Development of a Microplate Fluorescence-Based Assay for Biofilm Forming Strains of *Listeria monocytogenes*

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Story in Brief

The objective of this study was to develop a fluorescence-based assay for detection of *L. monocytogenes* cells entrapped in biofilms as a means of screening isolates for ability to form biofilms. A 96-well microplate format was used for our biofilm assay. Strains of *L. monocytogenes* were tested at various incubation intervals (4 hours to 4 days) and temperatures (25, 30, and 37^{o}C) to determine a good screening regimen. A plate washer was used to remove planktonic cells using several wash cycles. Retained cells were then incubated at different times or temperatures with 5, 6-carboxyfluorescein diacetate (5,6-CFDA) using four strains of *L. monocytogenes*. The internalized and hydrolyzed substrate generates 5,6-carboxyfluorescein (5,6-CF) that is highly fluorescent which was detected with a fluorescent plate reader. Analysis of biofilm formation among strains of *L. monocytogenes* isolated from various processing environments may provide a better understanding of molecular mechanisms involved in attachment and for solutions to help detach and eliminate them from food processing facilities.

Key Words: *Listeria monocytogenes*, Biofilm, 5, 6-Carboxyfluorescein Diacetate, CFDA

Introduction

Listeria monocytogenes is a significant foodborne pathogen that is difficult to eliminate from processing environments producing ready-to-eat (RTE) meats. *L. monocytogenes* is also capable of producing biofilms on processing equipment making its eradication even more difficult and allowing for potential contamination of RTE food products. *L. monocytogenes* presents a formidable problem to the RTE meat industry, as both USDA and FDA have established a 'zero-tolerance' for its presence in RTE foods. Biofilms have also been studied and shown to be resistant to antibiotics and sanitizers as reported by Chae et al. 2000. The purpose of this study was to develop an assay that would identify biofilm-forming strains that have been isolated from production animals, raw retail meats, and RTE meat processing environments for subsequent molecular and physiological studies related to biofilm formation by *L. monocytogenes*.

Materials and Methods

A microplate assay was used as the format to screen for cells of *L. monocytogenes* attaching as a biofilm layer in the plate wells. We chose a culture incubation temperature of 37^{o}C to promote attachment because we have observed the best results at this temperature, although there is reason to consider testing at lower temperatures that simulate near-refrigeration temperatures inside meat processing facilities where many strains have been isolated. In addition, three temperatures were examined for subsequent incubation of attached cells with the fluorescent substrate, 5, 6-carboxyfluorescein diacetate (5,6-CFDA) that is easily absorbed by bacterial cells and converted to the fluorescing 5,6-carboxyfluorescein (5,6-CF) form by cytoplasmic esterases (Hoefel et al. 2003). We tested the three temperatures (25^{o}, 30^{o}, and 37^{o}C) at incubation time

intervals of 15, 30, 45, 60, and 90 min. A Biotec Elx405 Magna plate washer was used to remove planktonic cells from 96-well microplates after incubation and then used to rinse and replace wash buffer in the same wells. A Tecan GENios fluorescent plate reader was used to detect fluorescence levels (excitation at 485 nm and detection at 535 nm) in 96-well microplates after incubation with the CFDA substrate. Various proteases (Sigma Chemical Co., St. Louis, MO) were tested for effect on viability of *L. monocytogenes* and for potential use in released attached bacterial cells. Data are expressed as the means of triplicate replications ± SD. Statistical comparison of incubation time with CFDA was performed by one way analysis of variance (Sigma Stat 3.0, SPSS, Chicago, IL). Data were considered signicant when their computed probalities were less than 0.05 ($P < 0.05$).

Results and Discussion

CFDA-based fluorescence gave excellent correlation and linearity (r^2=0.9979) with cell numbers in a cell dilution series (data not shown). *L. monocytogenes* ScottA-2 was incubated in microplate wells at 37°C and examined at various incubation temperatures with CFDA substrate. The results show that low incubation times at all three incubation temperatures gave the highest fluorescence levels (Fig. 1). However, since the rate of decrease of fluorescence was least when the cells were incubated at 25°C, we chose that temperature as the preferred assay substrate incubation temperature.

