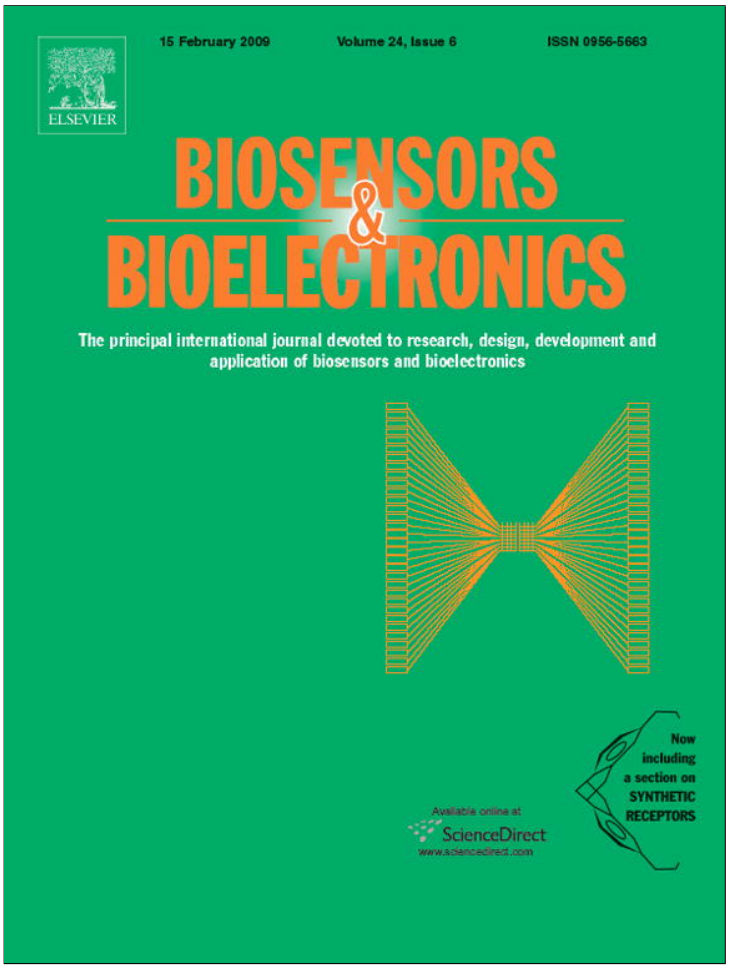

Biosensors and Bioelectronics 24 (2009) 1685–1692

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Label-free detection of multiple bacterial pathogens using light-scattering sensor

Padmapriya P. Banada[a], Karleigh Huff[a], Euiwon Bae[c], Bartek Rajwa[d], Amornrat Aroonnual[a], Bulent Bayraktar[e], Abrar Adil[a], J. Paul Robinson[d,f], E. Daniel Hirleman[c], Arun K. Bhunia[a,*]

[a] Molecular Food Microbiology Laboratory, Department of Food Science, Purdue University, IN 47907, USA
[c] School of Mechanical Engineering, Purdue University, USA
[d] Department of Basic Medical Sciences, Purdue University, USA
[e] School of Electrical and Computer Engineering, Purdue University, USA
[f] Weldon School of Biomedical Engineering, Purdue University, USA

ARTICLE INFO

Article history:
Received 11 June 2008
Received in revised form 21 August 2008
Accepted 28 August 2008
Available online 11 September 2008

Keywords:
Light scatterometer
Bacterial detection
Classification
Food
Clinical specimen

ABSTRACT

Technologies for rapid detection and classification of bacterial pathogens are crucial for securing the food supply. This report describes a light-scattering sensor capable of real-time detection and identification of colonies of multiple pathogens without the need for a labeling reagent or biochemical processing. Bacterial colonies consisting of the progeny of a single parent cell scatter light at 635 nm to produce unique forward-scatter signatures. Zernike moment invariants and Haralick descriptors aid in feature extraction and construction of the scatter-signature image library. The method is able to distinguish bacterial cultures at the genus and species level for *Listeria, Staphylococcus, Salmonella, Vibrio,* and *Escherichia* with an accuracy of 90–99% for samples derived from food or experimentally infected animal. Varied amounts of exopolysaccharide produced by the bacteria causes changes in phase modulation distributions, resulting in strikingly different scatter signatures. With the aid of a robust database the method can potentially detect and identify any bacteria colony essentially instantaneously. Unlike other methods, it does not destroy the sample, but leaves it intact for other confirmatory testing, if needed, for forensic or outbreak investigations.

1. Introduction

Outbreaks of methicillin-resistant *Staphylococcus aureus* in communities (Klein et al., 2007), *Escherichia coli* O157:H7 in spinach and ground beef (Heaton and Jones, 2008; Jay et al., 2007), *Salmonella* in peanut butter (Gerner-Smidt and Whichard, 2007), *Listeria monocytogenes* in ready-to-eat meats (Swaminathan and Gerner-Smidt, 2007), and *Clostridium botulinum* in canned chili sauce (CDC, 2007) are examples of recent public-health threats. In addition, concerns about intentional administration of pathogens to food or agricultural commodities (Manning et al., 2005; Relman et al., 2006) call for improvement in diagnosis and detection (Ligler et al., 2003; Lim et al., 2005).

Currently employed detection methods based on antibodies or DNA involve multiple steps and are labor intensive, time consuming, and often unable to detect low numbers of cells. Moreover, samples are terminally destroyed by the test and are thus unavailable for further confirmatory assessment. Though biosensor tools are able to improve sensitivity they must rely on the use of specific reporter molecules such as antibodies or nucleic acid probes coupled with fluorophores or enzymes, thus limiting their broad application for multipathogen detection. Additionally, direct detection of analytes from real-world samples and overall cost per analysis has not yet matched expectations (Bhunia, 2008).

