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Potential of the Melanophore Pigment Response for Detection of Bacterial Toxicity▽

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Chromatophore cells have been investigated as potential biodetectors for function-based detection of chemically and biologically toxic substances. *Oncorhynchus tshawytscha* (chinook salmon) melanophores, a chromatophore cell type containing brown pigment, rapidly detect the salmonid pathogens *Aeromonas salmonicida*, *Yersinia ruckeri*, and *Flavobacterium psychrophilum* and the human pathogen *Bacillus cereus*.

Chromatophores are a class of pigment cells present in amphibians, cephalopods, and fish that have been used as biodetectors to report toxicity (1, 2, 7, 8, 9, 14). Upon exposure to explicit toxic stimuli, chromatophores redistribute their intracellular pigment organelles in one of two directions. Aggregation describes pigment organelle movement to the perinuclear region, and conversely, dispersion indicates the relocation of pigment organelles to the outer periphery of the intracellular space. These pigment responses are monitored optically and can vary by the degree of aggregation or dispersion as well as the rate of pigment movement for different toxic substances.

We previously described the *Oncorhynchus tshawytscha* (chinook salmon) melanophore response and have shown that pigment dynamics of melanophores and *Betta splendens* (Siamese fighting fish) erythrophores are conserved in their responses to the environmental toxicants mercury and arsenic (2). Melanophores and erythrophores each belong to the chromatophore class and differ by containing brown and red pigment organelles, respectively. While erythrophores have been explored for their responsiveness to bacterial pathogens, such as *Bacillus cereus* and *Clostridium botulinum* (1, 7, 14), the potential for melanophores to be utilized in this capacity has not yet been investigated. This study addresses melanophores in their ability to respond to the salmonid bacterial pathogens *Yersinia ruckeri*, *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, and *Carnobacterium piscicola* and the human bacterial pathogens *Bacillus cereus* and *Clostridium botulinum*.

We hypothesized that melanophores would respond to *B. cereus* and *C. botulinum* through pigment aggregation, similar to observations previously reported for *B. splendens* erythrophores (7). The salmonid bacterial pathogens described in this study have not been analyzed with respect to *B. splendens* erythrophores or any other chromatophore detection system. However, a disease characteristic for both enteric red mouth disease, caused by *Y. ruckeri*, and bacterial cold water disease, caused by *F. psychrophilum*, is melanosis, or darkening of the tissues (4, 17). Therefore, we hypothesized that *Y. ruckeri* and *F. psychrophilum* would induce pigment dispersion in isolated melanophores. Ill fish, in general, have been observed to appear either pale or darker in color, and due to this dichotomy, no preconceived notions were made concerning the *O. tshawytscha* melanophore responses to *A. salmonicida* and *C. piscicola*.

The Oregon Department of Fish and Wildlife (ODFW) provided hatchery-raised *O. tshawytscha* for all melanophore preparations and analyses. Melanophores were extracted from the dorsal and tail fins of young salmon at 6 to 20 cm in length, used in melanophore response assays, and analyzed as previously described (2). Each melanophore assay was conducted in triplicate, and an end-point two-sample Student's *t* test was performed. Significance was declared at *P* values of less than 0.01.

Bacillus cereus ATCC 49064 (13), *B. cereus* ATCC 14579 (3), *B. cereus* ATCC 14579 Δ*plcR* (15), and *Bacillus subtilis* 1A1 (18) were cultured in brain heart infusion (BHI) broth as previously described (6). *Oncorhynchus tshawytscha* melanophores were exposed to two *B. cereus* strains that vary in toxin production (ATCC 14579 and ATCC 49064), as well as the nonpathogen *B. subtilis* 1A1. Changes in the melanophore pigment area in response to these bacteria were monitored over time. Figure 1A illustrates the numerical evaluation of change in the melanophore pigment area over time, while Fig. 1B illustrates an example of melanophores before (left) and after (right) exposure to *B. cereus* ATCC 49064. Melanophore exposure to *B. cereus* strains ATCC 49064 and ATCC 14579 resulted in pigment aggregation within 20 min, yet each *B. cereus* strain induced a different response curve. Pigment aggregated in melanophores to approximately −81% when exposed to *B. cereus* ATCC 49064, and this response achieved steady state after 5 min (where a negative change in the pigment area represents pigment aggregation and a positive change in the pigment area correlates to pigment dispersion). *Bacillus cereus* ATCC 14579 induced an end point of approximately −70% pigment aggregation in melanophores after 20 min. In contrast, melanophores exposed to *B. cereus* ATCC 14579 Δ*plcR*, a nonpathogenic mutant, exhibited very slight pigment aggregation of −9%. The melanophore pigment response to *B. subtilis* 1A1 was indistinguishable from the BHI control response. With the exception of *B. cereus* ATCC 14579 Δ*plcR*, the melanophore response to the *B. cereus* strains deviated significantly ($P < 0.005$) from the BHI control response.

