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Repression of flagella motility in enterohemorrhagic *Escherichia coli* O157:H7 by mucin components

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ABSTRACT

Whole genome-scale transcriptome analysis of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 EDL933 was performed to investigate the influence of mucin components on the EHEC gene expression. Here we report that the 732 candidate genes were differentially expressed by the presence of 0.5% porcine stomach mucin, including the 8 flagella-related genes. Quantitative real-time PCR analyses revealed that the transcription expression of the *flg* genes (encoding the structural components for flagella basal body) was down-regulated by the mucin components. Indeed, bacterial swarming motility was drastically reduced when grown on 0.3% trypton agar plates containing the mucin. These results imply that gastrointestinal (GI) mucin is a possible environmental signal which negatively regulates the flagellation of EHEC O157:H7 in the GI tract.

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1. Introduction

The gastrointestinal (GI) tract is lined by a continuously secreted mucus layer formed by high molecular mass (200–2000 kDa) oligomeric mucin glycoproteins that are composed of a peptide backbone linked to carbohydrates [1–3]. The mucins play an important role as receptors for bacterial adhesins as well as provide mucosal defense against infectious agents [4,5]. Most pathogenic bacteria that colonize the GI tract subvert the mucus barrier. For example, the StcE zinc metalloprotease secreted by enterohemorrhagic *Escherichia coli* (EHEC) is a mucinase [6].

EHEC cause outbreaks of hemorrhagic colitis and hemolytic uremic syndrome in humans [7]. They express many virulence factors, including adhesins, effector proteins and toxins, which are necessary to cause diseases and likely contribute to the extremely low infectious dose [8]. Commensal *E. coli* cells are dispersed throughout the mucus layer overlaying the intestinal epithelium [9]. As an enteric pathogen, intestinal colonization is the first step for EHEC infection. Although bacterial interaction with mucin during the initial infection of EHEC is very important, it has not been well characterized how EHEC competes with GI resident microbiota to colonize the intestine. Only a few molecular studies have been conducted to investigate the interactions between EHEC and mucins [10]. Moreover, any studies on the characterization of the global gene expression in EHEC by the presence of mucin have not been reported yet.

In this study, we investigated the effects of different growth conditions on the motility of the EHEC EDL933 strain. Here we report the flagella repression of *E. coli* O157:H7 by mucin components.

2. Materials and methods

2.1. Strains and culture conditions

We employed the genome sequenced EHEC strain EDL933, which belongs to the O157:H7 serotype. The strain was grown in LB (Luria–Bertani) medium at 37 °C for 18 h. To carry out a first screening of the culture conditions selected for the study, the growth rates of the strains in pure cultures were determined in the presence of individual culture conditions using M9 minimum medium modified by supplementation porcine stomach mucin (0.5% [v/v]) (type III from porcine stomach) (Sigma, USA). Each strain was cultured on M9 minimum medium with glucose (0.4% [v/v]) and sodium bicarbonate (44 mM [v/v]). The porcine mucin was added prior to autoclaving at 121 °C for 20 min.

2.2. RNA isolation

Bacterial RNA extracts were prepared using the Qiagen RNeasy midi-prep kit and RNA Protect Bacteria reagent (Qiagen, Germany), following the manufacturer's instructions for Gram-negative bacteria.

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2.3. Microarray analysis

Bacterial strains were routinely grown in LB broth containing 0.2% (w/v) glucose at 37 °C with aeration. To monitor bacterial growth, the optical density at 600 nm (OD_{600}) was measured by a GeneQuat™ Pro spectrophotometer. Total RNAs from the early stationary phase culture (~0.8 OD_{600}) were isolated using the RNeasy midi kit as the manufacturer's introduction (Qiagen, Germany). The resultant crude RNAs were treated with RQ1 RNase-free DNase I (Promega, USA) to eliminate all the contaminated DNAs. The *E. coli* O157:H7-specific whole genome-scale microarray analysis was performed using OciChip™ (*E. coli* O157 format, Korea) from E-Biogen (Seoul, Korea). The hybridization images were analyzed by GenePix Pro 6.0 (Axon Instruments, CA). The average fluorescence intensity for each spot was calculated and local background was subtracted. All data normalization and selection of fold-changed genes were performed using GeneSpring 7.3.1 (Agilent Technologies, USA). Intensity-dependent normalization (LOWESS) was performed, where the ratio was reduced to the residual of the Lowess fit of the intensity versus ratio curve. The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity.

2.4. Validation of microarray data using quantitative real-time PCR (qRT-PCR)

To confirm significant transcriptional differences between genes in both samples, qRT-PCR was performed on the genes determined by microarrays. The QuantiTect SYBR Green RT-PCR kit (Qiagen, Germany), along with the 7500 Fast Real-time PCR system (Applied Biosystems, Singapore), was used for RT-PCR. The parameters for RT-PCR included 30 min incubation at 50 °C for converting mRNA to cDNA. Subsequent amplification of cDNA was carried out by using an initial cycle of 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The final extension was carried out at 72 °C for 2 min. The reaction conditions for amplification and the parameters for fluorescence data collection were programmed into the Opticon Monitor Software (package 1.4).

