BARD Project Number: US 3864-06

Date of Submission of the report: December 2010

Project Title: A Whole-Cell Biosensor Panel for Agricultural Endocrine Disruptors

**Investigators**

**Principal Investigator (PI):**
Shimshon Belkin

**Institutions**
Hebrew University of Jerusalem

**Co-Principal Investigator (Co-PI):**
Sylvia Daunert (from 2008)  University of Kentucky
Mona Wells (to 2008)  Tennessee Tech University

**Collaborating Investigators:**

---

**Keywords** not appearing in the title and in order of importance. Avoid abbreviations.
Bioreporters; bioluminescence; environmental pollution; antibiotics.

**Budget:**
IS: $140,000  US: $140,000  Total: $280,000

---

Signature  Principal Investigator
Signature  Authorizing Official, Principal Institution
**Publication Summary** (numbers)

<table>
<thead>
<tr>
<th>Refereed (published, in press, accepted) BARD support acknowledged</th>
<th>Joint IS/US authorship</th>
<th>US Authors only</th>
<th>Israeli Authors only</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submitted, in review, in preparation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invited review papers</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Book chapters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Books</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Master theses</td>
<td></td>
<td>1 (Ruthi Kiro)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ph.D. theses</td>
<td></td>
<td>1 (Sahar Melamed)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Abstracts</td>
<td></td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Not refereed (proceedings, reports, etc.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Cooperation Summary** (numbers)

<table>
<thead>
<tr>
<th>Short Visits &amp; Meetings</th>
<th>From US to Israel</th>
<th>From Israel to US</th>
<th>Together, elsewhere</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longer Visits (Sabbaticals)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Postdoctoral Training:** List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

**Cooperation Summary** (numbers)

**Description Cooperation:**

Strong cooperative ties were kept between the Israeli and US partners. This cooperation was not hindered by the change of the US partner changed from Prof. Wells in Tennessee Tech to Prof Daunert in the University of Kentucky. Reciprocal working visits took place (Wells to HUJ, Belkin to UK), as well as a meeting organized in the course of a conference. During the entire period exchanges of results and experimental design were routinely conducted by email.

**Patent Summary** (numbers)

<table>
<thead>
<tr>
<th>Submitted</th>
<th>US inventor only</th>
<th>Joint IS/US inventors</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Issued (allowed)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Licensed</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Abstract (to 1 page, single spaced)

Objectives: The overall objective as defined in the approved proposal was the development of a whole-cell sensor panel for the detection of endocrine disruption activities of agriculturally relevant chemicals. To achieve this goal several specific objectives were outlined: (a) The development of new genetically engineered whole-cell sensor strains; (b) the combination of multiple strains into a single sensor panel to effect multiple response modes; (c) development of a computerized algorithm to analyze the panel responses; (d) laboratory testing and calibration; (e) field testing. In the course of the project, mostly due to the change in the US partner, three modifications were introduced to the original objectives: (a) the scope of the project was expanded to include pharmaceuticals (with a focus on antibiotics) in addition to endocrine disrupting chemicals, (b) the computerized algorithm was not fully developed and (c) the field test was not carried out.

Background: Chemical agents, such as pesticides applied at inappropriate levels, may compromise water quality or contaminate soils and hence threaten human populations. In recent years, two classes of compounds have been increasingly implicated as emerging risks in agriculturally-related pollution: endocrine disrupting compounds (EDCs) and pharmaceuticals. The latter group may reach the environment by the use of wastewater effluents, whereas many pesticides have been implicated as EDCs. Both groups pose a threat in proportion to their bioavailability, since that which is biounavailable or can be rendered so is a priori not a threat; bioavailability, in turn, is mediated by complex matrices such as soils. Genetically engineered biosensor bacteria hold great promise for sensing bioavailability because the sensor is a live soil- and water-compatible organism with biological response dynamics, and because its response can be genetically “tailored” to report on general toxicity, on bioavailability, and on the presence of specific classes of toxicants. In the present project we have developed a bacterial-based sensor panel incorporating multiple strains of genetically engineered biosensors for the purpose of detecting different types of biological effects.

Major achievements: (a) construction of innovative bacterial sensor strains for accurate and sensitive detection of agriculturally-relevant pollutants, with a focus on endocrine disrupting compounds (UK and HUJ) and antibiotics (HUJ); (b) optimization of methods for long-term preservation of the reporter bacteria, either by direct deposition on solid surfaces (HUJ) or by the construction of spore-forming Bacillus-based sensors (UK); (c) partial development of a computerized algorithm for the analysis of sensor panel responses.

Implications: The sensor panel developed in the course of the project was shown to be applicable for the detection of a broad range of antibiotics and EDCs. Following a suitable development phase, the panel will be ready for testing in an agricultural environment, as an innovative tool for assessing the environmental impacts of EDCs and pharmaceuticals. Furthermore, while the current study relates directly to issues of water quality and soil health, its implications are much broader, with potential uses is risk-based assessment related to the clinical, pharmaceutical, and chemical industries as well as to homeland security.
Achievements (to 3 page, 1.5 spaces)

Genetic engineering of novel bacterial sensor strains – Hebrew University: In the initial phase of the project we made preliminary evaluations of the detection capabilities of an existing panel of bacterial bioreporters towards target EDCs and antibiotic substances. It was clearly demonstrated that bacterial bioreporters do respond, in typical response patterns, to a variety of representaties of both groups that differ markedly in their modes of action.