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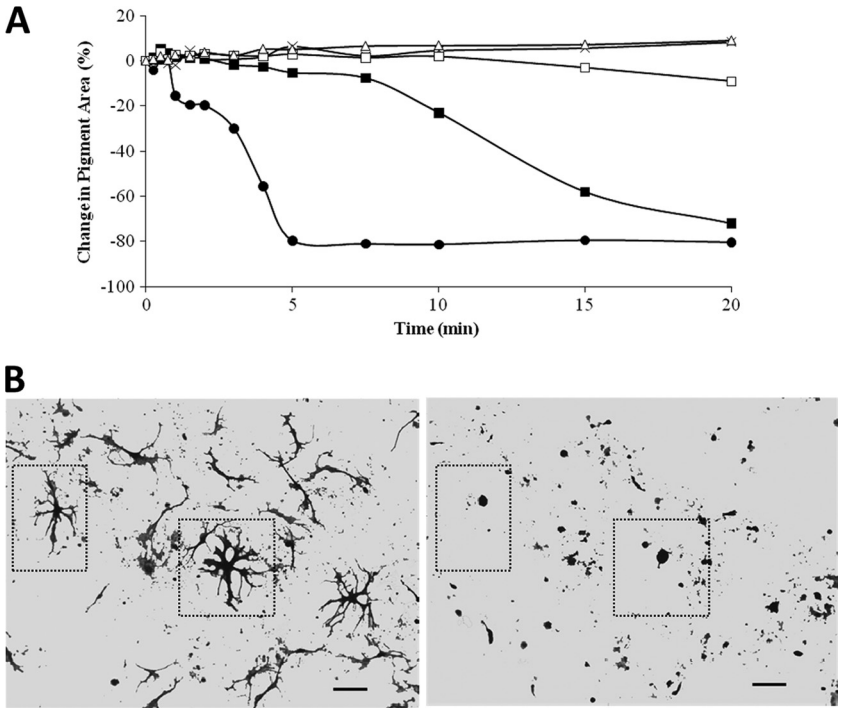

FIG. 1. (A) *Oncorhynchus tshawytscha* melanophore response to *Bacillus* strains. Melanophores were observed for 20 minutes following exposure to *B. cereus* ATCC 49064 (●), *B. cereus* ATCC 14579 (■), *B. cereus* ATCC 14579 Δ*plcR* (□), *B. subtilis* 1A1 (△), and BHI medium control (×). A negative change in the pigment area represents pigment aggregation, whereas a positive change in the pigment area correlates to pigment dispersion. Data represent the average results from three trials. Mean deviations are as follows: *B. cereus* ATCC 49064, 7.2%; *B. cereus* ATCC 14579, 5.5%; *B. cereus* ATCC 14579 Δ*plcR*, 3.4%; *B. subtilis* 1A1, 4.7%; and BHI broth, 6.1%. (B) Melanophore pigment organelles aggregate to the perinuclear region in response to *B. cereus* ATCC 49064. Melanophores are shown at time zero (left) and after 20 minutes (right). The size bar represents 100 μm, and dashed boxes reference specific melanophore cells.

