EXAMINING THE GENETIC BASIS FOR ALLYL ISOTHIOCYANATE RESISTANCE IN *E.COLI* O157:H7

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Abstract - The addition of deodorized mustard as a natural antimicrobial has been shown to control *E. coli* O157:H7 viability during dry fermented sausage manufacture. This control has been associated with the ability of *E. coli* O157:H7 to degrade glucosinolates (GSs) present in mustard by myrosinase-like enzyme activity to form lethal isothiocyanates (ITCs). The objective of this work was to investigate the *in vitro* susceptibility of *E. coli* O157:H7 to allyl isothiocyanate (AIT), an ITC, and assess the role of the response regulator gene BaeR in the development of resistance to AIT. The minimum inhibitory concentration (MIC) of AIT toward *E. coli* O157:H7 was 51 ppm. After growing *E. coli* O157:H7 in stepwise increased concentrations of AIT above its MIC, the strain grew at > 200 ppm AIT. The BaeR gene was deleted using a lambda-red recombination system. However, the Δ*baeR* and wild-type strains showed similar MICs toward AIT. Similarly, the MICs of penicillin and erythromycin were not different between both these strains. Thus, the BaeR gene by itself did not influence AIT resistance. Further work will explore the role of the signal transduction histidine protein kinase (BaeS) and multi-drug efflux proteins in the resistance of *E. coli* O157:H7 to AIT.

Key Words – Allyl isothiocyanate glucosinolate, lambda-red system.

I. INTRODUCTION

Escherichia coli O157:H7 has been responsible for several foodborne illness outbreaks associated with fermented meat products [1]. The addition of natural antimicrobials of plant origin, present in some sausage ingredients such as mustard, has shown promise for reducing the viability of *E. coli* O157:H7 during production of dry fermented sausages [2]. This reduction has been associated with the ability of the microorganism to degrade glucosinolates (GSs) present in mustard to form isothiocyanates (ITCs). It is suspected that *E. coli* O157:H7 degrades the GLSs present in deodorized oriental and yellow mustard powders in an attempt to acquire glucose as an energy source [3]. The BaeS/BaeR system serves as a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in many different environmental conditions [4]. Studies have shown that this two-component system is responsible for controlling efflux pump genes and stress pathways [5,6]. The objective of the present work was to investigate the in vitro susceptibility of *E. coli* O157:H7 to allyl isothiocyanate (AIT) and the role of the BaeR gene in the development of artificially stimulated resistance to AIT.

II. MATERIALS AND METHODS

The MIC of allyl isothiocyanate (AIT) against *E. coli* O157:H7 (strain 02:0304) was tested in broth using capped glass tubes. *E. coli* grew in Muller-Hinton broth (MHB) containing AIT concentrations ranging from 26 to 824 ppm, at 35°C and 200 rpm. Bacteria were plated on Violet Red Bile (VRB) agar after 18h and the MIC of AIT was calculated. After determining the MIC of AIT toward *E. coli* O157:H7, this strain was acclimated in concentrations below its MIC (26 ppm) and challenged with progressively increased concentrations of AIT in order to allow its adaptation to higher AIT levels. Genes associated with efflux proteins conferring multiple antibiotic resistance (*mdtA*, *mdtB*, *mdtC*, and *mdtD*), and the two-component regulatory system BaeS/R were amplified using specific primers designed with BioEdit Sequence Alignment Editor software. A lambda red-mediated gene replacement procedure was used to explore the role of BaeR in the resistance of *E. coli* O157:H7 to AIT [7]. The procedure involved two steps. For the first step,

primers were constructed in such a way that they had an internal overlap with the pKD3 chloramphenicol resistance marker (pKD3) and external overlap with the target knockout gene (baeR) (Table 1). Polymerase chain reaction (PCR) was conducted using PCR reaction volumes in 25µl containing 12.5µL Multiplex PCR Mastermix, 0.5µL of primer 1, 0.5µL of primer 2, 12µL bacterial DNA and 9.5µL water. All PCR amplifications were carried out using the following temperature program: initial denaturation at 94ºC for 5 min, 30 cycles at 94ºC for 1 min, annealing temperature for primers at 58ºC, an extension at 72ºC for 1 min, and a final extension step at 72ºC for 10 min. The amplification products were subjected to gel electrophoresis in a 1% agarose gel at 120 V for 60 min. PCR products were gel-purified, digested with *Dpn*I, repurified, and suspended in elution buffer (10 mM Tris, pH 8.0). Electroporation was done using 50 ul of eletrocompentent cells and 4ul of PCR product. Shocked cells were added to 1ml S.O.C medium, incubated for 3h at 39°C, and then plated on LB-Cm to select chloramphenicol resistant transformants. In the second step, the mutant Δ*baeR* was confirmed by PCR using primers that only flanked the target gene (baeR) (Table 1). PCR reactions followed the same parameters as described previously. All primers used in this study were manufactured by UCDNA Services, Faculty of Medicine, University of Calgary (Alberta, Canada). The MIC of AIT toward *E. coli* O157:H7 was determined as previously described to compare the AIT susceptibility of the mutant (Δ*baeR*) and wild-type strain. For controls, sterile 96-well microtitre plates were used to determine the MIC and minimum bactericidal concentration (MBC) of the antibiotics penicillin (β-lactam) and erythromycin (macrolide) against Δ*baeR* and wild-type *E. coli* O157:H7 strains. To each well 50 µL of MHB was added. Then 50 µL of separate antibiotic solutions were added to the first wells and serial two-fold dilutions were made to the desired concentration (0.125 to 256µg/mL), with the last well being discarded. Wells were separately inoculated with 50 µL of each bacterial suspension to give a total volume of 100 µL. Plates were covered and incubated overnight at 35°C. The MIC was considered to be the lowest antimicrobial concentration with no visible growth after 18h. Bacteria were plated on VRB agar to verify the MIC and MBC of the antimicrobials.