To allow screening and testing of antibiotic compounds, we replaced the standard antibiotic resistance selectivity marker with a newly constructed non-antibiotic selection system (auxotrophy to tryptophan). This development allowed the construction of a general purpose antibiotic bacterial biosensor. Once this was achieved, host bacteria harboring this selectivity marker were transformed with plasmids harboring varying promoter::lux fusions. We have cloned promoters of genes that were selected from several literature reviews for being responsive to antibiotic substances and/or EDCs. The overall effort resulted in a panel of bacterial bioreporters holding different promoter::lux fusions. This bioreporter panel was challenged with the tested antibiotics and characteristic response patterns were noted.

In the final phases of the project we concentrated on broadening the scope of tested promoters and antibiotics in order to obtain the most comprehensive results. We cloned and evaluated the response of additional promoters::lux fusions to eight antibiotic substances and also evaluated the effects of varying mutations (mostly in the cellular transport machinery) of our *E. coli* host on the performance of our bioreporters. We have further developed a protocol for the deposition of bioreporter bacteria as an array of drops on a glass slide, and demonstrated that the deposited bacteria remained viable for prolonged periods of time.

Genetic engineering of novel bacterial sensor strains – University of Kentucky: A whole cell sensing system for biphenyl was constructed employing elements of the *bph* operon from *Pseudomonas pseudoalcaligenes KF707*. In *P. pseudoalcaligenes*, expression of a gene cluster involved in the degradation of biphenyl is controlled by the regulatory protein expressed from the gene *bphR1* and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPD), the product of the enzymatic reaction catalyzed
by BphC (encoded by \textit{bphC}). The construction of a whole cell sensing system for biphenyl required cloning the promoter for the \textit{bphR1} gene into plasmid pQF50, which contains a promoterless \textit{lacZ} gene, coding for \(\beta\)-galactosidase. Plasmid pSD7000 was thus created, where the expression of beta-galactosidase is positively regulated by BphR1 and is dependent upon the biphenyl concentration. This sensing system was actually able to respond to biphenyl in a dose-dependent fashion (Xu et al., Toxicol. Environ. Chem. 87, 287-98, 2005). The possibility of using this whole cell sensing system for biphenyl for the detection of bisphenol-A, due to structural similarities between these two compounds, was explored.

In order to facilitate the use of whole cell sensing systems for field applications we developed a new strategy for the long-term preservation, storage and transport of these sensing systems, which is based on the use of naturally sturdy bacterial spores. Specifically, we showed that spore-based genetically engineered whole-cell biosensing systems retain their sensing ability and analytical performance up to 2 years when stored at room temperature in the form of dormant spores (Date et al., Adv. Biochem. Eng./Biotechnol., 117, 57-75, 2010). We also proved that these spore-based sensing systems can be effectively applied to the detection of target analytes in biological and environmental samples, such as serum and freshwater, by direct incubation of the dormant spores with the samples, with quantitative data obtained in 2.5 h or less. Arsenic and zinc were chosen as model analytes. The detection limit for arsenic is within the 10 ppb standard set by EPA for drinking water, while the detection limit for zinc is within physiological and pathological serum levels in humans (Date et al., Anal. Chem., 82, 6098-6103, 2010). Additionally, we showed the adaptation of the spore packaging procedure to micro-total analysis systems by incorporating the spore-based sensing systems into centrifugal microfluidic platforms, thus achieving portable miniaturized sensing devices suitable for rapid, on-site analysis (Date et al., Anal. Bioanal. Chem., 398, 349-356, 2010).

\textbf{Algorithm development for analysis of reporter response patterns – Tennessee Tech:}

Work in this direction was terminated prematurely due to Prof. Wells leaving the project. Nevertheless, progress during the first three years was substantial. Several mathematical and statistical directions were followed in order to construct an artificial neural network (ANN) that will serve to reliably predict sample composition for the
panel’s responses. An ANN that is referred to as “IDENT” had been developed, and this algorithm showed tremendous discernment as to identification of single effectors from biosensor panel data based on previously existing reporter strains. Various nets were tested for suitability with respect to discrimination of panel response for single effectors of varying concentration. The best method found to date involves three sequences of training: (a) training a net for a panel with five effectors, each at a concentration provoking a strong response in an individual panel strain activated by the effector, (b) retraining with a series of panel responses, each of which represents response for a single effector, but including multiple concentrations, then (c) retraining again but with noise injected into the signal. The ANN now discriminates concentration, more accurately for higher concentrations than lower, and importantly does not falsely identify other effectors as being present.

**Agricultural and/or economic impacts of the research findings:** At the present stage, no immediate agricultural/economic impacts can be foreseen. Nevertheless, following an appropriate field-oriented development stage, the bacterial sensor panel may have an impact as a new and efficient tool for environmental monitoring. Longer range prospects may include industrial and homeland security-related applications.
Details of cooperation:
Strong cooperative ties were kept between the Israeli and both US partners – initially Prof. Mona Wells in Tennessee Tech and later Prof Sylvia Daunert in the University of Kentucky. The nature of the joint work, however, shifted with the change of American PIs: while Wells’s contribution mostly focused on reporter testing and algorithm development, Daunert’s efforts were generally directed towards sensor design and construction. In both cases, however, continuous email contacts were maintained. Reciprocal working visits took place (Wells to HUJ, Belkin to UK), as well as a meeting organized in the course of a conference.

List of Publications: