the tests are displayed below.

Figure 1. In-vivo phosphorylation. 7/21/00
6911 30x >10 kDa 7/5/00

11 12 13 14 15 16 17 18 19



20 21 22 23 24 25 26 +C -C

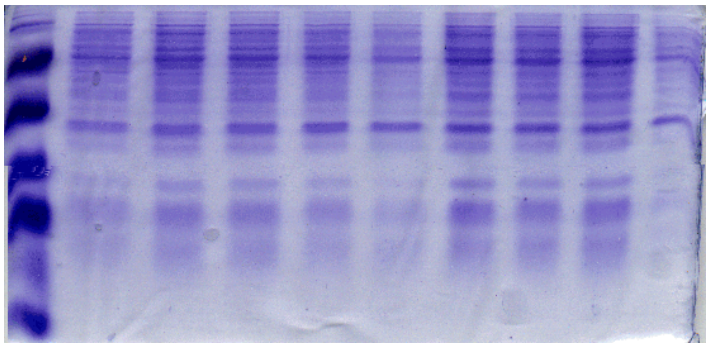


Fraction #17 contains a band around 21 kilodaltons that correspond to that of positive control (total supernatant added to 6390B cells)

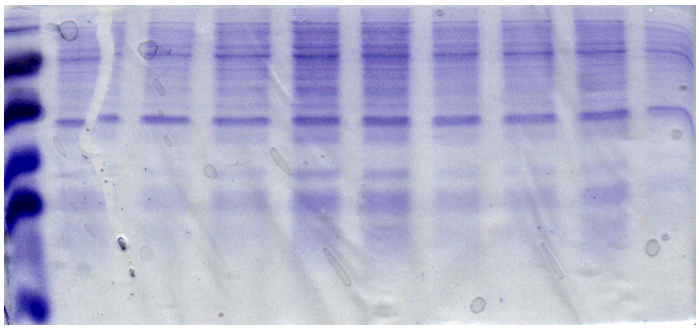
Figure 2. Coomassie Staining. 7/21/00
6911 30x >10kDa 7/5/00

The same gels above are subjected to Commassie staining.

Mw 11 12 13 14 15 16 17 18 19



Mw 20 21 22 23 24 25 26 27 28



The amount of cells in each column are roughly similar, indicating that fraction #17 (in in-vivo phosphorylation) did not appear more phosphorylated due to more cells being applied.

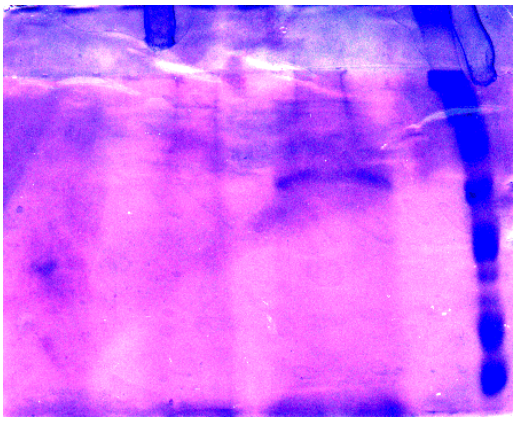
Figure 3. Silver staining
6911 30x >10kDa 7/26/00

13 14 15 16 17 18 19 20 21 mw

Fraction #19 contains a distinct band between 30 and 46 kilodaltons.

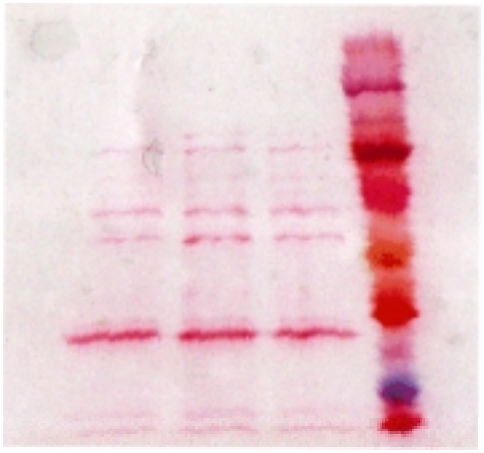
Figure 4. Coomassie staining (for sequencing)

17 18 19 mw



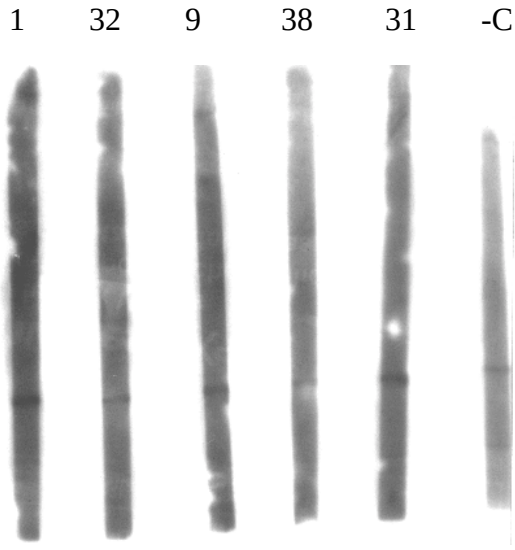
Large band on fraction #19 is cut and being sent to sequencing.

Figure 5: Western blot (stained with ponceau) 7/31/00
"L2" eluted pH 4 by Sen



No band is actually present between 30 and 46 kilodaltons, suggesting that L2 was not present in the sample. However the procedure was contined. The result is indicated in Figure 6.

Figure 6: Western blot 8/4/00
"L2" eluted pH 4 by Sen



Mouse 1 and 9: injected with RAP
Mouse 32, 31: injected with PBS

Mouse 38: untreated
C: no first antibody

All the strips exhibit one single band. Since the band exists in positive and negative samples alike, this suggests that either 1) RAP is not L2 or 2) L2 was not purified. The results of S. Virudachalam's tests indicate that suggestion #2 was correct.