The conventional culture-based detection method is highly reliable and is still considered to be the "gold standard" for microbiological analysis. For high-profile investigations such as those related to biosecurity (Kiratisin et al., 2002; Bhunia, 2006) or foodborne outbreaks (Jay et al., 2007), results obtained by rapid methods must always be confirmed by culture-based techniques (CDC, 2006; Lim et al., 2005). Therefore, sensors that incorporate a traditional culture-based approach as a part of the detection procedure are highly desirable. In culture methods, clinical or food specimens are diluted in buffer and distributed onto appropriate solid agar plates

* Corresponding author. Tel.: +1 765 4945443; fax: +1 765 4947953.
E-mail address: bhunia@purdue.edu (A.K. Bhunia).

0956-5663/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2008.08.053

to allow individual bacterial cells to grow and form a colony. A colony consisting of progeny (millions to billions) of a single parent cell is considered pure and can be subsequently tested by metabolic or genetic fingerprinting, immunoassays, or polymerase chain reaction (PCR) assays. However, all the aforementioned techniques require extensive sample handling and 3–24 h before a definitive result can be obtained (Bhunia, 2008). These cumbersome multistep processes require specific reagents, such as a panel of carbohydrates or proteins as substrates for bacterial metabolism to produce metabolic fingerprint patterns, or nucleic acid restriction enzymes, primers, and labeled DNA probes for genetic fingerprinting or PCR. Owing to the nature of the detection modality, immunoassays require specific enzyme- or fluorescence-labeled antibodies. An ideal sensor devoid of these problems and capable of direct, nondestructive, and label-free identification would revolutionize routine microbiological analysis of food, agricultural, environmental, and clinical samples.

Light-scattering technology has been used before to interrogate bacterial cells in suspension (Wyatt, 1969; Wyatt and Phillips, 1972) and characteristic angular distribution was exploited to provide information on the metal toxicity on bacteria, size distribution, shape, and refractive index (Bronk et al., 2001; Perkins and Squirrell, 2000; Wyatt, 1969). The scope of this approach is very narrow and only a limited number of bacterial species could be detected successfully. In a recent work, we found that interrogation of individual bacterium on surface of agar in a semi-solid state could provide a possible differentiation (Nebeker et al., 2001) and this work was extended to analyzing bacterial colonies of different species which provided distinctive forward-scattering pattern although their visual morphology looked similar (Bae et al., 2007; Banada et al., 2007; Bayraktar et al., 2006). Here, we report the design and construction of a laser light-scattering analyzer that detects and identifies bacterial colonies growing on a Petri dish in seconds without destroying the colony. The system was tested for its ability to distinguish bacterial species and strains from five different genera; *Listeria, Staphylococcus, Salmonella, Vibrio,* and *Escherichia* in pure form and from inoculated food matrices and experimentally infected animal.

2. Materials and methods

2.1. Light-scattering instrumentation

Initial light-scattering instrument setup was on an optical bread board (Banada et al., 2007). In this study, we designed an automated BARDOT (Bacterial Rapid Detection using Optical scattering Technology) system and a local start up manufacturing company (En'Urga Inc., W. Lafayette, IN) worked together with our team to build two prototype units (Fig. 1). The system uses a laser (635 nm) to illuminate individual colonies and create a forward-scatter signature that is collected and subsequently analyzed. The working prototype of a fully automated system consists of a sample locator (laser line scanner), a forward-scatter measurement system (an interrogating source and a CCD sensor), and a sample control (x–y motorized stage). The additional system components are described in Supporting information.

2.2. Bacterial cultures and growth condition

Representative species and strains of *Listeria*, *Escherichia, Salmonella, Staphylococcus* and *Vibrio* used in this study and their culture conditions are listed in Tables S1–S6. Preparation of these bacterial cultures for light-scattering experiments was done following the protocol described before (Banada et al., 2007). Each

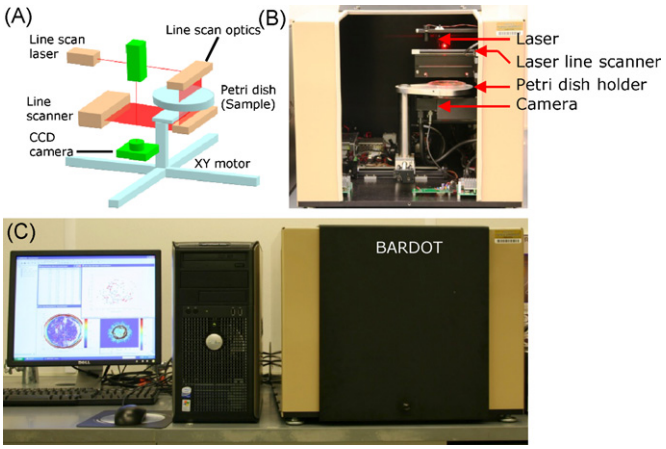

Fig. 1. (A) Components and schematics of the automatic light scatterometer, BARDOT (Bacterial Rapid Detection using Optical light-scattering Technology). The system integrates 3 major components: scatterometer, colony counter/locator, and motion control. (B) The photo shows a Petri dish on the BARDOT system (dimension: 24 in. × 20 in. × 17 in.; weight: 75 lbs) ready to capture the forward-scattering patterns. The laser line scanner acquires the transmission characteristics of the bacterial colonies (40 s per plate) and the colony center coordinates is computed via quadrant balancing of the scattering pattern. A laser diode (635 nm) is incident on top of the single bacterial colony and the forward-scattering signature is captured by a CCD image sensor (requires 5 s/colony). (C) The complete BARDOT setup.

bacterial culture, stored in −80 °C in BHI (brain-heart-infusion) broth-glycerol stock were sub-cultured twice in BHI (Difco, Sparks, MD) broth for 18–24 h at 30 or 37 °C depending on the culture and were surface plated onto BHI agar (1.5% w/w) plates. The plates were incubated at 30 or 37 °C until the colony size reached 1.3 ± 0.2 mm in diameter. BHI agar plates were prepared with 15 mL/plate of sterile tempered to 45 °C media (original volume before solidification).

