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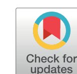
Detection and discrimination of *Shigella sonnei* and *Shigella flexneri* based on vacuolar responses in *Saccharomyces cerevisiae*



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ABSTRACT

This study provided a system for bacteria detection based on a lysosome-like-vacuole response in the yeast *Saccharomyces cerevisiae*. Vacuoles are factors known to activate the immune system in the presence of foreign substances. Here, *Shigella sonnei* and *Shigella flexneri* were exposed to yeast to analyze the alteration of vacuolar enzymes. The ability to detect the bacteria was evaluated by confocal microscopy after exposing and staining vacuoles with LysoTracker. Results showed that the treatment of yeast with these bacteria increased the number of red vacuole-like organelles surrounding yeast nuclei. Thus, vacuole alteration can be used as a biomarker for bacteria detection. Next, the expression of vacuolar enzymes under the influence of bacteria was examined using two-dimensional gel electrophoresis (2-DE) method for screening specific biomarkers for each *Shigella* strain. Finally, the recombinant yeasts that contained biomarkers fused to different fluorescent proteins confirmed the ability of yeast to detect these two *Shigella* strains at concentrations ranging from 10 to 100 CFU/mL.

1. Introduction

Shigella is a species of gram-negative enteric bacteria that causes diseases worldwide, especially in children living in developed countries (Von Seidlein et al., 2006). Shigellosis generated by *Shigella* is an infectious disease that presents with symptoms including diarrhea, fever, abdominal pain and tenesmus. Every year, there are approximately 500,000 cases of shigellosis in United States (US) (Scallan et al., 2011), and in 2013, the average annual occurrence of shigellosis in the US was 4.82 cases per 100,000 persons (Control and Prevention, 2013). Thus, it is necessary to develop a rapid and sensitive sensor for the detection and monitoring of this pathogenic bacteria in harsh conditions and at trace levels.

Traditional methods, including colony counting, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR), have been applied widely to detect pathogens in food (Lindsay et al., 2013; You et al., 2011). Despite the sensitivity and specificity of these techniques, there are disadvantages that limit their use, such as the length of time to diagnosis, inaccuracy of results in harsh conditions, and the complexity of the processes (Das et al., 2014). Recently, many studies have developed novel sensors that utilize surface modification approaches (Chai et al., 2013), immobilized antimicrobial peptides (Mannoor et al., 2010), or lysozymes modified by fluorescein for the detection of gram-positive bacteria (Arabski et al., 2015). In this study, we describe a novel sensor based on the yeast vacuole's response to exposure to two *Shigella* strains. Yeast vacuoles were applied to be used as a multi-function organelles to detect toxic chemicals or antimicrobial agents in previous studies (Nguyen et al., 2014; Yoon et al., 2009). Here, the biomarkers of pathogens were screened and then fused with fluorescent proteins to form recombinant yeasts that can detect each strain. Finally, the presence of these pathogenic bacteria can be quickly detected at a trace level via exclusive fluorescent signals.

2. Materials and methods

2.1. Bacteria culture

Shigella sonnei (ATCC 25931), *Shigella flexneri* (KCTC 2517), *Salmonella chloraesuis* (ATCC 10708), *Salmonella enteritidis* (ATCC 13076), *Salmonella typhimurium* (KTCC 2053) and *Salmonella dublin*

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Table 1
Characteristics of plasmids and oligonucleotides used in this study.

Strain/Plasmid	Relevant genotype	Reference
Plasmid		
pYES2.0	*URA3*	Invitrogen
pYES2::VPS9::GFP	*URA3 expression of VPS9 gene and GFP gene*	This study
pYES2::ENO2::BFP	*URA3 expression of ENO2 gene and BFP gene*	This study
Oligonucleotides		
VPS9-F	GCGAAGCTTATGACTGATG ATGAAAAG	F. primer for pYES2::VPS9
VPS9-R	CTACT TTCTCTGTCA GAAGGATCCAGG	R. primer for pYES2::PRB1
GFP-F	ATAGGATCCGTGAG CAAGGGCGAG GAG	F. primer for pYES2::PRB1::GFP
GFP-R	CTA CTTGTACAGCTCGTCGAATTCAGC	R. primer for pYES2::PRB1::GFP
ENO2-F	GGAGAGCTCATGGCTGTCT CTAAAGTT	F. primer for pYES2::ENO2
ENO2-R	ACCACG GTGACAAGTT GGGATCCTGT	R. primer for pYES2::ENO2
BFP-F	GCGGGATCCGTGAGCAAGGGCGAGGAG	F. primer for pYES2::ENO2::BFP
BFP-R	GACGAGCTGTACAAGTAACTCGAGGAT	R. primer for pYES2::ENO2::BFP

(ATCC 15480) were provided by the Department of Microbiology, Chungbuk National University, Korea and grown in LB medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) at 37 °C and 180 rpm.

