Supplementary material MICROBIO-2010-019 (revised)

Experimental procedures

Media, culture conditions, and acquisition of scatter patterns for Vibrio *species*

The bacterial cultures used (Table 1) were prepared as follows unless otherwise stated. Cultures from frozen stock were grown in tryptic soy broth (TSB) containing 1% sodium chloride (NaCl) for 18−24 h at 30°C and subcultured in brain-heart infusion broth (Acumedia, Neogen, MI) containing 1% NaCl (BHI-NaCl). The cultures were serially diluted and spread-plated on the surface of BHI-NaCl agar (BHI-NaCl with 1.5% agar), Heart Infusion (HI: Acumedia), or *Vibrio* selective Thiosulphate Citrate Bile salts Sucrose (TCBS; Acumedia) plates to obtain 30-100 colonies per plate. The plates were incubated at 30°C until the diameter of each colony reached 1.3 ±□ 0.2 mm (typically 12-18 h). Since growth rates are variable among species, colony size was used as a fixed parameter. Scatter patterns of colonies were acquired and images were analyzed using the BARDOT (Advanced Bioimaging Systems, West Lafayette, IN). The identity of bacterial cultures was confirmed by Gram-staining, testing with API 20E (BioMerieux, Hazelwood, MO), and 16S rDNA sequencing.

Bacterial confirmation by 16S rDNA sequencing

Cultures of *Vibrio* species (Table 1) were confirmed by 16s rDNA sequencing of PCR-amplified products (Nilsson *et al.*, 2003). 16S rDNA-specific primer pairs, UFUL and URUL were used to amplify the target gene. The PCR conditions include: an initial denaturation at 94°C for 2 min, followed by 30 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 5 min. PCR products were sequenced at the Purdue Genomics Core

Facility (Purdue University, W. Lafayette, IN) and the 16S rDNA nucleotide sequences were compared to the NCBI database by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Effect of physiological stress on forward light scattering patterns

Cultures of *V. parahaemolyticus* CECT511, *V. vulnificus* MLT362, and *V. anguillarum* were subjected to the following stresses for 3 h: low pH (4.0), osmotic stress (5% NaCl), or heat stress (42°C)(Hahm and Bhunia, 2006). Overnight cultures were inoculated into BHI-NaCl broth after adjusting to pH 4.0 using concentrated HCl or into BHI broth containing 5% NaCl and held at 30°C for 3 h. For heat stress, cultures in BHI-NaCl broth were incubated at 42°C for 3 h. Each culture was washed and resuspended in 20 mM phosphate buffered saline (PBS, pH 7.2) and plated onto BHI-NaCl agar plates for BARDOT analysis.

Detection of bacteria recovered from viable but non-culturable (VBNC) state

VBNC state in *V. vulnificus* strains (MLT 362 and MLT 364) were achieved by following procedure described before (Smith and Oliver, 2006). Briefly, cultures were grown in HI broth at room temperature (RT) for 24 h with agitation, subcultured in HI until log phase (OD_{610} = 0.13-0.2), and inoculated into pre-chilled (4°C) ½ strength artificial sea water (ASW; (NaCl 24.7g/L, KCL 0.67g/L, $CaCl{\cdot}H_2O$ 1.36g/L, $MgCl2{\cdot}6H_2O$ 4.66g/L, $MgSO_4{\cdot}7H_2O$ 6.29g/L, $NaHCO_3$ 0.18g/L) (Wolf and Oliver, 1992) and maintained under that condition. Entrance to VBNC state was monitored by analyzing 0.1 ml of the sample (serially diluted in ASW) by plating on HI agar plates and by staining cells by LIVE/DEAD stain (BacLight stain kit: Molecular Probes). When no culturable cells were detected in 0.1 ml test sample, then 5 ml sample was passed through a 0.22 um nucleopore filter, placed on HI and TCBS agar plates for 2 h then aseptically removed. Plates were incubated at RT for 24 h and monitored for growth. Cultures were deemed VBNC when there was less than 0.1 CFU/ml for each strain (Fig. S2). To resuscitate cells, cultures (5

ml) were incubated at RT for 24 h, plated on HI and TCBS, incubated at 30°C for about 12 h and analyzed by BARDOT.