2.3. Effect of bacteriological growth media on scattering patterns

Samples of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* were prepared as described above. In addition to surface plating on BHI agar, cultures were plated on Tryptic soy agar (TSA) and a selective differential media, xylose-lysine deoxycholate (XLD) for *Salmonella*, Modified Oxford media (MOX) for *Listeria* and Cefixime-Tellurite-sorbitol MacConkey agar (CT-SMAC) for *E. coli*. Plates were incubated until colonies reached appropriate size (1.3 ± 0.2 mm). Incubation times were identical for cultures on BHI or TSA. Longer times were necessary for cultures on selective media (16 h for *E. coli* on CT-SMAC, 16 h for *Salmonella* on XLD, 42 h for *Listeria* on MOX) to achieve colony diameters of 1.3 ± 0.2 mm.

2.4. Effect of pre-exposing bacteria to different stressors on scattering patterns

Cultures of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* were exposed to several stress inducing conditions such as temperature (42 °C), pH 4.0 and osmotic stress (5% NaCl) for 3 h (Hahm and Bhunia, 2006). Stress-exposed cultures were plated on BHI agar plates and incubated at 30 or 37 °C and scatter images of at least 20 colonies were taken using BARDOT. Control cultures did not receive any stress (see Supporting information).

2.5. Food sample testing

Food samples (3–4 each) tested in this study included ready-to-eat hotdog and shredded beef, raw ground beef and chicken, frozen and fresh spinach, and fresh tomato. Several 25 g portions

Table 1
Detection of bacterial pathogens from inoculated food samples using BARDOT

Bacteria	Food sample	Total detection time using BARDOT starting with the cell number[a]			
		1 (3–5) cfu/25 g	10 (7–15) cfu/25 g	100 (93–145) cfu/25 g	1000 (724–1051) cfu/25 g
E. coli O157:H7 (EDL933)	Frozen spinach	21 h (8 + 13)	21 h (8 + 13)	19 h (6 + 13)	17 h (4 + 13)
	Raw spinach	29 h (16 + 13)	29 h (16 + 13)	21 h (14 + 13)	NT
	Precooked shredded beef	ND	ND	23 h (10 + 13)	21 h (8 + 13)
	Raw ground beef	ND	21 h (8 + 13)	19 h (6 + 13)	17 h (4 + 13)
L. monocytogenes (F4244)	Processed Hotdog	38 h (12 + 26)	36 h (10 + 26)	36 h (8 + 26)	34 h (8 + 26)
S. Typhimurium (Tennessee)	Fresh Tomato	25 h (12 + 13)	23 h (10 + 13)	21 h (8 + 13)	17 h (4 + 13)
S. Enteritidis (PT1)	Raw chicken	31 h (18 + 13)	29 h (16 + 13)	27 h (14 + 13)	21 h (8 + 13)

[a] Food samples were inoculated with bacterial cells either at 1 (actual counts: 3–5) cfu/25 g, 10 (7–15) cfu/25 g, 100 (93–145) cfu/25 g, or 1000 (724–1051) cfu/25 g sample. The numbers in parenthesis indicate the enrichment time (h) + time (h) on BHI plate to form measurable colonies. This experiment was repeated with 3–4 of each food sample. Data with 1 cfu/25 g inoculation levels were in agreement with PCR assay (Table S7) performed in parallel. ND, none detected even after 30 h of enrichment in selective broth possibly due to the presence of inhibitors including background microflora; NT, not tested.

of each were inoculated either with *L. monocytogenes*, *S.* Enteritidis, *S.* Typhimurium, or *E. coli* O157:H7 at a concentration of 1, 10, 100 and 1000 cfu (Table 1). Actual inoculum counts were determined by plating the serial dilutions of cultures on the BHI agar plate. After inoculation, samples were placed at room temperature for 1 h before mixed with 225 ml of appropriate selective enrichment broths (modified EC broth with 20 mg/L novobiocin (mEC + *n*) for *E. coli*; Fraser broth for *Listeria*; Rappaport-Vassiliadis R10 for *Salmonella*) (BAM, 2008) and blended in a stomacher (Seward, Norfork, UK). Inoculated and uninoculated samples were enriched (incubated for variable times) at 37 °C (Table 1), diluted and plated onto BHI agar plates and also on selective agar plates (CT-SMAC for *E. coli*, MOX for *Listeria*, XLD for *Salmonella*). At least two plates per sample with 20 colonies per plate (total 40 colonies) were examined by BARDOT and scatter images were compared with the database for identification.

2.6. Mice experiment

A/J mice (8 weeks old females) were purchased from Jackson Lab (Bar Harbor, Maine) and were used as a model animal to acquire clinical specimens (Czuprynski et al., 2003) and this experiment was conducted in accordance to an institutional approved procedure. *L. monocytogenes* cells (10^7 cfu/mouse) were administered intragastrically in 12 mice using feeding needle (Popper) and each mouse was caged separately without bedding materials. Mice were sacrificed at 24, and 48 h by CO_2-asphyxiation and internal organs (liver, spleen, and intestine – from proximal duodenum to rectum) and blood from heart were collected aseptically, blended in buffered *Listeria* enrichment broth (BLEB) and diluted samples were surface plated onto BHI agar plates. Feces collected from each mouse were collected and processed in BLEB as above. Plates were incubated at 37 °C for 30–36 h and colonies were examined by BARDOT.

Identity of each bacterial colony from food or clinical specimens whose scatter signatures were used for identification was further confirmed by a multiplex PCR assay using specific primers (Table S7) or an automated Riboprinter (Qualicon) (Jaradat et al., 2002).

2.7. Extracellular polysaccharide and protein isolation

Discrete colonies (1–1.2 mm in diameter) of *L. monocytogenes* and *L. innocua* from BHI agar plates were streaked onto fresh BHI agar plates and incubated for 30–36 h to obtain lawn cultures. The cultures were scraped from the plates (5 plates/culture) using sterile cotton swabs, resuspended in PBS, vortexed and absorbance were adjusted to uniformity (OD_{600}). Equal volume of the cultures was incubated in a shaking incubator at 200 rpm for 2 h at 37 °C. The cultures were centrifuged and the supernatant was filter sterilized. Exopolysaccharide (EPS) extraction was carried out following the procedure of Van Geel-Schutten et al. (1999), quantified by measuring the final dry weight and carbohydrates were estimated by phenol:sulfuric acid method (Dubois et al., 1956). The purity of the polysaccharide samples was confirmed by NMR spectroscopy (Varian, Inc., Palo Alto, CA)

2.8. Image analysis

The scatter patterns, created upon illumination of individual colonies, were pre-processed by histogram equalization. Subsequently, a number of features were extracted for further analysis and classification. Two groups of features were used: rotation-invariant features were characterized using magnitudes of Zernike moments; and texture features were calculated using Haralick gray-level co-occurrence matrices (GLCMs) (Bayraktar et al., 2006; Haralick et al., 1973; Khotanzad and Hong, 1990; Mukundan and Ramakrishnan, 1995).

