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Erythrophore cell response to food-associated pathogenic bacteria: implications for detection

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Summary

Cell-based biosensors have been proposed for use as function-based detectors of toxic agents. We report the use of *Betta splendens* chromatophore cells, specifically erythrophore cells, for detection of food-associated pathogenic bacteria. Evaluation of erythrophore cell response, using *Bacillus* spp., has revealed that this response can distinguish pathogenic *Bacillus cereus* from a non-pathogenic *B. cereus ΔplcR* deletion mutant and a non-pathogenic *Bacillus subtilis*. Erythrophore cells were exposed to *Salmonella enteritidis, Clostridium perfringens* and *Clostridium botulinum*. Each bacterial pathogen elicited a response from erythrophore cells that was distinguished from the corresponding bacterial growth medium, and this observed response was unique for each bacterial pathogen. These findings suggest that erythrophore cell response has potential for use as a biosensor in the detection and toxicity assessment for food-associated pathogenic bacteria.

Introduction

Pathogenic bacterial infections afflict countless numbers of individuals annually, with outcomes ranging from minor and treatable complications to death. Food- and water-associated bacterial illnesses, estimated at 76 million illnesses per year, cost the US economy well in excess of $10 billion annually (Buzby and Roberts, 1997; Mead *et al*., 1999). Besides accidental contamination, food and water supplies are also vulnerable to intentional tampering. Development of accurate approaches for detecting and identifying diverse groups of pathogenic bacteria is essential for diagnosis and treatment of bacterial infections as well as assessing the toxicity potential of bacterial contamination. Despite enormous advances in molecular and immunological (DNA and antibody) detection technologies, pathogenic bacteria still escape detection. Detection methods based on protein or DNA structure specific to an organism often cannot distinguish between live and dead bacteria or the potential of these organisms to cause disease (McKillip *et al*., 1998; Sheridan *et al*., 1998; Nogva *et al*., 2003).

Previously, a cell-based biosensor consisting of cultured primary chromatophore pigment cells, specifically erythrophores, from *Betta splendens* (Siamese fighting-fish), was described in terms of its function-based detection of toxic agents such as herbicides, fungicides, genotoxins (Mojovic *et al*., 2004), cell receptor agonists and purified toxins (Dierksen *et al*., 2004; Mojovic *et al*., 2004). Erythrophore cells are specialized pigmented nerve cells that can change appearance when physiologically stimulated (Elwing *et al*., 1990; Karlsson *et al*., 1991; Danosky and McFadden, 1997). Erythrophore cell response to toxic agents can be easily detected by monitoring changes in optical density due to movement of pigment organelles (chromatosomes) within the erythrophore cell. Pigment organelle movement can be dispersive (pigment organelles move outwards toward the cell edge) or aggregative (movement towards the centre of the cell). The movement of pigment organelles along a radically arrayed microtubule network achieves visual changes in erythrophore cell morphology. Pigment organelle movement is thought to be controlled through signal transduction pathways and other regulatory mechanisms in response to triggering events at various cell surface receptor sites, including G-protein coupled receptor-binding events (Danosky and McFadden, 1997). The movement of the pigment organelles within erythrophore cells can be measured by recording the change in pigment area occupied by the erythrophore, allowing real-time assessment of erythrophore cell response to toxic conditions (Dierksen *et al*., 2004; Mojovic *et al*., 2004).

Little is known about the potential of erythrophore cells to detect biologically active food-associated bacterial pathogens. *Bacillus cereus* was selected as the initial model system because of its increasing implication in emetic and diarrheal forms of food poisoning (Turnbull, 1986; Kramer and Gilbert, 1989). In addition to its link to gastrointestinal infections, *B. cereus*, an opportunistic pathogen, has been linked to numerous

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non-gastrointestinal infections including, osteomyelitis, pulmonary and wound infections, bacteremia and septicemia (Kramer and Gilbert, 1989; Turnbull, 1986). In this study, we present findings in support of the potential for erythrophore cells to be used to detect, as well as potentially assess toxicity for, the food-associated pathogenic bacteria, *B. cereus*, *Clostridium perfringens*, *Clostridium botulinum* and *Salmonella enteritidis*.