III. RESULTS AND DISCUSSION

The MIC of AIT toward *E. coli* O157:H7 was 51 ppm. After being challenged with increased AIT concentrations the wild-type *E. coli* O157:H7 strain was able to grow > 200 ppm AIT, suggesting that the pathogen was able to overcome its inhibitory effects. In order to understand the resistance response expressed by *E. coli* O157:H7, a lambda-red gene-knockout was performed to delete the response regulator BaeR. Previous studies have shown that BaeR modulates the expression of *mdtABC*, which encodes multi-drug exporter systems [5]. Also, the two-component system BaeS/R seems to be associated with the regulation of stress responses in *E. coli* [6]. The deletion of the BaeR gene was confirmed using PCR assays (Fig. 1) and MIC assays were conducted again to compare the AIT resistance of the *E. coli* O157:H7 wild-type and the Δ*baeR* strain. The MIC value of AIT toward both strains was the same. Further, no difference was found in the MIC or MBC of penicillin and erythromycin toward the wild-type and Δ*baeR* strains (Table 2). Thus, the response regulator BaeR seems not to be the sole gene regulating drug resistance in this *E. coli* O157:H7 strain. It is likely that the expression of multi-drug resistance genes in *E. coli* O157:H7 may be modulated by other regulators, enabling this organism to overcome higher concentrations of AIT, as well as penicillin and erythromycin challenges.

Table 1: Primers used here for different PCR protocols

Name	Sequence 5' – 3'
baeR.F	*ATGACCGAGTTACCAATCGA*
baeR.R	*CTAAACGATGCGGCAGGCGT*
	CTAAACGATGCGGCAGGCGTCGGCT
baeRpkD3.F	TCCCAGCGGTAACCGACGCCGTAAA
	GTGTAGGGCTGGAGCTGCTTC
	TATGACCGAGTTACCAATCGACGAA
baeRpkD3.R	AACACACCGCGTATTTTGATCGTGGA
	A**CATATGAATATCCTCCTTAGT**

Italicized letters represent primers that only flank the target gene. Bold letters represent primers that have

internal overlap with the resistance marker (pKD3), while non-bold letters represent primers having external overlap with the target knockout gene (baeR).

Table 2: MIC and MBC of penicillin and erythromycin towards *E. coli* O157:H7 (02:0304)

E. coli O157:H7	MIC (MBC) values (μg/mL)	
	Penicillin	Erythromycin
Wild-type	64 (256)	32 (256)
Δ*baeR*	64 (256)	32 (256)

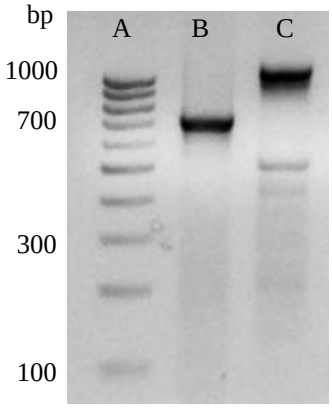

Figure 1: Gel-electrophoresis of PCR amplification products obtained from the knockout of the response regulator BaeR in *E. coli* O157:H7 (strain 02:0304). Lane A: DNA marker. Lanes B: 723bp PCR product from wild-type *E. coli* O157:H7. Lane C: 1000bp from Δ*baeR*.

IV. CONCLUSION

Results from this study showed that the DNA-binding transcriptional regulator (BaeR), by itself, did not influence the resistance of *E. coli* O157:H7 to AIT, penicillin and erythromycin. Further work will examine the role of the signal transduction histidine protein kinase (BaeS) and multi-drug efflux proteins (*mdtA*, *mdtB*, *mdtC*, and *mdtD*) in the resistance-response expressed by *E. coli* O157:H7 to AIT.

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