For the training of the recognition system, datasets containing 100 or more scatter patterns per class of bacteria (strain, species, or genus) were used. Each observation was represented by 120 features, which were selected on the basis of Fisher's criterion from a 240-element feature vector. The complexity of the classification problem was initially visualized using a linear discriminant analysis (LDA) plot; however, the classification was performed by support vector machine (SVM)-based algorithm (Chang and Lin, 2001), not by LDA. SVMs are able to construct decision hyperplanes in a multidimensional space that separates cases of different class labels, effectively allowing for non-linear decision boundaries in the parameter space (Burges, 1998; Vapnik, 1998).

The quality of the classifier and the evaluation of the classification were performed using cross-validation (Baldi et al., 2000). The confusion matrix resulting from the cross-validation was subsequently used to calculate sensitivity and precision of classifiers. Quality of a classifier was also estimated using a generalized squared correlation, GC^2 (see Supporting information).

3. Results

3.1. Light scatterometer and bacterial scatter image library and classification

Diagram and the picture of a prototype laser light-scattering analyzer are presented in Fig. 1. To explore the method's capability in detection and identification of pathogenic bacteria, we chose 5 major genera (Fig. 2A): *Listeria* (Fig. S1), *Salmonella* (Fig. S2),

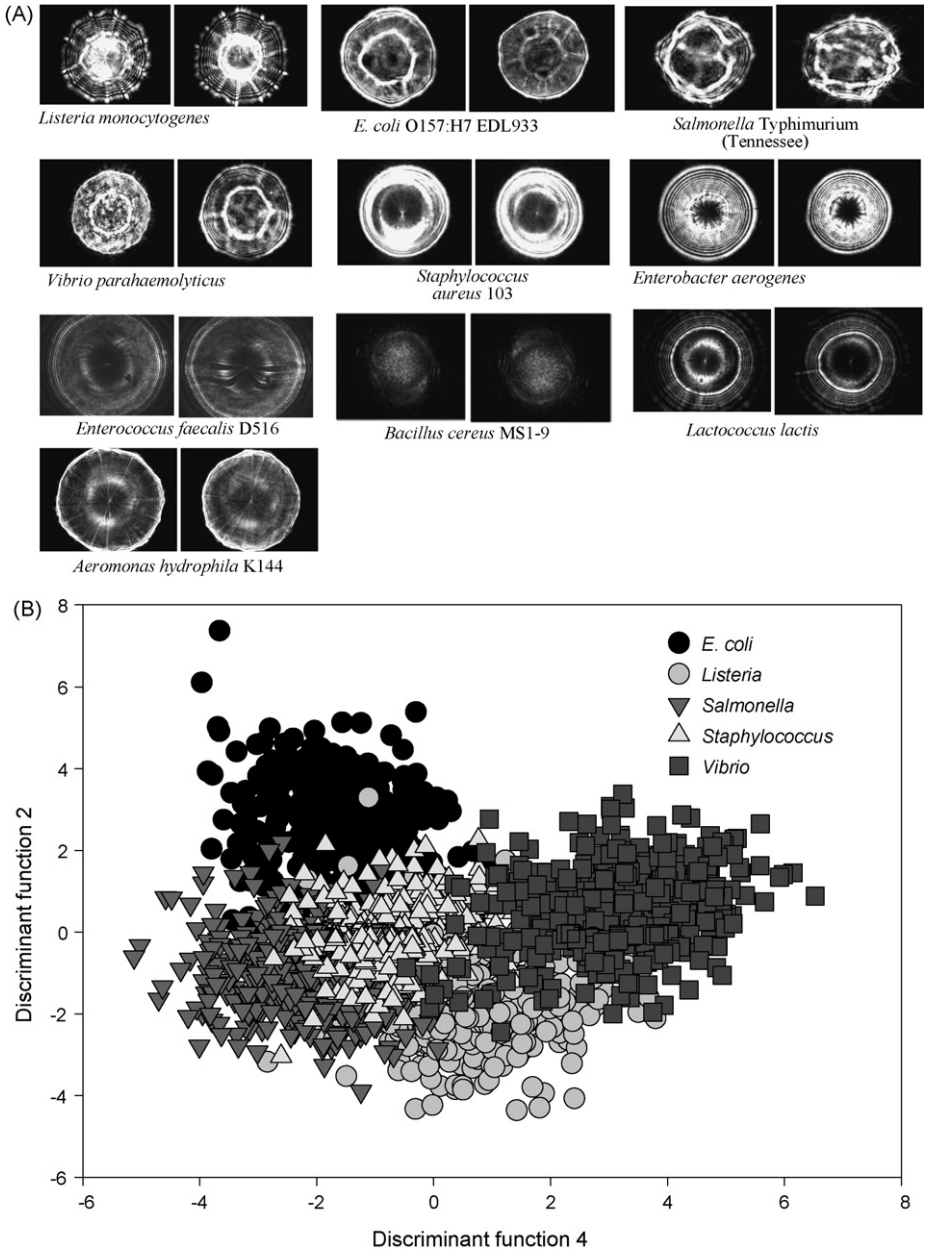

Fig. 2. (A) Forward-scatter images of colonies of representative species of genus; *Listeria*, *Staphylococcus*, *Salmonella*, *Vibrio*, and *Escherichia*. (B) Visualization of BARDOT scatter-patterns originating from five bacterial genera using linear discriminant functions.