Results

A desirable feature of an erythrophore cell-based biosensor would be the ability to distinguish uninoculated bacterial culture medium (representing the negative control) from the cultured bacterial strains, thus erythrophore cell response to culture medium was included in this analysis. Erythrophore cell response was tested against a set of characterized *Bacillus* spp. cultured in BHI (Brain Heart Infusion) medium: *Bacillus subtilis* 1A1 (a laboratory reference strain; Yoshida *et al*., 1995), *B. cereus* ATCC 49064 (identified in a gastroenteritis outbreak and produces diarrheogenic enterotoxin; Midura *et al*., 1970; Taylor and Gilbert, 1975; Melling *et al*., 1976), *B. cereus* ATCC 14579 (Frankland and Frankland, 1887) and a *B. cereus ΔplcR* deletion mutant strain that is a derivative of ATCC 14579 (Salamitou *et al*., 2000). The negative control (uninoculated BHI media) caused slight dispersion of pigment organelles within the erythrophore cell (Fig. 1A and D). The observed dispersion was slow, but stabilized 10 min after exposure. Erythrophore cell response to *B. subtilis* 1A1 culture was similar in strength and rate to the response elicited by the negative control (Fig. 1B and D), and this response was found to be statistically insignificant ($P = 0.635$). Addition of *B. cereus* ATCC 49064 (Fig. 1C) or ATCC 14579 induced erythrophore pigment organelles to rapidly aggregate; this response was detected within two to 5 min after addition of the bacterial culture (Fig. 1D). By 10 min, erythrophore cells remained in the aggregated state indefinitely. Erythrophore cell response to *B. cereus* ATCC 49064 or ATCC 14579 was found to be statistically significant compared with the negative control ($P < 0.001$ and $P < 0.001$ respectively).

To investigate the potential mechanism of erythrophore cell response to *B. cereus*, erythrophore cells were tested against a *B. cereus* ATCC 14579 strain lacking the global gene regulator, *plcR* (Salamitou *et al*., 2000). PlcR controls the expression of numerous secreted factors, including known virulence factors. The *B. cereus ΔplcR* deletion mutant strain induced a similar response (slightly dispersive rather than aggregative) in erythrophore cells as the negative control (BHI) ($P = 0.0754$; Fig. 1D), and this response was found to be statistically insignificant.

To further evaluate erythrophore cell response, we investigated the response of erythrophore cells to additional food-associated pathogenic bacteria including, the Gram-negative bacterium, *S. enteritidis* ATCC 4931 and the Gram-positive spore forming bacterium, *C. perfringens* SM101 (Zhao and Melville, 1998). Erythrophore cells were exposed to *S. enteritidis* ATCC 4931 grown in Luria–Bertani broth (LB; Fig. 2A). Pigment organelles began to aggregate slowly at 10 min and continued to aggregate. The difference between *S. enteritidis* and the negative control (LB media) was statistically significant ($P < 0.001$). *Clostridium perfringens* SM101 was cultured in Duncan-Strong sporulation medium to allow production of the potent enterotoxin CPE (Elwing *et al*., 1990; Karlsson *et al*., 1991). *Clostridium perfringens* induced aggregation of erythrophore pigment within 4 min followed by slow dispersion for the remainder of the assay (Fig. 2B). This response was very different from the dispersive response of the negative control (Duncan-Strong sporulation medium) and was statistically significant ($P < 0.004$).

Clostridium botulinum was chosen as a second representative of a Gram-positive, spore forming, yet anaerobic bacterium associated with food contamination and potential use as a biological weapon. Erythrophore cells were challenged with *C. botulinum* NCTC 7272 (Type A) or *C. botulinum* NCTC 7273 (Type B) cultured in BHI. Erythrophore cell response was monitored for 1 h. Very little response to *C. botulinum* was observed during this hour; therefore monitoring time was extended to 6 h. Two hours after exposure to *C. botulinum* NCTC 7272 or NCTC 7273, pigment organelles began to slowly aggregate (Fig. 3A) and aggregation of pigment organelles was complete at approximately 5 h. The erythrophore response to both *C. botulinum* cultures was statistically significant compared with the negative control (BHI) (*C. botulinum* NCTC 7272 $P < 0.005$ and *C. botulinum* NCTC 7273 $P < 0.006$ respectively). The observed morphological changes of the erythrophore cells were unique from changes induced by other bacterial pathogens. Extensive dendrite formation was observed in erythrophore cells treated with *C. botulinum* (Fig. 3C), while other bacterial pathogens induced pigment organelles to centrally localize within the erythrophore cell (Fig. 1C).