2.5. Motility assay

Trypton agar plates containing 0.3% (w/v) agar were used in motility assays. The plates were inoculated with a single colony of each bacterial strain from an overnight culture using a sterile toothpick. Plates were incubated at 37 °C for 24 h. Motility was assessed by examining circular swarms formed by the growing motile bacterial cells. The diameter of each motility halo was measured at 4, 7, 10, and 24 h.

3. Results and discussion

3.1. Bacterial growth characteristics with and without 0.5% porcine stomach mucin

Mucin is a component of mucus which covers most of the surface of the GI tract [1]. Cell-surface mucins extend further than most other cell surface structure and are likely to play an important role in mucosal defense as a barrier [11]. The thickness of the human intestinal mucus barrier has been estimated to centre around 150 μm [12]. In order to cause disease, EHEC must overcome the mucus barrier. However, only a few molecular studies have been conducted to investigate the interactions between EHEC and mucins [6]. Moreover, very little is known regarding the mechanism that EHEC can overcome mucus barrier in the early stage of infection. To address the question whether mucin influences on the growth of *E. coli* O157:H7 EDL933, we determined the bacterial growth in the M9 minimum medium with or without 0.5% porcine stomach mucin. As shown in Fig. 1, *E. coli* O157:H7 EDL933 strain showed a similar growth kinetic in both defined media, implying no obvious growth inhibition by the presence of 0.5% porcine stomach mucin. Therefore, we have used this culture condition for the further experiments.

3.2. Influence on the gene expression of E. coli O157:H7 EDL933 by the presence of mucins

Microbial products and inflammatory cytokines stimulate increased production of mucins by mucosal epithelial cells [13,14]. However, the interactions of bacteria with mucin are not well characterized at the molecular level. *Campylobacter jejuni* utilizes mucin as an environmental cue for the modulation of expression of genes with various functions including colonization and pathogenicity [15]. Several studies have indicated that proteases such as Hap and StcE may be important in the association of bacterial pathogens with the gut epithelium [6,16]. In order to determine the effect of mucin on the gene expression of *E. coli* O157:H7 EDL933, therefore, transcriptome profiles were examined in the absence or presence of 0.5% porcine stomach mucin using a whole genome-scale microarray technique. The results revealed the mucin-mediated regulation of numerous genes. A total of 732 of 6213 genes were regulated by the presence of mucin (at least 2 folds). Among them, the 320 candidate genes were up-regulated whereas the 412 genes were down-regulated when compared to the control without mucin (Supplementary Tables 1 and 2). The significance and differences in transcript levels for all genes are depicted as a scatter plot (data not shown). As shown in Table 1, we observed that several genes related with intestinal adherence were up-regulated. For example, 1 Type III secretion system-related gene (*escR*), 2 curli-related genes (*csgA, crl*), 2 fimrial genes (*fimB*, *fimZ*), and 3 genes in relation with LPS biosynthesis (*waaD*, *waaP*, *waaY*) were differentially expressed by the mucin components (Table 1). Among the subset of the genes identified from the microarray analysis, surprisingly, it was found that the 8 genes associated with flagella biosynthesis were all down-regulated by the presence of mucin (Table 1).

In order to verify the results obtained from the microarray, the qRT-PCR analyses were carried out (see Section 2). The 9 genes,

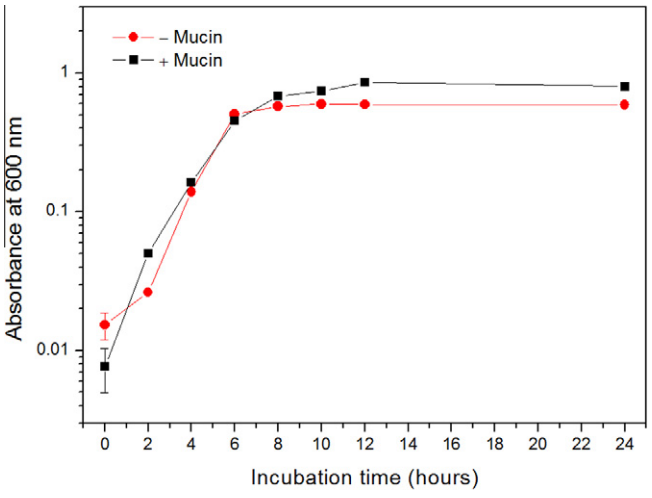

Fig. 1. Bacterial growth with or without 0.5% mucin. The bacterial growth rates were determined in the M9 medium containing 0.4% glucose and 44 mM sodium bicarbonate with or without porcine stomach mucin.