Application of BARDOT to detect and identify Vibrio *from oyster and water samples*

Two dozen oysters (prime grade) from North Atlantic coast were purchased from a local fish monger and transported on ice to the laboratory. Six oysters were discarded because they were either too small or dead. Upon arrival they were placed immediately inside a clear plastic bag and kept in ASW for 30 min. Oysters were rinsed in deionized water, scrubbed, and washed in 70% ethanol, and divided into two groups (9 per group): control and experimental, and placed in two separate 5-gallon bucket containing 7 L sterile ASW, and continually aerated with flowing air. After 2 h, the experimental group received mixed (1:1) culture of *V. vulnificus* MLT 364 and *V. parahaemolyticus* CETT5711 to obtain a final concentration of 10^5 CFU/ml and both groups were maintained at RT for 24 h with aeration. Active filtering by oysters were noticed as the ASW became cloudy then cleared multiple times. Tissue and liquor from each oyster were harvested, weighed, placed in stomacher bag built with filter mesh (Nasco Whirlpak®, Fort Atkins, WI) containing alkaline peptone water (pH 8.5) with 1% peptone (Becton Dickinson) to achieve 1:10 dilution, homogenized (for 2 min in a stomacher; Seward, Norfork, UK) and incubated at 30°C for 6 h. Enriched samples were diluted, plated on TCBS and HI, incubated at 30°C for 12 h, and plates were scanned by BARDOT. Colony scatter images were compared with scatter image library consisting of *V. vulnificus*, and *V. parahaemolyticus*. Select colonies from representative plates were further verified by colony PCR using primers specific for *V. vulnificus* and *V. parahaemolyticus* described below. Entire oyster experiment was conducted under a biosafety cabinet.

Similarly, *V. parahaemolyticus* CECT511 was also inoculated into 500 ml sterile tap water at about 2×10^3 CFU/ml and the samples were passed through a membrane filtration

system using 0.45-µm filters (Millipore). The filter was then placed in a centrifuge tube with 10 ml sterile PBS, vortexed for 2 min, and centrifuged at 5000 × g for 10 min. The cell pellet was resuspended in 100 µl PBS and plated onto BHI-NaCl agar. Plates were incubated at 30°C for 12 h or until colony size reached 1.3 ± 0.2 mm, and colonies were subsequently analyzed using BARDOT.

PCR verification of vibrios

Polymerase chain reactions were used to confirm the identity of colonies on BHI-NaCl plates. Colonies of *V. parahaemolyticus* were confirmed by using VPM1 and VPM2 primers specific for the metalloprotease gene (Luan *et al.*, 2007). *V. vulnificus* was confirmed by using Cyt1 and Cyt2 primers targeting the hemolysin/cytolysin gene (Morris *et al.*, 1987). A standard amplification condition was used consisting of initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 5 min. The amplicons were analyzed by agarose gel electrophoresis.

Pattern analysis and classification

Each scatter signature was analyzed using an automated pattern processing and classification system (Bayraktar *et al.*, 2006, Banada *et al.*, 2009), which accompanied the BARDOT hardware. A set of radial orthogonal pseudo-Zernike polynomials, as well as Haralick texture features, was computed for every scatter pattern. The feature reduction and selection procedure was based on three independent wrappers whose output was combined. One hundred ninety features were analyzed using sequential floating forward selection (SFFS), which is a combination of the sequential forward and the sequential backward methods. The SFFS-based wrapper was used with the k-nearest neighbor, LDA, and recursive-portioning algorithms. The number of selected features was 23.

Subsequently, the selected best features were used to train the pattern-recognition system. Three different classifiers were used: linear Fisher's discriminant (LFD), support vector machine with linear kernel (SVM-L), and support vector machine with radial-basis function kernel (SVM-RBF). The classifiers were optimized using standard 10x cross-validation. The quality of detection and classification was evaluated by computing values of sensitivity and specificity, and area under receiver operating curve (AUC).

For the purpose of supervised classification four classes were created: *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus,* and "Mixture" class containing colonies of *V. alginolyticus*, *V. anguillarum*, *V. mimicus*, and *V. orientalis*. The classifiers were optimized to detect and recognize patterns belonging to three important classes of known pathogens: *V. cholerae, V. parahaemolyticus,* and *V. vulnificus*.