Discussion

This study demonstrates the potential of a novel bacterial biosensor populated with *B. splendens* erythrophore cells. When erythrophore cells are exposed to a bacterial pathogen, the response is easily observed and quantified. Erythrophore cells respond to selected food-associated bacteria, *B. cereus, S. enteritis*, *C. perfringens* and *C. botulinum*, and this response is different from the respective culture medium (negative control). Erythrophore cells were unable to distinguish the non-pathogenic bacterial strains, *B. subtilis* and the *B. cereus ΔplcR* mutant

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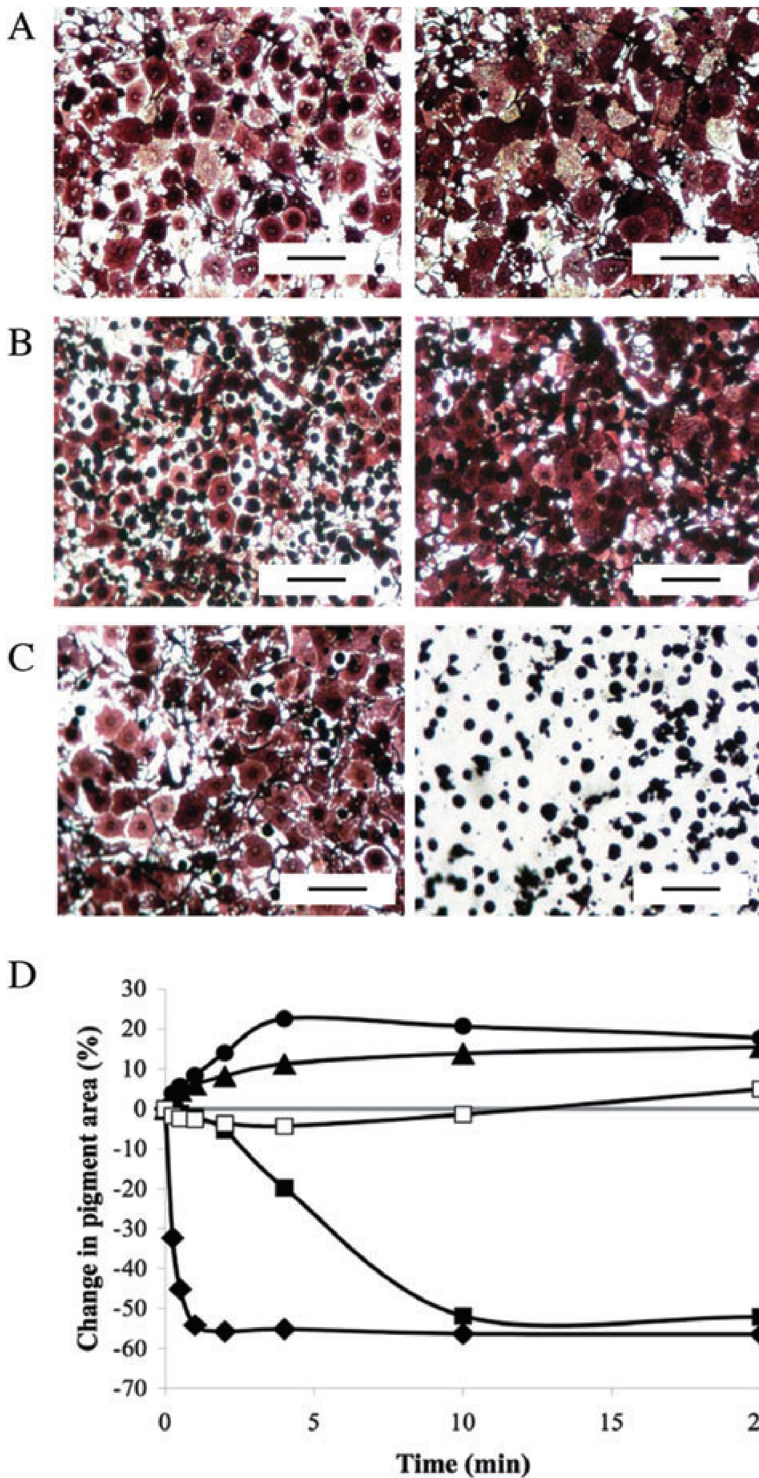