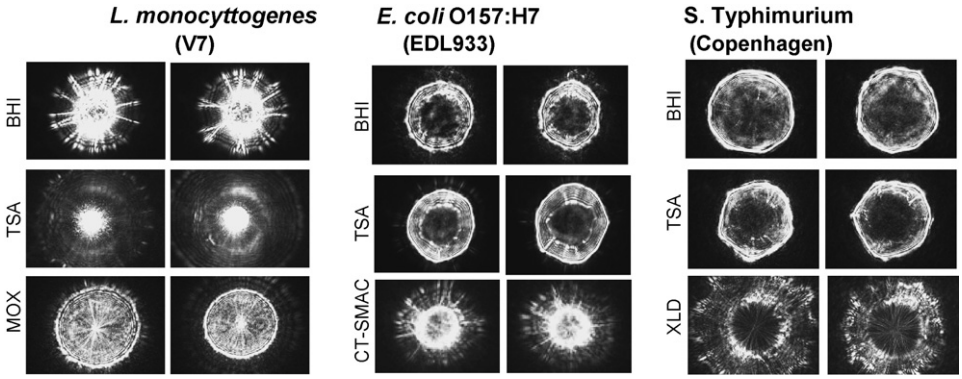

Fig. 3. Effect of nonselective (BHI, TSA) or selective (MOX, CT-SMAC and XLD) growth media on light-scattering images of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* Typhimurium.

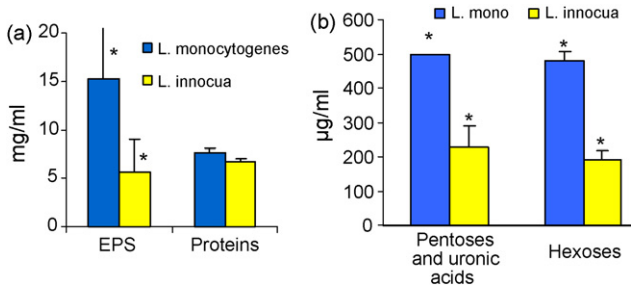

Fig. 4. Quantitative estimation of total (a) exopolysaccharides (EPS) and proteins and (b) pentose and hexose sugar fractions of EPS in *L. monocytogenes* F4244 and *L. innocua* F4248. Values are presented as mean ± S.D. *Denotes significant difference ($P < 0.05$).

Escherichia (Fig. S3), *Staphylococcus* (Fig. S4), and *Vibrio* (Fig. S5), representing 56 species and 95 strains/serovars. About 9000 scatter images were collected (Figs. 2A and S1–S5). Fast-growing bacteria such as *Vibrio*, *Aeromonas*, and *Escherichia* were detected as early as 12–16 h, *Salmonella* and *Staphylococcus* in 18–20 h, while slow-growing *Listeria* could be detected after 30–38 h. In addition, scatter patterns from colonies of *Enterococcus faecalis*, *Enterobacter aerogenes*, *Bacillus* spp., and *Lactococcus lactis* were generated.

The classification success for five bacterial genera (Fig. 2B) was 90–99% and for species within genus *Staphylococcus* was 99.35%, *Listeria* 95%, and *Vibrio* 94.5%, *Salmonella* 95.64% and *E. coli* 90.46% (Fig. S6 and Table S9).

3.2. Effect of physiological stress and media on scatter patterns

The method's ability to classify bacteria that have been exposed to various physiological stresses naturally encountered during their existence in food or water samples was also investigated. As expected, the patterns for stress-recovered organisms and controls were identical (Fig. S7), suggesting the suitability of the method in recognizing colonies formed by stress-exposed bacteria. Further, we have shown that bacteria (*Salmonella*, *Escherichia* and *Listeria*) grown either at 30 or 37 °C did not affect the scatter patterns of colonies formed on BHI or respective selective agar plates.

In a subsequent analysis the effect on the scatter image patterns of composition of the growth medium in the presence or absence of selective antimicrobial agents was examined. Use of selective agents in media is a common practice in microbiology to suppress the growth of undesirable background resident microflora. The collected scatter signatures from selective media were substantially different from their counterparts acquired employing nonselective media for all tested bacterial cultures (Fig. 3). This property could be advantageously exploited to further differentiate cultures that may exhibit similar or ambiguous patterns when grown on nonselective media seen in *Salmonella* (Fig. S2) and *E. coli* (Fig. S3). The media dependence reduces the chance of obtaining false results and provides another feature for classification. In fact, this strategy allowed us to detect *E. coli* O157:H7 on CT-SMAC agar plate from spiked raw spinach and ground beef that contained high background microflora (Table 1 and Fig S8).

Next, we investigated some possible relationships between the biochemical and physical determinants of colony structure and the observable forward-scatter properties by examining the production of EPS and protein contents in representative cultures of *Listeria* (*L. monocytogenes*, a pathogen; and *L. innocua*, a non pathogen). Although no significant difference in total protein contents between the two cultures was observed, the total EPS content in the pathogenic species was two- to three-fold greater than in the non-pathogenic one (Fig. 4). Further chemical analysis of EPS revealed that both pentose and hexose sugars were abundant in *L. monocytogenes* compared to *L. innocua* (Fig. 4).

3.3. Scatterometer application with real-world samples

To validate application of the light-scattering method with food, various representative vegetable and meat samples (hotdog, spinach, poultry, tomato, and ground beef) were spiked separately with *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* (Table 1 and Fig. 5A) and analyzed. In each agar plate, 20 colonies were examined and presence of a single positive colony (i.e., target pathogenic bacterium) among 20 analyzed colonies was used as a criterion for sample positivity (Table 1 and Figs. S8–S11). This arbitrary criterion was not very conservative. Owing to the method's speed and accuracy, one can potentially analyze a much higher number of colonies per sample to improve the robustness of the testing protocol. US governmental regulatory agencies have established zero-tolerance policies for *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* in certain ready-to-eat foods. In agreement with these stringent criteria, forward scattering is able to detect the presence of contamination even for samples inoculated with a single cell per 25-g portion of test specimen. *E. coli* O157:H7 was detectable in spinach at 1 cfu/25 g and ground beef at 10 cfu/25 g after 21–29 h despite the presence of high background bacterial counts (Fig. S8). *L. monocytogenes* and *Salmonella* were also detected at levels as low as 1 cfu/25 g in hotdog, tomato, and chicken samples. As expected, samples containing a higher initial pathogen load can be detected earlier than samples with a low initial inoculum. On average a result can be obtained within 24 h and food-testing data obtained from the scatterometer were in agreement with PCR assays performed in parallel (Table 1). The colonies identified by light scatterometer were further confirmed by PCR (Figs. S12–S15) or ribotyping (Supporting information).