References

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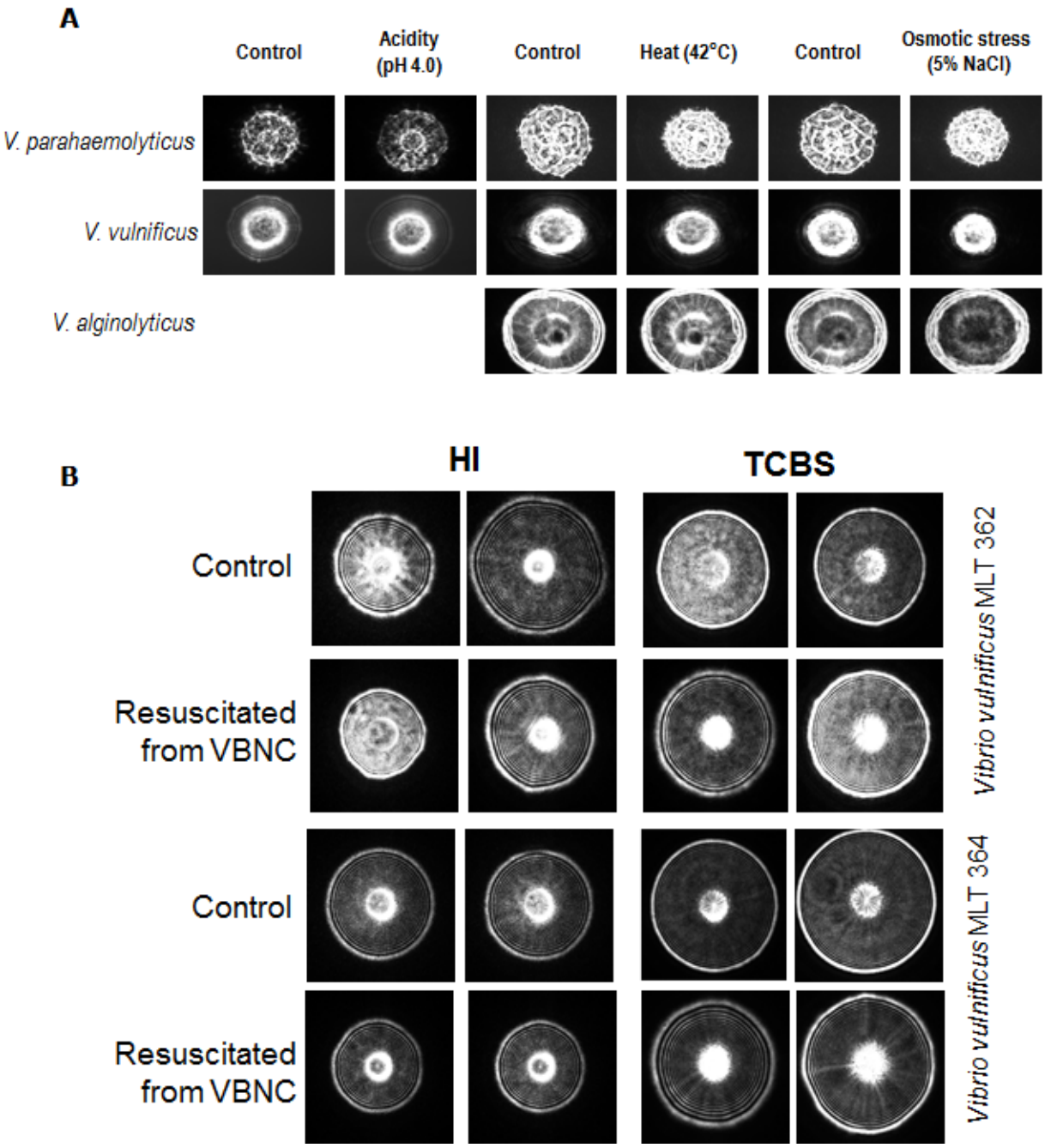
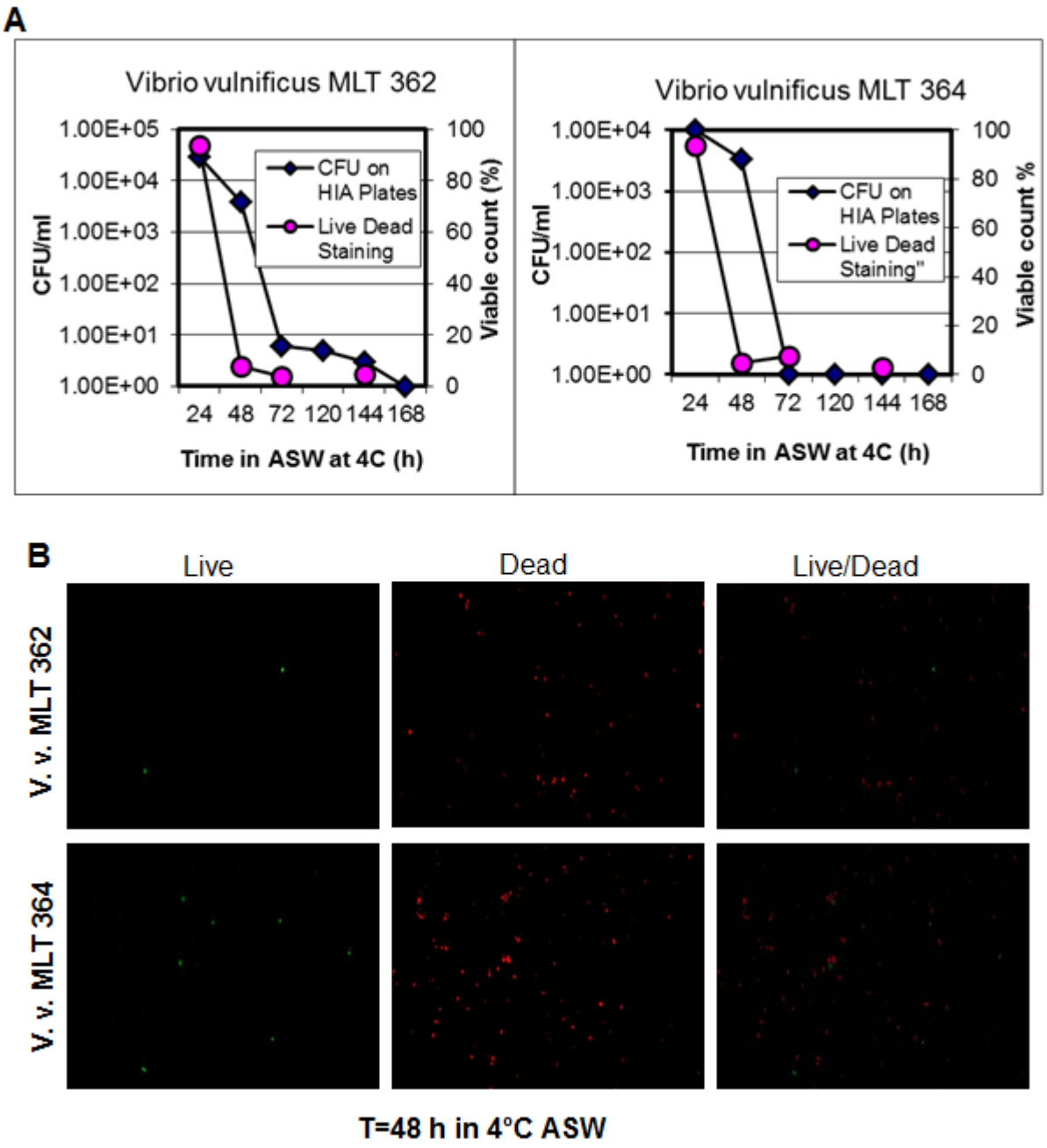