Fig. 1. *B. splendens* erythrophore cell response to *Bacillus* spp. Erythrophore cells appearance at Time 0 (left) and Time 20 min (right) upon exposure to: (A) BHI medium, (B) *B. subtilis* 1A1, (C) *B. cereus* ATCC 49064. Images are at 100×, the size bar represents a length of 100 μm. (D) Graphical display of erythrophore cell pigment area change in response to *Bacillus* spp. A negative change in pigment area is indicative of pigment aggregation in erythrophore cells whereas a positive change in pigment area represents pigment dispersion. (▲) BHI medium; (●) *B. subtilis* 1A1; (◆) *B. cereus* ATCC 49064; (■) *B. cereus* ATCC 14579; (□) *B. cereus ΔplcR* deletion mutant strain that is a derivative of ATCC 14579. Data represent the mean values of three trials.

strain from the negative control. The statistical and graphical evidence suggests that erythrophore cell response has potential for use as a biosensor in the detection or toxicity assessment for food-associated pathogenic bacteria.

What is it about a bacterial pathogen, such as *B. cereus*, that causes erythrophore cells to respond by relocating pigment organelles to the centre of the erythrophore cell? The failure of erythrophore cells to respond to the non-pathogenic *B. cereus ΔplcR* mutant strain provides insight to this intriguing question. PlcR, a pleiotropic transcriptional regulator, controls the expression of numerous extracellular compounds, including several

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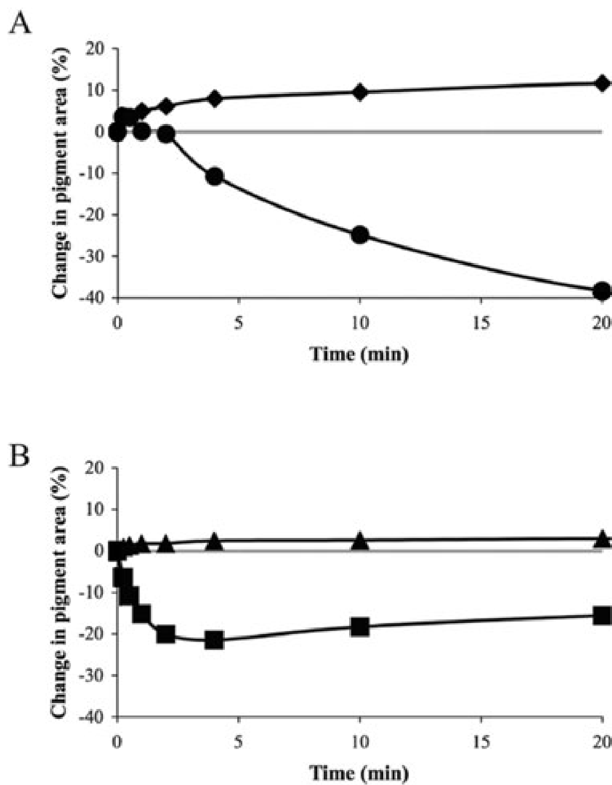

Fig. 2. *B. splendens* erythrophore cell response to *Salmonella enteritidis* and *Clostridium perfringens*. A negative change in pigment area is indicative of pigment aggregation whereas a positive change in pigment area represents pigment dispersion.
A. (◆) LB medium; (●) *S. enteritidis* ATCC 4931.
B. (▲) Duncan-Strong sporulation medium; (■) *C. perfringens* SM101.
Data represent the mean values of three trials.

virulence factors (Agaisse *et al.*, 1999; Økstad *et al.*, 1999; Salamitou *et al.*, 2000; Slamti and Lereclus, 2002). The observation that *B. cereus* lacking *plcR* does not induce pigment aggregation in erythrophore cells suggests that failure to express one or several of the PlcR regulated genes results in the failure of *B. cereus* to induce a response in erythrophore cells. Lereclus and co-workers have shown that *B. cereus* pathogenicity is dependent on PlcR (Lereclus *et al.*, 2000; Salamitou *et al.*, 2000), suggesting that erythrophore cells may interact with bacterial virulence traits or other gene products dependent on PlcR. Identification of *B. cereus* mutants that no longer induce a response in erythrophore cells is currently underway. Identifying the mutant bacterial gene products will define the mechanism(s) by which this bacterial pathogen induces a change in pigment organelle location within erythrophore cells.