Similar to results obtained with *B. splendens* erythrophores (7), *O. tshawytscha* melanophores respond differentially to *B. subtilis* and *B. cereus*, suggesting that melanophore pigment aggregation in response to *B. cereus* may be mediated by one or more virulence factors. Further, a *B. cereus* Δ*plcR* mutant alters the melanophore response dramatically. PlcR is a transcriptional regulator that influences expression of many secreted products, including enterotoxins, hemolysins, and additional proteases (5, 11, 12). We hypothesize that the various melanophore response curves observed in this study are dependent upon the specific *plcR*-regulated expression patterns of each *B. cereus* strain. Importantly, the *O. tshawytscha* melanophore responses to *B. cereus* ATCC 49064, ATCC 14579, and ATCC 14579 Δ*plcR* and *B. subtilis* 1A1 are similar to the responses previously described for *B. splendens* erythrophores (7), indicating that the pigment responses to these *Bacillus* species are conserved between these chromatophore cell types.

Clostridium botulinum NCTC 7272 and *C. botulinum* NCTC 7273 were cultured in BHI broth, as previously described (6). Melanophores were exposed to each *C. botulinum* strain, and pigment responses were observed for 6 h. Neither of the *C. botulinum* strains induced a melanophore response that differentiated from that of the BHI medium control, and the responses were insignificant ($P > 0.1$) (data not shown). Previous work has shown that *B. splendens* erythrophores aggregate when exposed to these same *C. botulinum* strains under the same conditions (7). It is interesting that while some pigment responses are conserved between melanophores and erythrophores (2), differences do arise.

The melanophore responses to salmonid bacterial pathogens *Y. ruckeri*, *A. salmonicida*, *F. psychrophilum*, and *C. piscicola* were investigated. *A. salmonicida* SD2 and *Y. ruckeri* LP4 were cultivated in tryptone yeast extract salts (TYES) medium at 26°C for 48 h without aeration. *F. psychrophilum* LP5 was incubated in TYES medium at 16°C for 48 h without aeration. Additionally, *C. piscicola* SD2 was cultured in Trypticase soy broth (TSB) at 26°C for 48 h without aeration. All salmonid bacterial pathogens were isolated as natural outbreak strains from Pacific Northwest fish-rearing environments (provided by ODFW). The melanophore response to each bacterial pathogen (Fig. 2) was significant ($P < 0.01$) compared to that of the respective control (TYES medium or TSB), with the exception of the response to *C. piscicola* ($P > 0.1$), which closely resembled the TSB response. *Y. ruckeri* caused the most rapid pigment aggregative response in melanophores, reaching steady state at 10 min, with a final aggregation of −80%. Similarly, *A. salmonicida* resulted in pigment aggregation of approximately −55% within 15 min, and this response reached steady state at 40 min, with a final aggregation of −80%. The melanophore response to *F. psychrophilum* initiated with pigment dispersion (13%), followed by pigment aggregation after 25 min of exposure, ultimately leading to a −84% change in the pigment area after 45 min. *C. piscicola*, TYES medium, and TSB induced

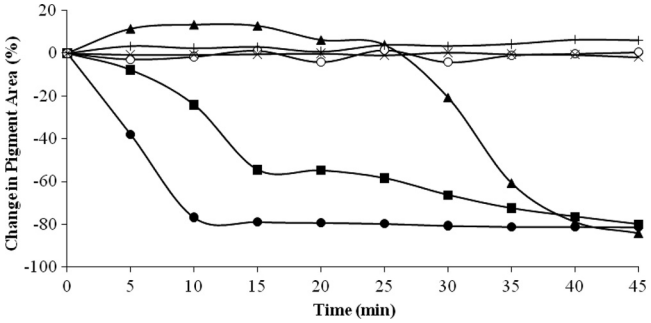

FIG. 2. *Oncorhynchus tshawytscha* melanophore response to salmonid bacterial pathogens. Melanophores were exposed to *F. psychrophilum* (▲), *C. piscicola* (○), *A. salmonicida* (■), and *Y. ruckeri* (●). The medium controls included TYES medium (×) and TSB (+). *Carnobacterium piscicola* was grown in TSB, and the remaining pathogens were grown in TYES medium. A negative change in the pigment area represents pigment aggregation, whereas a positive change in the pigment area correlates to pigment dispersion. Data represent the average results from three trials. Mean deviations are as follows: *F. psychrophilum*, 9.1%; *C. piscicola*, 2.2%; *A. salmonicida*, 6.0%; *Y. ruckeri*, 9.2%; TYES medium, 1.7%; and TSB, 1.6%.