Conclusion.

Reviewing the Specific Objectives of this experiment, it can be seen that
Objective 1 is achieved
Objective 2 will be achieved as soon as the sequence comes back
Objective 3 is achieved by Senbagam Virudachalam
Objective 4 will be achieved in the coming week after this Fellowship
Objective 5 was undertaken but not achieved because L2 was not purified. It will be achieved in the coming week after this Fellowship.

Protocol.

Bacterial Strains

- wild-type *S. aureus* strain RN 6309 B
- *agr*-null *S. aureus* mutant strain RN 6911. 6911 produces no toxins and therefore the supernatant will be relatively free of contaminants, making the purification of RAP more probable.

bacteria are grown in CY broth supplemented with beta-glycerophosphate at 37 degrees Celsius with shaking from early exponential phase

RAP purification

6911 cells are grown for six hours in blood agar plates in CY broth, beta-glycerophosphate, and tetracycline (an antibiotic to which 6911 strain is resistant), till cells reach post-exponential phase. The mixture is centrifuged 10 minutes at speed 6.5 at 4 degrees Celsius and the supernatant is obtained. The supernatant is frozen in liquid nitrogen and lyophilized. The liquid thus obtained is applied to a 10 kDa cutoff membrane (Centriprep 10 (Amicon)) to concentrate material and to remove material smaller than 10 kDa. The resultant concentration is 30X. To apply to FPLC column, 100 microL of sample is obtained, centrifuged at speed 14 for 5 minutes to ensure no pellet is left. If pellet is found, the supernatant is further obtained and centrifuged at speed 14 for 1 minute. The sample is fractionated in FPLC column (Superose 12, Pharmacia) in 0.1 X PBS (1mM phosphoate buffered saline pH 7.2), at flowrate of 0.5 ml/ min and collected in 1 mL fractions. The O.D. readings of fractions are observed on UV detector and the O.D. patterns of flow are recorded on chromatopac. In order to collect large enough volume to run assays, 7 runs with similar chromatopac graph are combine tube by tube, with result of each tube having 7 mL. Liquids obtained from combined runs are lyophilized and the resultant solids are resuspended in 700

microL ddH2O with the final concentration obtained being 10X. Fractions are tested for presence of RAP by the procedures of in-vivo phosphorylation followed by Comassie staining, Northern blotting, and silver staining

In-vivo phosphorylation

Due to the radioactive nature of this experiment, it was performed by Dr. Naomi Balaban.

A rough description of the methods follow:

- Grow cells in the presence of radioactive phosphoate
- Collect cells
- Run cells on 15% SDS PAGE
- Radioactivity is exposed to film

Northern blotting

- 900 microL of 6390 B OD600 = 0.200 (early exponential phase) are combined with 100 microL FPLC fractions in scintillation tubes and incubated at 37 degrees C for 40 minutes. Procedure is stopped by ice. The cell samples are centrifuged for 2 minutes and the pellet is retained. To each pellet was added 50 microL of lysostaphin in TES (10 mg / mL). The mixture is then incubated 10 minutes at room temperature. 50 microL, 2% SDS, Proteinase K (50 microG / mL) is added and the mixture is vortexed at high speed until clear. The mixture is then frozen and thawed two times. 15 microL of the sample thus obtained is mixed with 11% deionized glyoxal, 16 mM phosphate buffer at pH 7.0, 55% DMSO and incubated at 65 degrees C for 1 hour. RNA loading buffer (Ambion) is added.
- RNA Sample is loaded in 1% agarose gel in buffer (10 mM phosphate buffer pH 7.0, 5 mM Iodoacetic acid (Sigma). Gel is dry transferred to nitrocellulose overnight which is wet with 1 X SSC. Membrane is UV crosslinked, stained with methylene blue to visualize the presence of RNA and to note the relative amount obtained. Membrane is then rinsed with deionized, distilled H2O and destained with SDS 1X SSPE DEPE. Membrane is prehybridized with Rapid-hyb buffer (Amersham) for 1 hour then hybridized with PCR radioactive RNA III-specific DNA (32P) for two hours. It is then put on film and expose overnight

Silver Stain

Follow directions in Bio-Rad kit for silver stain.

Western blot

- Sample of "L2" is inserted into plasmid, amplified with E. coli and histidine-tagged. Sample is eluted through nickel column by Sen Virudachalan
- L2 sample is run on 12% SDS PAGE (0.35 microG / microL): 6 microL sample, 3 microL sample buffer, 5 microL ddH2O with 1 X running buffer. Gel is transferred onto membrane by secondary, horizontal electrophoresis for 1 hour, 100 volts in 1X transfer buffer and stirred. The membrane is taken out, rinsed in ddH2O, stained with ponceau to visualize the presence of L2, rinsed again with ddH2O, and destained

with 1X PBS + 0.05 % Tween to remove ponceau. Membrane is blocked in 3% bovine serum albumin in 1X PBS + 0.05% Tween overnight (1 hour is sufficient). BSA is then discarded and membrane is incubated in mice serum (anti-RAP, previously made by injecting HPLC-purified RAP to mouse) for 2 1/2 hour at room temperature. Membrane is rinsed in PBS 3 times at 15-minute session, incubated in secondary antibody (peroxidase-conjugated anti-mouse antibody: 1:2000 dilution in 1X PBS + 0.05% Tween, or 3 microL in 6 mL). The secondary antibody binds to H2O2 to give off fluorescence

- Membrane is rinsed in 1X PBS + 0.05% Tween. ECL (chemoilluminescence) is performed: strips are dipped in detection agent and a film is pressed over strips in dark room. The film is developed the next day.

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