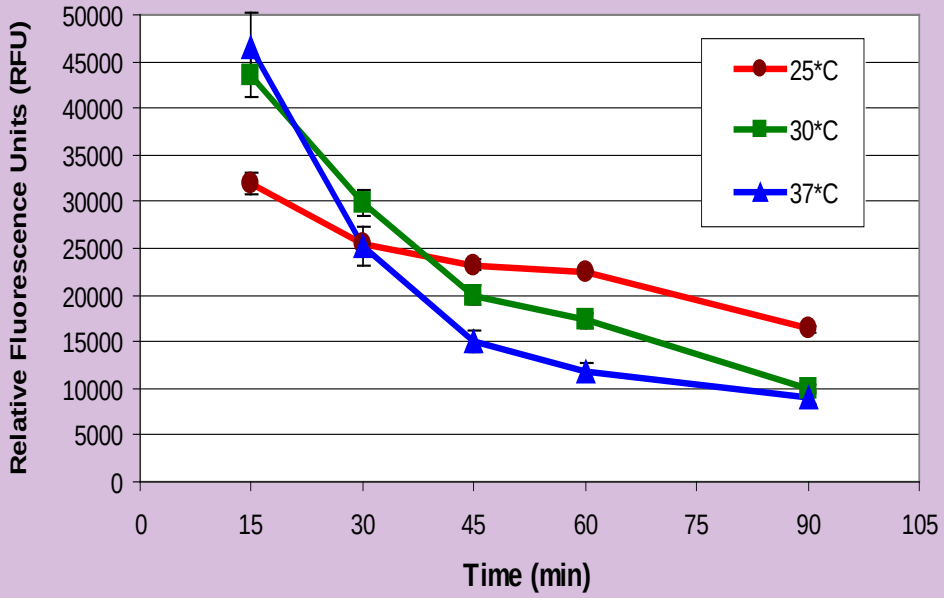

Figure 1. Fluorescence from *L. monocytogenes* ScottA-2 cells incubated at various times and temperatures with 5,6-CFDA. Data are the means of duplicate replications.

Current results with four test strains of *L. monocytogenes* (V7-2, Scott A-2, 383-2, and 39-2) have indicated that incubation at 37°C for 48 hours yields adequate cell attachment to establish strong fluorescence signals and we have therefore started to screen several hundred strains in our

culture collection using these biofilm attachment conditions. However, one consideration that we will also address in future studies is the potential for biofilms to form at lower incubation temperatures, as many of the strains of *L. monocytogenes* have been isolated from processing plants that are held at 45°F or below, and therefore may provide a selective environment for low temperature biofilm formation.

From our earlier results, we examined smaller substrate incubation periods at 25°C and found that a 15 min incubation period provided higher fluorescence levels from attached cells than either shorter or longer incubation periods (Fig. 2). Any shorter time span would present a problem because of the need to wash the plates and prepare them for the plate reader. We therefore standardized our biofilm assay to incubate cells from fresh cultures in microplates for 48 hr at 37°C, followed by washing with the plate washer, then 15 min incubation with CFDA substrate at 25°C, and again followed by washing, buffer replacement, and detection with the fluorescent plate reader. Plates were standardized to be read at 5 min after the substrate incubation period to insure that cellular fluorescence is read at the same time after the end of substrate incubation.

