

## Guidelines for MAFMA Final Report

Final Reports due 45 days after completion of project  
(4-5 pages)

Project Title Minimizing *Pseudomonas* spp. Biofilms in the Food Environment Using the  
Cleaning-In-Place (CIP) System

PI (s) Hua Wang

Co-PI (s) \_\_\_\_\_

Academic Institution The Ohio State University

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### Please complete all questions below and attached form

#### 1. Objective Summary (1-2 sentence summary)

The **Objectives** of this project are 1) to identify key bacterial molecules involved in spoilage *Pseudomonas* spp. biofilm development, and 2) to screen for potential functional ingredients minimizing spoilage *Pseudomonas* biofilm buildup and application of the CIP regime on removal of biofilms formed by the wild-type and mutant strains of *Pseudomonas*.

#### 2. Objective Accomplishments

Although we have isolated a large number of bacteria isolated from the food environment, but only a small portion turned out to be real *Pseudomonas* by 16S rRNA gene sequence analysis, and we were not successful in conducting with genetic manipulation on these strains. Meanwhile, we were able to conduct genetic analysis on a laboratory isolate of *Pseudomonas* sp., and proteinase activity and milk protein can affect its biofilm formation, and EDTA can serve as an active ingredient interfering its biofilm development. Molecular studies further characterized a PA5022 gene, likely encoding a novel regulator involved in modulating *Pseudomonas* quorum sensing system, swimming motility and biofilm formation.

*Strain isolation.* Isolation for possible *Pseudomonas* spp. from food and food-processing equipments was done by plating environmental swabs and homogenized meat samples on *Pseudomonas* Isolation Agar. Most environmental swabs taken from the dairy- and meat-processing equipments were negative for growth on the selective medium. Representative microorganisms growing on the selective plates were identified as those belonging to the genera *Pseudomonas*, *Citrobacter*, *Aeromonas*, and *Stenotrophomonas* by amplifying the partial 16S rDNA fragment using PCR, determining the DNA sequences using the ABI Prism® 3700 (Applied Biosystems, Foster City, CA) at the Plant-Microbe Genomics Facility of the Ohio State University, followed by comparing the sequences with those in the GenBank database. Out of 61 isolates obtained from raw meat, 33 tested positive for protease production based on their

ability to liquefy 10% gelatin. *Pseudomonas* sp. GRD-1 and *Stenotrophomonas* sp. GRD-2 isolated from meat grinding equipment were negative for gelatinase production. Several strains previously isolated in our laboratory, including *Pseudomonas* sp. 35 and 40 (Luo and Wang, 2003 IFT annual meeting), were also examined and used in the following study.

*Biofilm formation.* The different strains tested did not show visible attachment on the surface of both treated and untreated polystyrene 24-well plate cultures when grown in either tryptic soy broth (TSB) or Luria-Bertani (Miller) broth for 2-3 days. However, when grown in UHT milk, selected strains produced a visible biofilm. *Pseudomonas* sp. 40 curdled the milk and formed a very thick film attached to the slide even after the washing steps. A control set-up to which no bacterial culture was added was completely removed from the slide after washing.

*Ingredients interfering Bfm.* When 50 and 100 mM EDTA was added to the milk, biofilm formation was not observed for *Pseudomonas* sp. 40. Moreover, the addition of either 50 or 100 mM EDTA to TSB inhibited the growth of most *Pseudomonas* strains including the control strains *P. aeruginosa* ATCC 27853 and *P. aeruginosa* PAO1. Among the various strains tested, only *Pseudomonas* sp. 35 was resistant to the inhibitory action of EDTA.

*Establish protocols for genetic manipulation of Pseudomonas sp.* Attempts to transform *P. aeruginosa* ATCC 27853, *Pseudomonas* sp. 35, *Pseudomonas* sp. GRD-1 and *Stenotrophomonas* sp. GRD-2 using both chemical treatment and electrotransformation have not been unsuccessful. Transformation of strain *Pseudomonas* sp. 40, however, was successful. A derivative of this strain carrying UV-excitable green fluorescent protein was constructed using plasmid pGFPuv (Clontech Laboratories, Inc., California, USA) to which a tetracycline resistance cassette was added. Because the strain can form biofilm and the attachment can be monitored under UV excitation without the complicated microbial plate counting verification, it will further be used to examine the efficacy of the CIP system for biofilm removal.