To validate application of the light-scattering method with clinical specimens, we examined organs, blood, and fecal samples from experimentally infected mice. A uniform population of visually indistinguishable colonies from liver, spleen, and blood was observed on BHI plates. Each of the colonies was correctly identified as *L. monocytogenes* by the instrument (Fig. 5B). As expected, samples of intestine and feces homogenates produced visually distinguishable mixed colonies on BHI agar plates. One to 5% of the total colonies per plate were identified as *L. monocytogenes*. These data demonstrate the ability of the method to recognize colonies formed by target bacteria in the presence of natural background microflora in clinical specimens.

4. Discussion

Light scattering is a fundamental optical process whereby electromagnetic waves deviate from a rectilinear path as a result of non-uniformities in the medium that they traverse. The light scatterometer, BARDOT (Fig. 1) is capable of rapid detection and identification of multiple bacterial pathogens with a high degree of accuracy and is a *sine qua non* for control measures to safeguard the food supply from accidental or deliberate microbial contamination. Automated label-free fingerprinting and identification of bacterial colonies on Petri dish in real time has a chance not only to extend the well-established traditional approach to pathogen identification, but also to substantially impact currently employed protocols owing to the amenability to automation and high throughput. It is evident from the current study that this technology can be expanded to bacterial cultures beyond the ones tested in the present study. In our earlier works we have demonstrated that scattering properties of *Listeria* colonies grown on agar

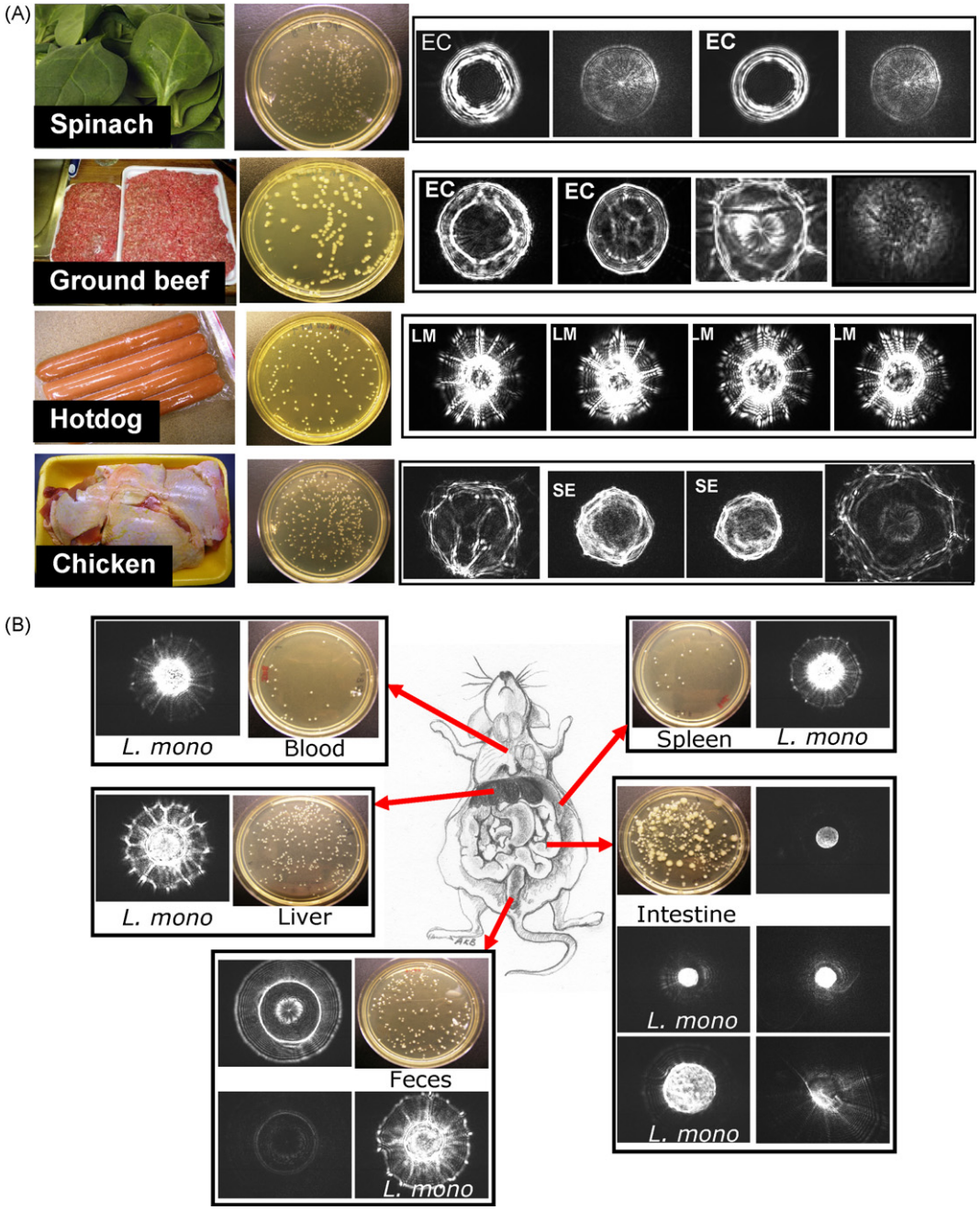

Fig. 5. BARDOT analysis of bacterial colonies from (A) food and (B) clinical specimens. (A) Each food sample was spiked before analysis; spinach and ground beef with *E. coli* O157:H7 (EC) EDL933, hotdog with *L. monocytogenes* (LM) F4244, and chicken with *Salmonella* Enteritidis (SE) PT1, enriched in the respective selective enrichment broth, and surface plated on BHI or selective media agar plates. Scatter images of representative colonies are presented; images marked with initials are identified from the database, while remainders are considered unknown background microflora. (B) Scatter images of representative colonies from *L. monocytogenes* F4244 infected mouse specimens. Bacterial colonies from blood, spleen, liver, intestine, and fecal matter were positive for *Listeria* (though *Listeria* patterns are visibly heterogeneous but maintained key features for positive identification by the image analysis program). Only feces and intestine contained bacteria other than *Listeria*.

