I. Anti-*V. vulnificus* Oyster Defensin. Its Synthesis and Use to Reduce the *V. vulnificus* Load in Oysters Destined to be Eaten Raw.

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II. Abstract

Our aim was to develop a safe effective way to reduce the *V. vulnificus* load in oysters destined to be eaten raw to levels deemed safe by the USFDA. This is being achieved with the isolation of a small peptide with defensin-like properties from tissue extracts of the oyster (*Crassostrea virginica*) found to specifically inhibit *V. vulnificus*. Currently, this peptide is being characterized and sequenced. Using these data, the peptide will be synthesized. This would allow it to be produced in bulk and available for use by the shellfish industry. Alternatively and perhaps more advantageous, is the direct production of this peptide, now called "Anti-*V. vulnificus* oyster defensin" (AVvOD), by oyster hemocytes in continuous culture (presently available) in response to exposure to *V. vulnificus.* Other than the need to define extraction, and perhaps concentration procedures, AVvOD could be produced in bulk and at a minimum cost.

III. Executive Summary

A factor that was specifically bacteriocidal to *V. vulnificus* was reported present in tissue extracts from the oyster, C. virginica. It was also showed that this factor could be used to reduce the *V. vulnificus* load in oysters that were to be eaten raw. Current studies found this factor to be a small peptide with defensin-like properties. As a consequence, this peptide was called "Anti-*Vibrio vulnificus* oyster defensin" (AVvOD). While being isolated and sequenced inpreparation for its synthesis and subsequent production in bulk, it was also learned that AVvOD was secreted into the hemolymph by hemocytes in response to the presence of *V. vulnificus*. In addition, to the studies conducted with respect to. AVvOD, hemocytes from the eastern oyster (*C. virginica*) were successfully propagated in vitro. These have been in continuous culture for nearly two years. Details regarding the methods and procedures used for their isolation, growth, maintenance, etc. are presented as are the observations and characterizations relating to the different hemocytes. Planned is the large scale use of these cells as a means to produce AVvOD in bulk.

IV. Purpose

A. The Problem: During the past three decades, there has been an overage of 10 deaths per year attributed to having eaten raw oysters harvested from the Southeastern Atlantic and Gulf coastal waters. This is where oysters become exposed to and contaminated with *Vibrio vulnificus*.

Public pressure generated by bad press motivated the USFDA to mandate the posting of warnings about the dangers of eating raw oysters in all commercial establishments where raw shellfish are sold and/or eaten, and on all containers holding the raw flesh from sucked animals. As a consequence, both the Gulf and Southeastern Atlantic coast shellfish industries suffered a major economic impact that not only depressed local sales, but also the sales to oyster dealers in other states. This impact still persists to this day.

B. Objective: The objective of this investigation was to develop a safe effective way to reduce the *V. vulnificus* load in oysters destined to be eaten raw to levels deemed safe by the USFDA.

V. Approach

A. Work Performed: Our initial efforts to reduce the *V. vulnificus* load in raw oysters made use of pools of nine *V. vulnificus*-specific bacteriophage that we had isolated from water samples collected at oyster reefs in the Gulf. While this approach achieved our objective, we concluded that it would not lend itself for mass application because of the costs for the technical support that would be needed. In the interim, we encountered a protein factor in crude oyster tissue extracts. When mixed with *V. vulnificus,* then followed by an overnight exposure of the mixture to a temperature of 4°C, the protein factor would significantly reduce the number of micro-organisms in 100grams of oyster flesh by 5 to 7 logs. This reaction between protein factor-positive oyster extracts and *V. vulnificus* was reproducible no matter whether direct exposure to *V. vulnificus* was involved, whether live oysters were first contaminated with the bacterium, the tested, or whether naturally contaminated oysters were challenged. We called this protein factor "Anti-*Vibrio vulnificus* Oyster Protein" (AVvOP).

Carrying this a step further, a combination of pooled phage and AVvOP-positive oyster tissue extract was added to oysters that had first been deliberately contaminated with *V. vulnificus*. Following incubation, *V. vulnificus* in oyster homogenates were quantitated. The results showed that the combination of pooled phage and AVvOP-positive oyster extract was even more effective in reducing the *V. vulnificus* load.