### 3. Unexpected findings, if any

For *Pseudomonas* sp. strains that having trouble colonizing to the surface, adding external proteins such as casein can significantly change the situation. The finding indicates that the presence of exogenous food ingredients play a role in facilitating/enhancing the biofilm built-up in the food processing environment.

Because we are unsuccessful in isolating spoilage *Pseudomonas* sp. from the food environment that actually can be transformed for further genetic manipulation, we have also started to characterize genetic elements that are involved in biofilm formation by *Pseudomonas aeruginosa*, a closely related organism that has an established system for genetic manipulation, and hope results from the study can provide insight on biofilm formation by other *Pseudomonas* species, and assist us in developing new mitigation strategies. In collaboration with Dr. Minting Qiao from Tianjing University, Dr. Yingli Li (Ph.D. graduate from Qiao lab, continued with post-doctoral work in Wang's Lab) we actually discovered a new regulatory gene in *Pseudomonas aeruginosa*, PA5022, isolated from Mu transposon mutagenesis library of *P. aeruginosa* strain PA68, has impaired swimming motility ability and increased biofilm formation. The promoter-*lacZ* fusion plasmids ( $P_{lasI}$ -*lacZ*,  $P_{rhII}$ -*lacZ*,  $P_{rpoN}$ -*lacZ*,  $P_{rpoS}$ -*lacZ*,  $P_{qscR}$ -*lacZ*,  $P_{vqsR}$ -*lacZ*) were introduced into PA68 and the mutant PA5022 respectively. The result of  $\beta$ -galactosidase activity

assay showed a decreased transcription of *vqsR* and *lasI* promoters in the mutant PA5022 comparing with the wild type PA68. It suggested that the putative PA5022 protein may be essential in the process of regulating quorum sensing system, swimming motility and biofilm formation.

4. Practical impacts of research efforts. Include: implementation of accomplishments by industry partners (if any), identification of economic impacts, and any further pursuit by PI of research in area of this project whether MAFMA or not.

Long term impact: Microbial biofilm formation involving spoilage and pathogenic organisms is the leading cause for persistent contamination in the food environment and is a major threat to food safety and public health. The proposed study addresses this industrially significant problem and uses innovative approach through minimizing backbone *Pseudomonas* biofilms to achieve the goal of improving sanitation. Revealing the molecular mechanism involved in spoilage *Pseudomonas* biofilm development establishes the basis for developing effective counter approaches, and incorporating the CIP system directly connect laboratory molecular mechanism study to the food industrial applications. Results from the proposed study not only will contribute to improved scientific knowledge on biofilm formation in the food environment, but have direct practical significance to improve the performance of the automated CIP sanitation unit widely used in the food processing industry.

Identification of novel biofilm regulator in *Pseudomonas* and revealing its involvement in quorum sensing system, swimming motility and biofilm formation contributed to the scientific understanding of the complicated cascade of network involved in biofilm development, and can help with developing further mitigation strategies.

5. If you are also making reports to other funding agencies in the course of this research work, please include a copy of that report.

N/A.

6. a. If any publications resulted from the research, a copy must be included. Please note we were notified by the USDA/CSREES National Program Leader for the Midwest Advance Food Manufacturing Alliance (MAFMA) that all publications resulting from research that was funded by MAFMA must include the following wording **“The project was supported by the USDA Cooperative State Research, Education and Extension Service, special research grant number 200X-34328-xxxxx.**

We are still collecting more data for the paper, and will include the acknowledgement when the paper is ready to be published.

**b. If any patents (pending or granted) resulted from the research, please include the patent information.**

N/A/

7. Budget summary of actual expenditures

(See attached form) Include actual matching funds received and/or in-kind. Remember no more than 50% can be in-kind.