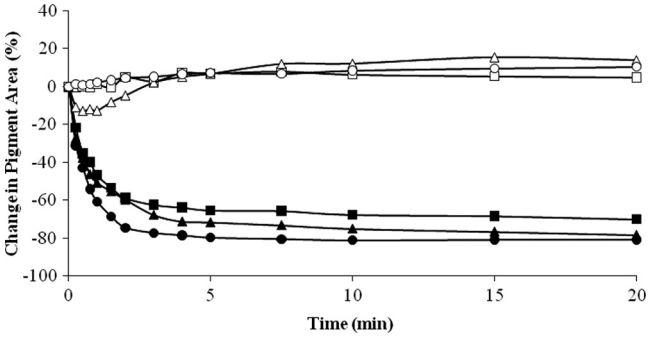

FIG. 3. *Oncorhynchus tshawytscha* melanophore response to healthy and diseased tissues. Organs from healthy rainbow trout and rainbow trout infected with *F. psychrophilum* were collected. Melanophores were exposed to healthy livers (□), healthy spleens (○), and healthy kidneys (Δ) as well as diseased livers (■), diseased spleens (●), and diseased kidneys (▲). A negative change in the pigment area represents pigment aggregation, whereas a positive change in the pigment area correlates to pigment dispersion. Data represent the average results from three trials. Mean deviations are as follows: healthy livers, 3.7%; healthy spleens, 4.2%; healthy kidneys, 7.4%; diseased livers, 3.2%; diseased spleens, 2.2%; and diseased kidneys, 7.1%.

minimal changes in the pigment area. Interestingly, the melanophore response to *Y. ruckeri* and *F. psychrophilum* opposed previously described disease signs (10, 16, 17), but notably, *F. psychrophilum* initially did cause pigment dispersion.

Melanophores were exposed to healthy spleens, livers, and kidneys isolated from rainbow trout, and these responses were compared to those obtained by exposure to spleens, livers, and kidneys from rainbow trout infected with *F. psychrophilum* (provided by ODFW). Spleens, livers, and kidneys were dissected from healthy and diseased trout and submerged in 200 μl L15 (without antibiotic). To prepare the tissues for analysis, 5 spleens, 5 livers, and 5 kidneys obtained from healthy and diseased trout were pooled and homogenized (separately). Melanophores were exposed to collected supernatants, and images were captured over 20 min. Rapid pigment aggregation within melanophores was observed in response to all diseased organs (Fig. 3), and these responses were significant ($P < 0.005$) from their respective healthy organ responses. Diseased spleens resulted in the most rapid pigment aggregation and reached steady state at 3 min, with a final aggregation of −81%. Diseased kidneys and livers reached steady state at 7.5 min and resulted in −78% and −70% final changes in pigment areas, respectively. Healthy kidneys initially induced slight aggregation (−12%) but reached steady state at 10 min, with a final dispersion of 14%. Healthy livers achieved steady state at 2 min, with a final percent area change of 5%. Spleens caused pigment dispersion, with a final change in the pigment area of 10%, and took 15 min to reach steady state. Melanophores aggregate in the presence of *F. psychrophilum* whether in culture or an infected tissue sample, and the rate of this aggregation is greatly accelerated for the infected tissues.

In conclusion, this study highlights further stimuli that result in the conservation of the pigment response between *O. tshawytscha* melanophores and *B. splendens* erythrophores. Interestingly, chromatophores obtained from fish species inhabiting two different ecological niches share similar responses. We give particular evidence for *O. tshawytscha* melanophores to be exploited as biodetectors for *B. cereus*, *A. salmonicida*, *Y. ruckeri*, and *F. psychrophilum*. Biodetectors represent a novel technique based on function-based detection. Instead of responding to the mere presence of conserved nucleic acid or antibody structures, biodetectors relay a signal based on the physiological function of a particular analyte. As a result, biodetectors are capable of assessing the viability and toxicity of a sample rather than just the present or absence of a sample. Given the potential to use chromatophores, such as melanophores and erythrophores, as biodetectors of bacterial toxicity, further investigation is warranted to better understand the underlying mechanisms contributing to melanophore and erythrophore aggregation in response to certain bacterial pathogens.

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