Erythrophore cell response to *S. enteritidis* ATCC 4931 and *C. perfringens* SM101 cause a slower rate of aggregation and less overall aggregation when compared with *B. cereus* ATCC 49064 and ATCC 14579, suggesting that bacterial pathogens may interact with erythrophore cells through different mechanisms. Additionally, when erythrophore cells are exposed to *C. botulinum*, the erythrophore cell morphology in response to this bacterium is different from reactions with other bacterial pathogens. The observation of specific erythrophore cell morphology in response to specific bacterial pathogens as well as differences in the kinetics of erythrophore cell response may provide another means to measure and characterize bacterial detection.

Reports of *Salmonella* contaminated tomatoes, peanut butter and spinach and *C. botulinum* contaminated canned meats indicates the ease by which food-associated bacterial pathogens evade detection, often resulting in massive recalls of popular food items. Given the economic impact of massive recalls, as well as the lack of effective detection methods to enforce the USA's and UK's zero tolerance policy for certain food-associated pathogenic bacteria, suggests a need for detection methods based on parameters different from those describing bacterial presence. Although newer detection methods can identify single microbes, thus meeting zero tolerance requirement, many technical challenges remain for these methods. These challenges often include time intensive sampling and testing practices, long culture times to increase the number of bacteria to detectable levels, and costly shipment methods to move samples to a central laboratory for analysis (Cady *et al.*, 2005; Batt, 2007). Furthermore, many of these methods rely on detecting a structure through the use of a DNA or antibody probe, and thus are limited in the evaluation of toxicity and the potential of bacteria to cause disease. Additionally, DNA- or antibody-based detection methods are limited in the detection of unidentified (e.g. genetically rearranged) or newly emerging (e.g. new toxin producing variants) pathogenic bacteria. While DNA technology such as PCR may be able identify offending bacteria in the food, it cannot assess if the detected bacteria are alive and exhibiting the pathogenic behaviour that make them a health risk. For example, the bacteria may be non-viable and not exhibiting pathogenic behaviour, but their DNA may still be detectable by PCR technology, thus suggesting that information gathered from DNA characterizing techniques, if taken alone, may be seriously misleading. The *B. splendens* erythrophore cell response described here represents a unique class of cell-based biosensors with potential to complement current detection methods as well as circumvent a number of limitations and challenges with the available technology.

Experimental procedures

Bacterial culture preparation

All *Bacillus* spp. [*B. subtilis* 1A1 (Yoshida *et al.*, 1995), *B. cereus* ATCC 49064 (Midura *et al.*, 1970), *B. cereus* ATCC 14579 (Frankland and Frankland, 1887) and a *B. cereus*