surfaces can be used to differentiate the species occurring in food samples (Banada et al., 2007; Bayraktar et al., 2006). This setup was employed as a basis for a semi-automated robust system using automated sample handling and image capture via a monochromatic CCD detector. The method offers a novel, rapid, non-invasive, label-free optical detection capability for bacterial colonies with specificity as high as 100% even in the presence of other significant background. The conceptual simplicity of the measurement process makes the approach especially attractive for integration into highly automated systems.

Images of the unique scatter signatures created upon illumination of individual colonies with the laser beam were treated as “fingerprints” in order to identify the colony-forming microorganism. The analysis system was designed under the assumption that the unique morphotype of the colonies revealed by the scatter patterns may contain enough information to be unequivocally and reproducibly linked with the genotype of the colony-forming organisms. This notion was based on known observations that the formation of bacterial colonies requires coordinated gene expression, regulated cell differentiation, autoaggregation, and

intercellular communication (Daniels et al., 2004; Engelberg-Kulka et al., 2006). Therefore, the colonies can be considered multicellular organisms or "superorganisms," and the data analysis leveraged the consistency, uniqueness, and reproducibility of the colony signatures (Aguilar et al., 2007; Bayraktar et al., 2006; Shapiro, 1998).

Bacterial colonies are comprised of about a billion cells and their morphology (appearance and composition) is broadly dependent on the genetic make-up of the bacteria as well as on the nutrients supplied. Changes in the environmental conditions may also indirectly affect colony morphology, owing to the adaptations to new environment that bacteria achieve (Enos-Berlage and McCarter, 2000; Monk et al., 2004). For example, scatter pattern for same culture was remarkably different when grown on media containing selective antimicrobial agents from the media devoid of selective agents (Fig. 3). Differences in scatter patterns are thought to occur due to the phase variation, which alters expression of certain cell surface–associated components like flagella, membrane proteins, and exopolysaccharides and lipopolysaccharides (Enos-Berlage and McCarter, 2000; Henderson et al., 1999). Those changes may impact the composition of the colony and its resultant scatter properties owing to the modification of cellular arrangement and/or accumulation of bacterial by-products inside the colony dome (Bae et al., 2007; Banada et al., 2007). Our study indicated that there was a significant increase in the total EPS production by pathogenic bacteria than the non-pathogenic species (Fig. 4). Polysaccharide, being a major structural component, is speculated to be an important contributing factor influencing scattering signatures via optical amplitude/-phase modulation (Banada et al., 2007). The other important physical relationship between the colony morphology and observed scatter patterns was the impact of overall colony shape. The semi-transparent colonies act as biological microlenses; therefore the scatter patterns are affected by the elevation of colonies, i.e., convex colonies interact differently with the interrogating laser beam than do flat colonies.

Pathogenic bacteria surviving in food are often subjected to many stresses such as acid, osmotic stress and heat during food processing. Often stressed cells have altered physiological properties and are difficult to detect (Hahm and Bhunia, 2006; Lathrop et al., 2008). We demonstrated that the colony patterns of bacterial cultures exposed to osmotic (5% NaCl), acidic (pH 4), and thermal (42 °C) stress were identical to the non-exposed colonies (Fig. S7). The sample preparation strategy employed here allowed resuscitation of stressed cells and provided unambiguous results for stress-exposed pathogens.

We also applied the light scatterometer for detection of pathogens from food and experimentally infected clinical samples. Results showed that the method is able to detect most pathogens at a concentration of as low as 1 cfu/25 g of sample in less than 24 h in the presence of natural background bacteria in most food and clinical specimens (Fig. 5).

Since the proposed technique relies on the biophysical properties of the bacterial colonies, rather than on genetic or biochemical markers, it can be readily adapted to recognize any new forms of the pathogens of interest by simply retraining the classifier on a new set of scatter patterns. This is an important property for biodetection techniques as some infectious agents are characterized by a high mutation rate, making the application of well-established molecular biology techniques problematic owing to their dependence on very specific reagents.

5. Conclusion

The results show that BARDOT is a semi-automatic, novel, non-invasive, label-free detection and identification system for bacterial colonies originated from five genera (*Escherichia, Salmonella, Listeria, Staphylococcus*, and *Vibrio*) and considered a next-generation biological detection tool. A single colony can be identified and differentiated from the pool of other bacterial colonies with very high specificity (up to 100%). BARDOT was able to detect most pathogens at 1 cfu/25 g sample in less than 24 h in presence of background microflora. Drawbacks of this system include its inability to detect colonies growing on membrane filters (typically practiced for water sample testing) and lack of defined scatter signatures for rare but highly transparent colonies. Nevertheless, further expansion of the scatter-pattern database would enable screening of various bacterial pathogens originating not only from food samples, but also from clinical specimens, animals, soil, air, or water. The potential scope of the method as an automated pathogen-recognition tool appears to be limitless.

Acknowledgements

We thank M.G. Johnson and A. Aronson for critical reading, B. Reuhs for assistance with EPS analysis, and G. Lawler for technical editing of the manuscript. The project was supported by funds from the Agriculture Research Service of the US Department of Agriculture project number 1935-42000-035 and the Center for Food Safety Engineering at Purdue University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.08.053.