AVvOP was found to have some of the characteristics attributed to defensins. Autoclaving (15 lbs/ 15 min.) does not inactivate it. AVvOP can pass through a membrane filter with an APD of 0.22 ?M (Millipore Corp., Bedford, MA), but not through a membrane filter with an APD of 0.10 ?M. We found it to be small peptide consisting of two molecular species with masses, 1.9 kD and 3.2kD. this peptide is now called "Anti-*Vibrio vulnificus* Oyster Defensin" or AVvOD.

We became interested in propagating cells from oyster tissue following our findings relating to the pros and cons associated with the use of pooled *V. vulnificus*-specific bacteriophage to treat *V. vulnificus*-contaminated oysters as well as from the basic information that came to light in the course of our investigation.

A search of the literature revealed numerous references to the propagation of cells from several bivalve species including oysters.

Efforts have been made to establish cell lines with marine invertebrate tissues with a number being successful with different cells from a variety of species including oyster heart cells, gill cells, etc. There have been no reports regarding the long-term propagation *in vitro* of hemocytes from the oyster, *C. virginica*.

While continuing our literature search, a report was encountered describing the production and excretion of several kinds of antimicrobial peptides including defensins by cells present in the hemolymph of mussels (*Mytilus galloprovincialis* and **Mytilus edulis**) (1). Since AVvOD was defensin-like with respect to its activity, it was speculated that it may also be produced by similar cells in oyster hemolymph. A search uncovered several reports describing the propagation of cells from different oyster tissues. Of these, only two related specifically to hemocyte propagation (2,3).

Crude AVvOD-positive oyster tissue extracts served to obtain preliminary baseline data

for this investigation. However, to have been able to use AVvOD for treating *V. vulnificus*-contaminated oysters on a commercial scale, methods that would successfully produce this peptide in bulk first had to be given consideration.

We concluded that there were two alternatives. The first would have involved attempts to synthesize the peptide, i.e. where one seeks to prepare artificially, a biologically active product of nature.

The second option would search fro a system of cells that grew *in vitro* and also secreted AVvOD. Envisioned were large populations of AVvOD-secreting cells being maintained as a suspension culture for an appropriate period of time then harvested. AVvOD present in the culture medium would be collected using appropriate extraction procedures, yielding a product in quantities sufficient for commercial use. To assure our success, it was decided to pursue both options.

The role that seasons play with respect to AVvOD activity in tissues of oysters is not surprising. Ecological studies have shown repeatedly that *V. vulnificus* population numbers vary directly with the temperature of the waters in which they are present. During the summer months the temperatures of the marine waters of the gulf often reach 30°C. Similarly, the serious human infections and deaths have resulted from eating raw oysters have occurred during the summer months when *V. vulnificus* populations and water temperatures reach their peaks.

Our data has shown that the presence of AVvOD in oyster tissue and in the hemolymph in particular, is a reaction to the presence of *V. vulnificus*. To support of this finding, we examined Japanese oysters (*C. gigas*) and Washington oysters (*C. lurida*) for AVvOD activity on two occasions. These were harvested in and shipped from the state of Washington to New Orleans. We found both species to be negative for AVvOD. There in Washington State, the marine waters are cold and *V. vulnificu*s populations are virtually non-existant.

To confirm that an extreme difference did not exist between *C. gigas* and *C. virginica*, newly received *C. gigas* were randomly placed into each of two aquaria, one of which we supplemented with a saline suspension of *V. vulnificus*. These were held for one week after which animals in each of aquaria were sacrificed, their tissues pooled, processed, and each assayed for AVvOD. The results showed that the pooled tissue extract from animals not exposed to *V. vulnificus* was negative for this peptide, while the pooled tissue extract from animals exposed to *V. vulnifcus* was positive.

B. **Project Management:** William Pelon, Ph.D. is the Principal Investigator for this project. Professionally, he is a virologist and microbiologist with extensive experience in cell culture. His responsibilities include Option 2.

Kenneth H. Johnston, Ph.D. is a Co-Investigator for this project. Professionally, he is a microbiologist and a protein chemist. He is responsible for the genetic and molecular biological phases and for Option 1.

Ronald B. Luftig, Ph.D. is and Advisor for the project. Professionally, he is a virologist with extensive experience as an electron microscopist.