2.2. Yeast culture and bacteria exposure

Saccharomyces cerevisiae 2805 (ATCC 208280) were provided by the Korean Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea) and grown in YPD medium (10 g/L yeast extract, 20 g/L bacto peptone and 20 g/L glucose) in a 250-mL flask at 30 °C and 180 rpm. The yeast cells were then exposed to bacteria at different concentrations in 15 min. For studying the alterations of vacuoles, the cells were stained with LysoTracker and then analyzed using a confocal microscope.

2.3. Cytological staining with LysoTracker

S. cerevisiae was grown in YPD medium, rinsed with 1x PBS, and then stained with 100 nM LysoTracker Red DND-99 in distilled water for 5 min at 30 °C. The samples were studied using a confocal laser-scanning microscope (LSM 510 META) at an excitation wavelength of 543 nm. Images were generated with the Zeiss LSM image Browser (Yoon et al., 2009).

2.4. Vacuole isolation from S. Cerevisiae

To investigate the alteration of vacuolar proteins in *S. cerevisiae*, yeast cells were exposed to the minimum detectable concentration of bacteria (20 CFU/mL) in 15 min. Vacuoles were isolated after exposure. After harvesting exponential growth cells, 5 mL of cells were added to 25 mL of Tris-SO_4 buffer (containing 1 mL of 1 M DTT solution per 100 mL) and then incubated for 15 min at 30 °C. The mixture was then centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was discarded, and the pellet was collected and suspended in 5 mL of breaking buffer (containing 4 mL of 1 M Tris-Cl pH 7.4 and 50 mL of 2.4 M sorbitol in a total volume of 200 mL). In the next step, the cells were broken by mixing 1 g of glass beads with the cell mixture before being vortexed 5 times, 30 s each. The mixture was then centrifuged at 500 xg for 5 min before centrifuging the supernatant at 20,000 xg, 4 °C in 30 min. The vacuoles were then collected from the pellet.

2.5. Protein extraction for two-dimensional electrophoresis

Vacuolar proteins were extracted using the method that was described in a previous publication (Nguyen et al., 2014). The protein concentration was determined using a Bradford assay and the supernatants were stored at −70 °C until analyzed by 2-DE.

2.6. Two-dimensional electrophoresis (2-DE) – Silver staining – Spot analysis

The methods for 2-DE, gels silver staining and analysis of protein spots were described in a previous publication (Nguyen et al., 2014).

2.7. DNA isolation, manipulation and transformation

Chromosomal DNA was prepared from *S. cerevisiae* s2805 using a Wizard SV genomic DNA kit (Promega, Madison, US). Plasmid DNA, such as pYES2.0 (Invitrogen, US), pBFP and pEGFP-C1, were prepared from *E. coli* cells using an alkaline lysis technique with a QIA spin Miniprep kit (Qiagen, Germany). DNA modification, analysis by agarose gel electrophoresis, and ligation were performed using standard procedures (Sambrook et al., 1989). PCR was carried out using a T Gradient Thermocycler (Biometra, Germany), *Ex Taq*TM DNA polymerase (Takara Bio Inc., Japan), and chromosomal DNA as the template. The PCR products were purified using a QIA quick PCR purification kit (Qiagen, Germany). The oligonucleotides used for PCR amplification were purchased from Accu Oligo (Bioneer Co., Korea). Table 1 lists the primers used to introduce the base pair changes. The transformation of *E. coli* was performed by electroporation with an Electro Cell Manipulator (BTX Technologies Inc., US), and yeast transformation was performed using the lithium acetate method (Schiestl and Gietz, 1989).

2.8. Construction of pYES2::VPS9::GFP and pYES2::ENO2::BFP in S. cerevisiae

Plasmid pYES2::VPS9::GFP was constructed for expression VPS9 with GFP to create a green fluorescent signal specifically for the detection of *S. sonnei*. The coding regions of the VPS9 gene were amplified from the chromosomal DNA of *S. cerevisiae*, and the GFP (green fluorescent protein) gene was amplified from the pEGFP-C1 by PCR using the primer pairs in Table 1. The PCR products were digested with restriction enzymes HindIII/BamHI and BamHI/EcoRI and then ligated into the plasmid. Yeast transformation was performed by the lithium acetate method[18]. *S. cerevisiae* s2805 was transformed with pYES2::VPS9::GFP to construct a recombinant yeast strain. The empty vector pYES2.0 was transformed into *S. cerevisiae* s2805 to construct the control strain.

Plasmid pYES2::ENO2::BFP was constructed in the same way. The ENO2 gene was amplified from the chromosomal DNA of *S. cerevisiae*, whereas BFP (blue fluorescent protein) was amplified from plasmid pEBFP. The genes were then transformed into *S. cerevisiae* to generate the recombinant yeast with blue fluorescent signal for *S. flexneri* detection.

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