References

Aguilar, C., Vlamakis, H., Losick, R., Kolter, R., 2007. Curr. Opin. Microbiol. 10 (6), 638–643.
Bae, E., Banada, P.P., Huff, K., Bhunia, A.K., Robinson, J.P., Hirleman, E.D., 2007. Appl. Opt. 46 (17), 3639–3648.
Baldi, P., Brunak, S., Chauvin, Y., Andersen, C.A., Nielsen, H., 2000. Bioinformatics 16 (5), 412–424.
BAM (Bacteriological Analytical Manual), 2008. Food and Drug Administration, Bethesda, MD.
Banada, P.P., Guo, S., Bayraktar, B., Bae, E., Rajwa, B., Robinson, J.P., Hirleman, E.D., Bhunia, A.K., 2007. Biosens. Bioelectron. 22 (8), 1664–1671.
Bayraktar, B., Banada, P.P., Hieleman, E.D., Bhunia, A.K., Robinson, J.P., Rajwa, B., 2006. J. Biomed. Opt. 11 (3), 034006.
Bhunia, A.K., 2006. In: Amass, S., et al. (Eds.), The Science of Homeland Security: Advances in Homeland Security. Purdue University Press, West Lafayette, IN, pp. 109–149.
Bhunia, A.K., 2008. In: Taylor, S. (Ed.), Advances in Food Nutrition Research. Academic Press, London, pp. 1–44.
Bronk, B.V., Li, Z.Z., Czégé, J., 2001. J. Appl. Toxicol. 21 (2), 107–113.
Burges, C.J.C., 1998. Data Min. Knowl. Discov. 2 (2), 121–167.
CDC (Centers for Disease Control and Prevention), 2006. MMWR 55, 1042–1045.
CDC (Centers for Disease Control and Prevention), 2007. MMWR 56, 767–769.
Chang, C.C., Lin, C.J., 2001. LIBSVM: A Library for Support Vector Machines.
Czuprynski, C.J., Faith, N.G., Steinberg, H., 2003. Infect. Immun. 71 (2), 682–689.
Daniels, R., Vanderleyden, J., Michiels, J., 2004. FEMS Microbiol. Rev. 28 (3), 261–289.
Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Anal. Chem. 28 (3), 350–356.
Engelberg-Kulka, H., Amitai, S., Kolodkin-Gal, I., Hazan, R., 2006. PLoS Genet. 2, e135.
Enos-Berlage, J.L., McCarter, L.L., 2000. J. Bacteriol. 182 (19), 5513–5520.
Gerner-Smidt, P., Whichard, J.M., 2007. Foodborne Pathog. Dis. 4 (4), 391–394.
Hahm, B.K., Bhunia, A.K., 2006. J. Appl. Microbiol. 100 (5), 1017–1027.
Haralick, R.M., Shanmuga, K., Dinstein, I., 1973. IEEE Trans. Syst. Man. Cybern. 3 (6), 610–621.
Heaton, J.C., Jones, K., 2008. J. Appl. Microbiol. 104 (3), 613–626.
Henderson, I.R., Owen, P., Nataro, J.P., 1999. Mol. Microbiol. 33 (5), 919–932.
Jaradat, Z.W., Schutze, G.E., Bhunia, A.K., 2002. Int. J. Food Microbiol. 76 (1–2), 1–10.
Jay, M.T., Cooley, M., Carychao, D., Wiscomb, G.W., Sweitzer, R.A., Crawford-Miksza, M., Farrar, J.A., Lau, D.K., O'Connell, J., Millington, A., Asmundson, R.V., Atwill, E.R., Mandrell, R.E., 2007. Emerg. Infect. Dis. 13 (12), 1908–1911.
Khotanzad, A., Hong, Y.H., 1990. IEEE Trans. Pattern Anal. Machine Intell. 12 (5), 489–497.
Kiratisin, P., Fukuda, C.D., Wong, A., Stock, F., Preuss, J.C., Ediger, L., Brahmbhatt, T.N., Fishcher, S.H., Fedorko, D.P., Witebsky, F.G., 2002. J. Clin. Microbiol. 40 (8), 3012–3016.

Klein, E., Smith, D.L., Laxminarayan, R., 2007. Emerg. Infect. Dis. 13 (12), 1840–1846.
Lathrop, A.A., Banada, P.P., Bhunia, A.K., 2008. J. Appl. Microbiol. 104 (3), 627–639.
Ligler, F.S., Taitt, C.R., Shriver-Lake, L.C., Sapsford, K.E., Shubin, Y., Golden, J.P., 2003. Anal. Bioanal. Chem. 377 (3), 469–477.
Lim, D.V., Simpson, J.M., Kearns, E.A., Kramer, M.F., 2005. Clin. Microbiol. Rev. 18 (4), 583–607.
Manning, L., Baines, R.N., Chadd, S.A., 2005. Brit. Food J. 107 (4), 225–245.
Monk, I.R., Cook, G.M., Monk, B.C., Bremer, P.J., 2004. Appl. Environ. Microbiol. 70 (11), 6686–6694.
Mukundan, R., Ramakrishnan, K.R., 1995. Pattern Recognit. 28 (9), 1433–1442.
Nebeker, B.M., Buckner, B., Hirleman, E.D., Lathrop, A., Bhunia, A.K., 2001. Proc. SPIE 4206, 224–234.
Perkins, E.A., Squirrell, D.J., 2000. Biosens. Bioelectron. 14 (10–11), 853–859.
Relman, D.A., Choffnes, E., Lemon, S.M., 2006. Science 311, 1835.
Shapiro, J.A., 1998. Annu. Rev. Microbiol. 52, 81–104.
Swaminathan, B., Gerner-Smidt, P., 2007. Microb. Infect. 9 (10), 1236–1243.
Van Geel-Schutten, G.H., Faber, E.J., Smit, E., Bonting, K., Smith, M.R., Brink, B.T., Kamerling, J.P., Vliegenthart, J.F.G., Dijkhuizen, L., 1999. Appl. Environ. Microbiol. 65 (7), 3008–3014.
Vapnik, V., 1998. Statistical Learning Theory. Wiley, New York.
Wyatt, P.J., 1969. Nature 221 (5187), 1257–1258.
Wyatt, P.J., Phillips, D.T., 1972. J. Theor. Biol. 37 (3), 493–501.