VI. Findings

A. **Actual Accomplishments and Findings:** It is accepted that a function of defensin is to protect a host lacking humoral immunity against invasion of its tissues by a would be

pathogen. We found that the threat of contamination of infection by *V. vulnificus* is not restricted to oysters. *V. vulnificus* is also a threat to other marine species that inhabit the same ecological niche as does *V. vulnificus*. We have detected AVvOD activity in tissue extracts from a number of marine species that have same ecological requirements. In contrast, when those marine species harvested from marine environments known to be unfavorable for *V. vulnificus (*cold temperatures, excessive salinity, deep waters, etc.) were examined, AVvOD could not be detected. While contamination or infection of other species by *V. vulnificus* do occur as evidenced by the presence of AVvOD in extracts from their tissues it has not received the attention given to oysters. Human infections have not been reported from these sources since, as a rule, these animals are not consumed raw.

Option 1: Synthesis of AVvOD. While some success in sequencing, fragments was obtained, there is a question as to whether the peptide will lend itself to being synthesized. Studies were being conducted into the feasibility of synthesizing AVvOD as a means to produce this peptide in volume for commercial application.

As an example, we have been able to analyze, sequence, and identify AVvOD structurally as a defensin. We have successfully isolated and performed preliminary sequencing on two polypeptides, AVvOD1 and AVvOD2 obtained from the hemolymph of *Crassostrea virginica* that exhibited bacteriocidal activity against *V. vulnificus*. Most interestingly, these polypeptides have a significant degree of homology to a recently described hemolymph polypeptide, cavortin, isolated from *C. gigas* by a New Zealand group. However, this group did not characterize its biological or microbiological activity.

We also have identified similar but bio-chemically dissimilar polypeptides from oyster hemolymph exposed to *Aeromonas hydrophila* and *Vibrio parahemolyticus* that exhibited unique bacteriocidal activity against these two pathogens. We are in the process of subjecting them to amino acid sequencing analysis.

Previously we had indicated that smaller polypeptides had antibacterial properties identical to that described above. It is our belief that these are fragments of the native sequence molecule. We are investigating how homocytes upon exposure to the bacterial stimulus may process the parent molecule into specific defensins. It should be noted that thee polypeptides significantly differ from the cationic polypeptide (Lysozyme that h as been recently described (4).

Dr. Johnston has recently prepared a cDNA library of the oyster from hemocytes. He has identified the DNA domain which codes for this polypeptide. He is presently cloning and sequencing this DNA to determine the complete sequence as initial studies to determine the mode of action and domain specificity of the molecule.

Option 2: Production of Natural AVvOD. An alternate way to produce this peptide in bulk that was under consideration was the propagation of AVvOD producing and secreting oyster cells *in vitro*. We already have evidence of AVvOD activity in the hemolymph of oysters exposed to *V. vulnificus.* Hemocytes in the hemolymph, particularly the granulocytes are believed to be cells that produce the peptide and release it into the hemolymph. Described below in a pseudo-chronological order are the directions that were taken, the hurdles that were encountered along with the consequences of our efforts.

The medium used to propagate oyster tissue cells and hemocytes evolved following a series of experiments. Its composition can be seen in Table 1. Details relating to the various constituents and/or their preparations are presented below.

Leibovitz L-15 medium (5) was obtained as a powder from Sigma Chemicals, in quantities that make 1 liter. This was added to 1 liter of 1.5% saline and stored frozen at -20°C.

Eagles Minimal Essential Medium (MEM) (6) received as frozen stock concentrates were also obtained from Sigma chemicals. The following were added to an 800 ml aliquot of Hanks Balanced Salt Solution (BSS) (7) in the volumes indicated: 100 x vitamin mixture 10ml, 200 mM glutamine solution 10ml, 50 x essential amino acid mixture 20ml, 100 x non-essential amino acid mixture 10ml, NaCL 15 g, and $NaHCO_3$ 0.0035%. The mixture was brought up to a 1 liter volume with additional Hanks BSS. This was stored frozen at -20°C until needed.

TRIS-HCL buffer complex stock was prepared using 0.2M Tris (hydroxymethyl) aminomethane (24.2 g/L) and 0.2 M HCl made with activated carbon-filtered deionized water. This solution was supplemented with 2% NaCL, 2% glucose, and 0.5% EDTA. The pH was adjusted to 5.0. The buffer was sterilized by membrane filtration then stored at 4°C.