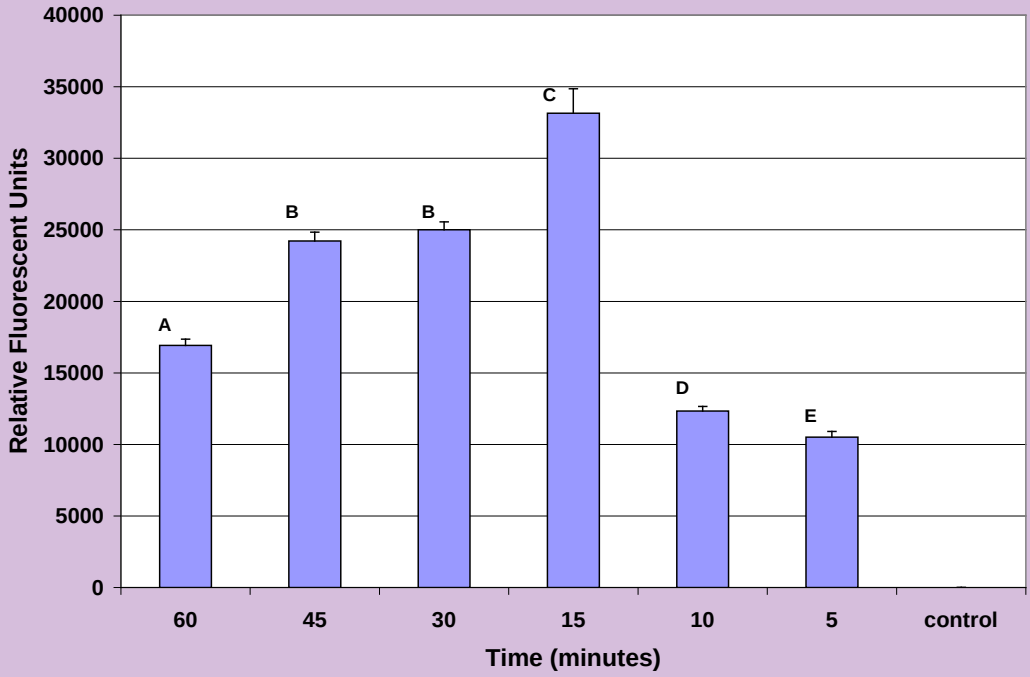

Figure 2. Effect of incubation time on fluorescence levels obtained from incubation of microplate-attached cells with the 5,6-CFDA fluorescence substrate at 25°C using *L. monocytogenes* strain ScottA-2. Bars are the means of five replications. Error bars represent standard deviation of the mean. Data bars with different letters are significantly different from each other ($P<0.05$)

We are currently screening several hundred strains of *L. monocytogenes* isolated from animal production lots, animal hides, RTE meat processing facilities, and retail raw and RTE meats for their ability to form biofilms. Of the > 30 strains tested to date during the development of the

current assay, more than a 50-fold difference was obtained in fluorescent signals between the strongest and weakest biofilm-forming strains (Fig. 3).

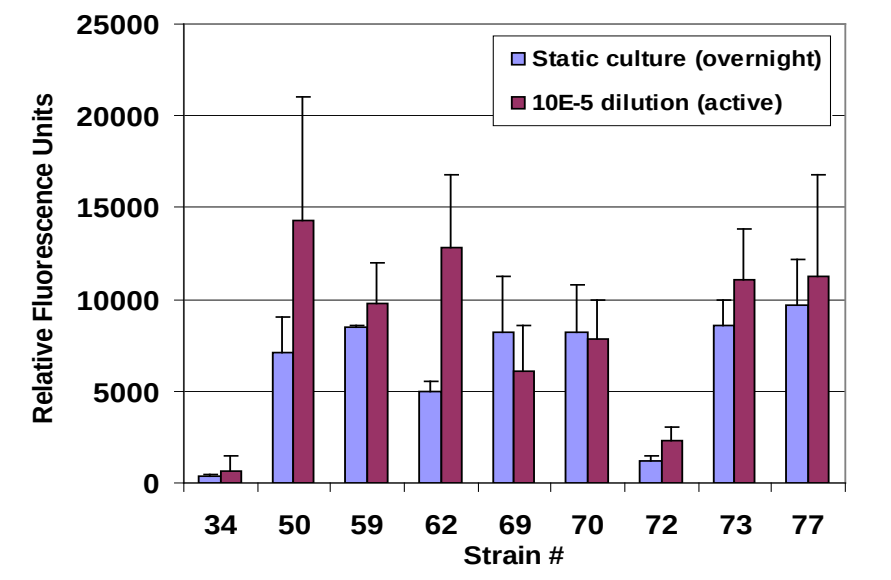

Figure 3. Fluorescence levels of various *L. monocytogenes* CW strains (isolated from retail franks). Cells were incubated at 37°C in microplates at either 10^9CFU/ml (stationary phase) or at 10^4CFU/ml (log phase) to promote attachment. Error bars represent standard deviation of the mean of triplicate replications