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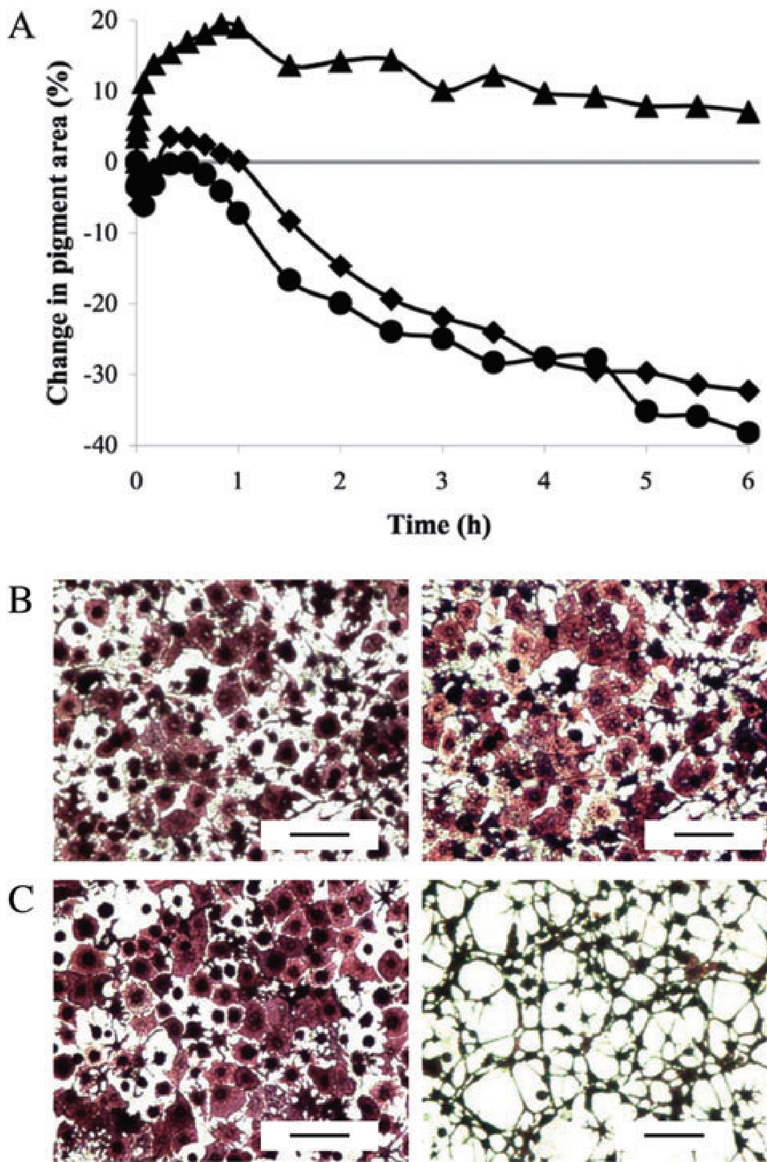

Fig. 3. (A) *B. splendens* erythrophore cell response after 6 h exposure to bacterial isolate. A negative change in pigment area is indicative of intracellular pigment aggregation whereas a positive change in pigment area represents pigment dispersion. (●) *C. botulinum* NCTC 7272; (◆) *C. botulinum* NCTC 7273; (▲) BHI medium. Data represent the mean values of three trials. (B) and (C) *B. splendens* erythrophore cells at 100×, the size bar represents a length of 100 μm; Left, time = 0 h, Right, time = 6 h, exposure to: (B) BHI medium, (C) *C. botulinum* NCTC 7272.

Δ*plcR* deletion mutant strain that is a derivative of ATCC 14579 (Salamitou *et al.*, 2000)] were cultured in BHI broth (Difco # 0418-17-7) at 37°C overnight. *Salmonella enteritidis* ATCC 4931 was cultured in LB overnight at 37°C. *Clostridium perfringens* SM101 (Zhao and Melville, 1998) was generously provided by Dr Mahfuzur R. Sarker. Briefly, a 0.1 ml aliquot of a frozen stock of *C. perfringens* SM101 was transferred into 6 ml of fluid thioglycollate (FTG) and then heat shocked for 20 min at 70°C. The heat-shocked culture was then incubated for 14 h at 37°C, and 0.4 ml of this starter culture was transferred to a second 6 ml FTG before this culture was incubated for 9 h at 37°C (Kokai-Kun *et al.*, 1994). An aliquot (0.4 ml) of this FTG culture was then added to 20 ml of Duncan Strong (DS) sporulation medium (McDonel *et al.*, 1988) and incubated for 8 h at 37°C (McDonel *et al.*, 1988). The presence of spores was verified using microscopy. *Clostridium botulinum* NCTC 7272 and *C. botulinum* NCTC 7273 were obtained from the National Collection of Type Cultures, PHLS Central Public Health Laboratory. A 0.1 ml aliquot of a cooked meat medium stock of *C. botulinum* NCTC 7272 and NCTC 7273 was transferred to 1.0 ml of BHI medium. The starter culture was then incubated for 24 h at 37°C. A 0.1 ml aliquot of the starter culture was transferred to a second 1 ml BHI tube before the culture was incubated for 24 h at 37°C. All *C. botulinum* culturing was done in an anaerobic chamber using AnaeroGen gas packs (Fisher). All bacterial cultures were pH controlled.