Newborn calf serum was obtained from Sigma Chemicals. It was heat-inactivated (56°C/30 min) prior to use and stored frozen at 20°C.

Sea clam juice in 46oz tins and under the brand name “Sea Watch” was donated by Sea Watch International Ltd., Milford, Delaware. Prior to use, the juice was centrifuged at 800 xG/1 hour, the supernatant sterilized by membrane filtration and the filtrate stored frozen at 20°C.

Artificial estuarine water was prepared with Instant Ocean Sea Salts (Aquarium Systems Inc., Mentor, Ohio). These were added to activated carbon-filtered demineralized water at a concentration that gave a salinity of 29 ppt.

1.5% saline solution was prepared by adding fifteen grams of NaCl to activated carbon-filtered deionized water. The volume was brought up to 1L then sterilized by autoclaving.

Time-lapse photography: Time-laps photographs were taken at a rate of one frame per hour with the use of a Leitz Fluorovent microscope in conjunction with a RT Color Spot digital camera and program. The finished product was reproduced at 4x and/or 20x magnification.

Oysters: Freshly harvested oysters, donated by the P&J Oyster Processor and Distributor (New Orleans, LA) were transported to the laboratory under wet ice. Individual animals were washed under running tap water, then scrubbed with a nylon-bristled brush, and rinsed under running activated carbon-filtered deionized water. Oysters were either sacrificed immediately or held in aquaria containing artificial estuarine water for future use.

As a filter feeder, the oyster was observed to pump 30 or so liters of water per hour. Under such circumstances, the animal is exposed to many microbial contaminants, both outside and inside its valves. Furthermore, the mantle of the oyster forms a sac with which the internal sea water flow is controlled and within which the internal organs are bathed. The contaminants are not restricted to microbial types, but also include molds and species of fungi, protozoa and marine worms.

In an effort to cope with these contaminants, a battery of antibiotics was incorporated into the outgrowth medium and into salt solutions used to wash the cells. These included penicillin G, streptomycin, colistin, polymyxin B, mycostatin, and amphoteracin B. An

effort was made to control the contamininating protozoa by incorporating metranidazole (Flagyl) into the culture medium. This however was not successful.

The presence of protozoal-like organisms as contaminants in cultures of oyster cells was not uncommon. The experiences of others revealed that 30% of cell cultures from oyster and 27% of those derived from clams were contaminated with these organisms and were discarded (3).

Experiences with oyster tissue cells. Freshly harvested oysters were cleansed as described above. Despite these preparations, it was assumed that the animals were still contaminated both externally with microorganisms common to the marine environment, and internally by similar organisms as the result of filter-feeding.

Initially, our efforts began with rinsing and scrubbing the valves, followed by a rinse with 70% ethanol, then shucking to remove the upper valves. Pieces of various tissues were randomly excised, pooled and minced. The tissue fragments were washed in 1.5% saline, then re-suspended in Eagles MEM containing 10% newborn calf serum placed into 25 cm2 flasks and incubated at ambient temperature. Examination of the flasks on the following day revealed gross contamination. The contaminants were not restricted to microbial types, but also included a spectrum of life forms.

Oyster hemocytes *in vitro*. Based upon this evidence and the realization that of all of the oyster tissues, the hemocytes and hemolymph were the most protected against contamination efforts were made to isolate and propagate *C. virginica* hemocytes.

To further reduce contamination, the cleansed oyster was placed into a fresh 10% solution of sodium hypochlorite (Chlorox) for ten minutes. This was followed by rinsing with a sterile solution of 1.5% NaCl. The oyster was then shucked by first breaking the ligament, then lifting the upper valve, cutting the adductor muscle with a sterile sharp oyster knife, and carefully removing the upper valve. The animal was placed into a fresh solution of 10% sodium hypochlorite for an additional 10 minutes. Upon removal, the animal was again rinsed with a sterile 1.5% NaCl solution. The hemolymph, together with the hemocytes, were aspirated from the pericardial sac within which the heart was suspended and from the adductor muscle sinus using a sterile 3.0 ml syringe and a 22 gauge needle. The hemolymph-hemocyte suspension was centrifuged at low speed (140 x g/6 minutes). The supernatant fluid was decanted and saved for future reference.