Fig. S1. (A) Scatter images of resuscitated colonies of *Vibrio* species that were exposed to various stresses (pH [4.0], heat [42°C] and osmotic [5% NaCl]) for 3 h. Cultures were exposed to stress, serially diluted and plated onto BHI containing 1% NaCl, and allowed to grow for 12 h before scatter images were acquired. (B) Scatter images of *V. vulnificus* MLT 362 and 364 recovered from viable but non-culturable (VBNC) state.



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146 **Fig. S2.** (A) Induction of VBNC state in *V. vulnificus* MLT 362 and 364 strains suspended in
147 artificial sea water (ASW). (A) Viable cell counts were determined by plating on HI agar (HIA)
148 plates and by staining cells with BacLight LIVE/DEAD staining kit. (B) Fluorescence microscopic
149 analysis of live, dead and live/dead *V. vulnificus* cells taken at 48 h indicating <1% cells are still
150 viable at that condition and are entering into VBNC state.

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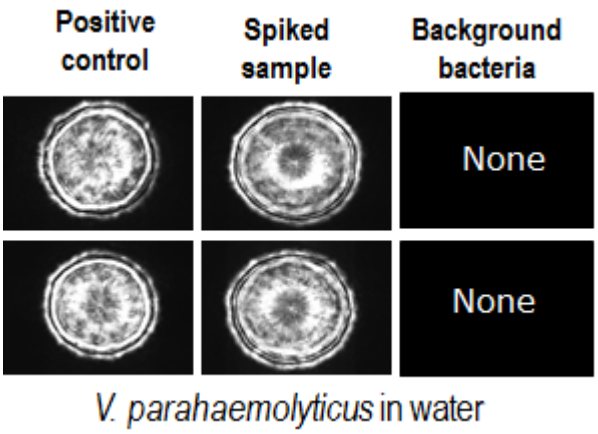

Fig. S3. Detection of *V. parahemolyticus* on BHI-NaCl agar plate from spiked water sample using BARDOT.