All Biosafety Level 2 (BSL2) bacterial agents were cultured in an approved BSL2 facility and performed under the requirements and regulations of and approved by Oregon State University's Institutional Biosafety Committee.

Erythrophore cell preparation

Erythrophore cells were isolated from the tails and fins of *B. splendens* fish as described previously (Mojovic *et al.*, 2004).

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Briefly, tissue was washed in skinning solution [1 mM NaEDTA, 5.6 mM glucose and penicillin–streptomycin mixture 1:100 (w/v) in calcium- and magnesium-free phosphate-buffered saline (PBS)], digestion with an enzyme solution [20–30 mg of collagenase type 1 (Worthington), and 1–3 mg of hyaluronidase (Worthington), in 7 ml of PBS]. After several washes, the cell pellet was re-supsended in a predetermined volume of Leibovitz L15 Medium+ (L15+, Gibco #21083-027, 500 ml of dye-free L15 was supplemented with 10 ml 1 M HEPES (Gibco-BRL 15630-080) buffer and 5 ml Antibiotic-Antimycotic (Gibco-BRL 15240-062) to ensure the desired plate density in the centre of each well of a 24-well microtitre plate or 48-well microtitre plate. The L15+ medium was added in each well to a final volume of 1.5 ml (24-well microtitre plate) or 0.75 ml (48-well microtitre plate) and 5% fetal bovine serum (HyClone #SH30071-01) was added to each well. Validation tests were performed for each preparation of erythrophore cells to ensure that isolated erythrophore cells were physiologically responsive. Erythrophore cells were treated with a known dispersive agent, melanocyte stimulating hormone (MSH), and a known aggregative agent, clonidine, to verify physiological responsiveness (Dierksen *et al*., 2004; Mojovic *et al*., 2004).

This effort was performed under the requirements and regulations of and approved by Oregon State University's Institutional Animal Care and Use Committee, approval #2979 and #3513.

Erythrophore cell response and computer analysis

Monitoring erythrophore cell response is based on observing and recording changes in the pigmented area of erythrophore cells exposed to bacterial agents and control agents as previously reported (Dierksen *et al*., 2004; Mojovic *et al*., 2004). Cell culture plates were mounted on the stage of an inverted microscope. A field of view containing approximately 100 erythrophore cells was monitored under a magnification of 100. This selected area was not moved throughout the course of the experiment. Bacterial suspension or physiological response testers (400 nM clonidine, Sigma C7897; 400 nM MSH, Sigma M4135, respectively) was added to each well to a final dilution of 1:10 in L15 medium (viable cell counts were ~10^7 for *Bacillus* spp. and ~10^6 cells *Salmonella* and *Clostridium* spp. respectively). Digital images (JPEG format) at 300×300 resolution were taken using a SPOT Insight 320 colour camera (Diagnostic Instruments, Sterling Heights, MI, USA) and SPOT software version 3.5.6.2 (Diagnostic Instruments), through a Leica (Leica, Wetzlar, Germany) DMIL inverted microscope (Bartels and Stout). Erythrophore response was recorded for 20 min; images were captured at 0, 0.25, 0.5, 1, 2, 4, 10 and 20 min. For experiments lasting 6 h, images were captured at the same time interval as the 20 min experiments, then every 10 min up to 60 min. After 60 min, images were captured every 30 min until the experiment was completed. The images were opened in Image Pro Plus 4.1 (Media Cybernetics), exported and processed into Excel. The total area in pixels (digital area units) for each captured image was determined and the percentage pigmented area change [cell area change (%) $= -(A_0 - A_x)/A_0 \times 100$, where A_0 is the initial area and A_x is the final area]. An aggregative response would result in a negative per cent area change and a dispersive response would result in a positive per cent area change.

Statistical analysis

The statistical analysis was carried out with S Plus statistical software (Insightful Technologies, Seattle, WA). All bacterial challenges were completed in triplicate using three different erythrophore cell preparations; reported values are the average of three trials. Final time points were analyzed using a two-sample *t*-test, all reported *P*-values were declared significant at $P < 0.05$.

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