The repeated use of a 10% sodium hypochlorite solution to reduce microbial contaminants on and in oysters used as sources for hemocytes may seem drastic, particularly following removal of the upper valve. However, it was not uncommon to see the hearts of such animals pulsate following the second sodium hypochlorite exposure. The use of sodium hypochorite (Chlorox) for the disinfection of oyster tissue has been described by others (3). While the objectives for using this chemical were identical, we differed in terms of the concentrations used (10% in our case versus 1% to 5%), the exposure time (10 minutes versus 1 minute), and the frequency of exposure (twice versus once).

In order to reduce the opportunities for microbial contamination further, the cell pellet was resuspended in the hemocyte medium (Table 1) and incubated at ambient temperature for 1 hour. This was followed once more by low-speed centrifugation. The decanting of the supernatant fluid, resuspension of cell pellet in medium and a second 1 hour incubation. The hemocyte suspension was centrifuged once more. After the supernatant fluid was decanted, the cell pellet was resuspended in hemocyte medium placed into a $25cm^2$ TC flask and incubated at ambient temperature.

Characteristics of *C. virginica* Hemocytes in our hands, cultured hemocytes were best described as a mixture of mostly spherical cells ranging in size from large to small. Studies by others, using electron microscopy, identified at least three types of agranulocytes and a single granulocyte type present in *C. virginica* hemolymph, based upon nuclear size and morphology, and cytoplasmic constituents (8). Type 1 agranulocytes were described as small, but having a large oval-shaped nuclei and scanty cytoplasms ("lymphocyte-like"). Type II agranulocytes have an oval-shaped nucleus, but also a large cytoplasm containing clusters of glycogen. Type III agranulocytes have a spherical nucleus containing dense chromatin material as well as a numerous localized vesicles and tubules. The single granulocyte type is described as having either a round or oval-shaped nucleus with or without a nucleus, and cytoplasm containing light or dark granules

Our studies detected at least four cell types by electron microscopy and by FACS analysis. In collaboration with Dr. Anthony Haag, UTMB, Galveston, TX, proteonomic analysis of hemocytes exposed to and not exposed to *V. vulnificus* is being performed with the purpose of using subtractive analysis to determine which oyster proteins are up-regulated on exposure to bacterial production of AVvOD.

In our experience, hemocytes in culture did not form monolayers, nor did they attaché to the surfaces of their containers regardless of the material with which the containers are fabricated (flint or Pyrex glass, plastics including polystyrene, polypropylene, and polycarbonate). Chemical pretreatment of surfaces with substances such as poly-d-lysine, collagen 1, fibronectin, and laminin prior to the addition of these cells did not result in cells adhering.

There was concern that EDTA as a component of the hemocyte medium may prevent adherence of the hemocytes. The use of an EDTA-free hemocyte medium did not enhance hemocyte adherence.

It was reported that hemocytes were capable of amoeboid movement (6). In observing cells in flasks held as stationary cultures and where the cells came at rest and in contact with a container's surface we found that most were stationary. However, it is possible that cell movement was so minimal as to be undetectable during the time periods when microscopic observations were made. Hourly time-lapse photographs of cells in flasks did reveal movement of some cells in the course of 24 hours.

Efforts were made to determine the time required for hemocytes to double in population size. Daily viable cell counts were made of hemocytes growing *in vitro*. The results from such counts revealed that a period of 48 hours was required for a doubling of the cell population.

Microscopic observations of hemocytes growing in vitro often revealed numerous cells in pairs. Other cells appeared to undergoing binary fission , although this has not been confirmed. To complicate matters more, some cells appear to be undergoing budding, analogous to that seen with yeast species. Scanning micrographs of hemocytes in culture also reveal some cells undergoing what we have interpreted again as budding. Our interpretation may be reasonable if the relative positions of innate cell-mediated immunity and humoral immunity systems on the evolutionary scale are considered.

We have been successful in maintaining oyster hemocytes in continuous culture for a prolonged period using the hemocyte culture medium in conjunction with the following protocol:

Every seven to ten days, individual flasks of hemocyte stock were shaken to disperse the

small clumps of cells that formed during incubation. The cell suspension from each flask was decanted into its own centrifuge tube (the size and number of tubes was contingent upon the volume of fluid in the flask). Centrifuging was carried out at low speed (140 x g) for 6-8 minutes. The supernatant fluids were decanted and either saved for study or discarded. The cell pellets are resuspended in equal volumes of fresh medium and returned to the original flask, or divided into equal volumes and added to both the original flask and a new flask. The cells are then incubated at ambient temperature.