Additional studies include protein analysis via SDS-PAGE gels, fluorescence microscopy of cells attached to slide chambers, cell viability and release from biofilms after treatment with proteases (Protease, Pepsin, Papain, Trypsin, and Thermolysin), and screening of *L. monocytogenes* strains for their ability to form biofilms. Initial studies have indicated that protease treatments show little or no loss of viability (data not shown). Cell viability after protease treatment is essential for quantitation after detachment from biofilms. We can currently reduce microplate well fluorescence to background levels by proteolytic release of attached *Listeria* (Fig. 4). Plate counts of *Listeria* released by this method will serve to complement fluorescence (indirect) and microscopy analysis (direct) of release of previously attached *Listeria* (data not shown).

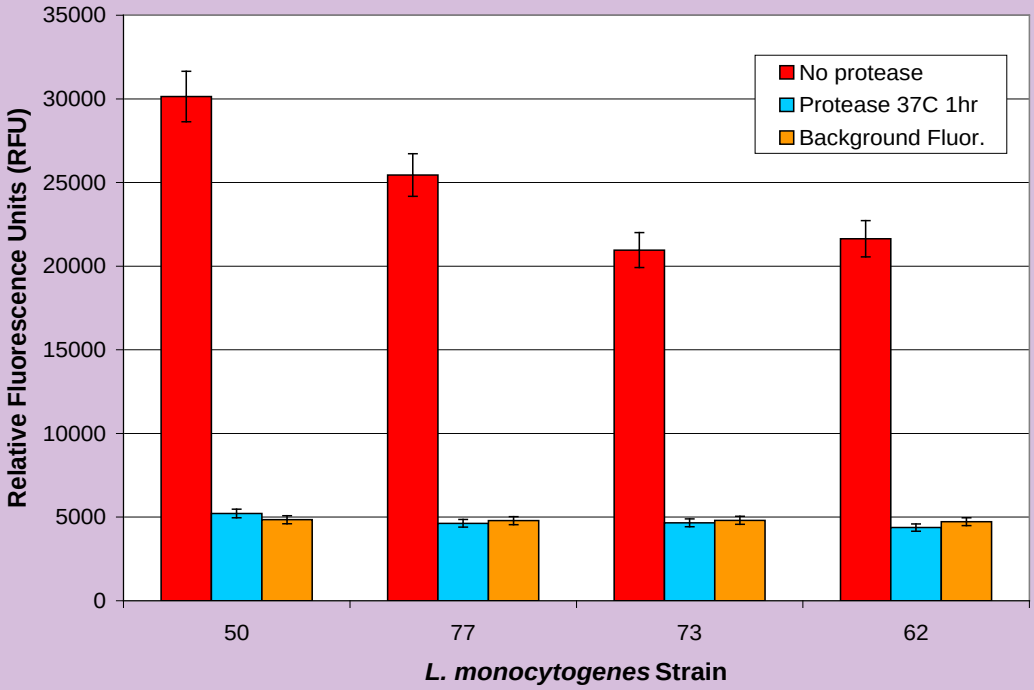

Figure 4. Effect of a generic protease on fluorescence detected from four attached strains of *L. monocytogenes*, with or without, protease treatment Error bars represent standard deviation from the mean

The work presented herein will hopefully identify both strong and weak biofilm-forming Listeria for further research studies. It will be interesting to see if the strains in our collection that have been repeatedly isolated from RTE meat processing plants are the result of persistant contamination due to strong biofilm tendencies. It is possible that the protease treatments we identify may help to remove these pathogens from meat processing facilities in which they are so difficult to eliminate.

Literature Cited

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