Efforts were made to preserve a portion of the oyster hemocyte cell stocks by freezing. While our method may appear primitive by today's standards, it did permit an initial period of slow freezing and was effective with respect to cell survival.

Dense cultures of oyster hemocyte were shaken, then centrifuged at low speed (140 x g) for six minutes, after which the supernatant was decanted. The cell pellet was resuspended to one quarter of the original volume of the culture using hemocyted medium supplemented 12% DMSO and 2% glucose. This was distributed into freezer vials (Nunc) in 1 ¼ ml volumes. The vials were tightly covered with non-absorbent cotton and placed into a small glass jar that was stuffed with additional cotton and sealed. This was placed into a mailing tube, stuffed with more cotton, sealed, and the tube stored in a -70°C electric freezer.

To determine the efficacy of our method, a vial of frozen cells was removed following one week of storage, quickly thawed at 45°C and planted using a 25 cm^2 TC flask containing 5.0 ml of hemocyte medium. This was incubated at ambient temperature. While no effort was made to quantitate surviving cells, these appear to be in the majority after 48 hours of incubation.

To ascertain the effect of prolonged freezing under the conditions described, a vial of hemocytes was quickly thawed after being frozen at
-70°C for 17 months, and planted into a 25cm^2 TC flask containing hemocyte medium. The cells looked healthy during the first seven days of incubation, at which time the hemocytes were divided into three flasks because of heavy growth. Growth continued and by day 14, it became necessary to transfer the cells from the small flasks to a large flask.

In past investigations of oyster hemocytes in culture, the number of mitotic figures that were seen served to reflect the health status of the cells (3). The more mitotic figures seen, the more successful was the culture. The absence of, or the presence of only a few mitotic figures was interpreted as a decline in the general state of the cells.

Mitotic figures were rare in our cultures when viewed microscopically even though increases in population densities could be seen with continued incubation. This may have been due to the low magnification needed when examining hemocytes though the bottom surfaces of the flasks. Also neither chromosome analysis nor chromosome spreads were made. On the other hand, we considered the presence of cells in pairs, or where paired cells appeared to be drawing apart as being indicative of cell replication. In addition, cells in the process of apparent budding comparable to that seen with some yeast species, were common.

Our strongest argument favoring the budding process by hemocytes in addition to usual binary fission was obtained from scanning electron microscopy where cells in the process of budding were obvious although some may interpret the buds as pseudopodia associated with hemocytes that are phagocytic.

Assuming that AVvOD continues to live up to our claims as being the panacera for the *V. vulnificus* problem, its use to treat oysters on a grand scale would minimize the threat of human infection. Since the oyster itself would not be affected by exposure to AVvOD, its flavor would be retained. Unlike other methods to ftreament used to eliminate contaminating *V. vulnificus*, exposure to AVvOD will no kill the oyster, hence there would be no need for the added expense associated with having to band each individual oyster prior to treatment in order to keep its valves shut. This would not only be a plus for the oyster connoisseur, but also the salvation for the shellfish industry.

B. Significant Problems of Negative Results: We have no significant problems to report. Those that we had encountered were of a technical nature in which we were able to resolve.

C. Need for Additional Work: We believe that by its very nature, research does not resolve problems but instead creates new questions. Hence, there is always the need for additional work.

VII. Evaluation

A. Attainment of the Objective: This project represents a simultaneous effort to solve a problems associated with public health to explore the production of these peptide by hemocytes in the hemolymph of oysters in response to exposure to *V. vulnificus* with information and data obtained from ongoing research. While we have not as yet completely attained the objective of the project per se, we have been able to accumulate a wealth of basic information that previously had not been known and which we are applying towards fulfilling this project's objective. The research data obtained thus far has confirmed the soundness of our approach, and has provided us with insight as to the direction that we should go to provide sufficient quantities of AVvOP to routinely treat and rid freshly harvested oysters that are to be eaten raw, of contaminating *V. vulnificus*.

B. Dissemination of Project Results: The progress that has been made relative to this project has been reported at national meetings (see below).

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