Table 1
A set of the EHEC O157:H7 genes differentially expressed by mucin components (>2 or <2 folds).

Gene ID	Gene Name	Delta fold (vs. no mucin)	Gene product or function
B1042	csgA	+5.6	Curlin major subunit
Z5050	waaD	+5.5	Putative LPS biosynthesis enzyme
Z5910	fimB	+4.4	Fimbrial B protein
ECS5191	ECs5191	+4.2	2′:3′-cyclic-nucleotide 2'-phosphodiesterase
Z0693	fimZ	+3.8	Fimbrial Z protein
ECS0014	ECs0014	+2.9	DnaK
Z5054	waaP	+2.8	Putative LPS biosynthesis enzyme
Z5127	cesD	+2.7	CesD
Z4154	aas	+2.7	2-Acyl-glycerophospho-ethanolamine acyltransferase
Z4966	Z4966	+2.3	Putative fimbrial protein
ECS2075	ECs2075	+2.3	IpaH-like protein
Z3041	rcsA	+2.3	Positive transcription regulator for ctr capsule biosynthesis
ECS2973	ECs2973	+2.2	Shiga toxin I subunit B precursor
Z1629 M	Z1629 m	+2.2	Glycosyl transferase
Z4759	envZ	+2.1	Protein histidine kinase/phosphatase sensor for OmpR
Z5052	waaY	+2.1	Putative LPS biosynthesis protein
B3630	rfaP	+2.1	Lipopolysaccharide core biosynthesis
B1609	rstB	−4.9	Sensor histidine protein kinase (RstA regulator)
Z4582	nanT	−4.9	Sialic acid transporter
ECS4395	ECs4395	−4.9	Putative ARAC-type regulatory protein
B3806	cyaA	−4.8	Adenylate cyclase
B1184	umuC	−4.7	SOS mutagenesis and repair
Z1710	flgA	−4.8	Flagellar biosynthesis
B0241	phoE	−4.7	Outer membrane pore protein E
B0240	crl	−4.6	Transcriptional regulator of cryptic csgA gene for curli surface fibers
Z1716	flgG	−4.6	Flagellar biosynthesis, cell-distal portion of basal-body rod
Z1719	flgJ	−4.4	Flagellar biosynthesis
B0374	yaiU	−4.3	Flagellar protein
Z3678	zipA	−4.3	Cell division protein involved in FtsZ ring
Z5055	rfaG	−4.2	Glucosyltransferase I
Z4541	ftsJ	−4.2	Cell division protein
B2565	recO	−4.1	DNA (gap) repair protein
B1610	tus	−3.9	DNA-binding protein
ECS1668	ECs1668	−3.9	MinE
Z1717	flgH	−3.8	Flagellar biosynthesis, basal-body L (LPS layer) ring protein
Z5047	rfaF	−3.8	ADP-heptose–lps heptosyltransferase II
B2573	rpoE	−3.8	RNA polymerase, sigma-E factor
B0889	lrp	−3.8	Regulator for leucine (or lrp) regulon
Z4198	Z4198	−3.6	Putative regulatory protein for type III secretion apparatus
Z4552	murA	−3.5	First step in murein biosynthesis
Z5135	escR	−3.5	EscR
B0987	ymcD	−3.1	Putative membrane glycoprotein
Z1711	flgB	−3.0	Flagellar biosynthesis, cell-proximal portion of basal-body rod
Z3038	fliP	−2.9	Flagellar biosynthesis
Z5748	mopA	-2.9	GroEL, chaperone Hsp60, peptide-dependent ATPase
ECS3549	ECs3549	−2.5	LuxS
Z1034	dps	−2.4	Global regulator, starvation conditions
B3621	rfaC	−2.1	ADP-heptose
Z1715	flgF	−2.0	Flagellar biosynthesis, cell-proximal portion of basal-body rod

including 3 up-regulated, 3 down-regulated, and 3 equally-expressed genes, were randomly chosen for validation along with 16S rRNA, a control gene that showed no differential expression. We have found that microarray analysis data and qRT-PCR data were identical (data not shown). This is the first study to characterize gene expression changes of EHEC O157:H7 grown in the presence of mucin, compared to bacteria cultured in mucin-free minimal medium.

3.3. Inhibition of flagella motility by the presence of 0.5% porcine stomach mucin

Flagella motility is a critical element in the virulence strategies of many bacterial pathogens [17–19]. The influence of mucin on bacterial motility, specifically on the expression of the genes encoding flagella subunits, has not been well reported. To confirm the results from the micoroarray data on the relationship between mucin and flagella motility, both qRT-PCR and swarming motility were performed. The qRT-PCR analyses showed that the 6 genes associated with flagella biosynthesis were down-regulated

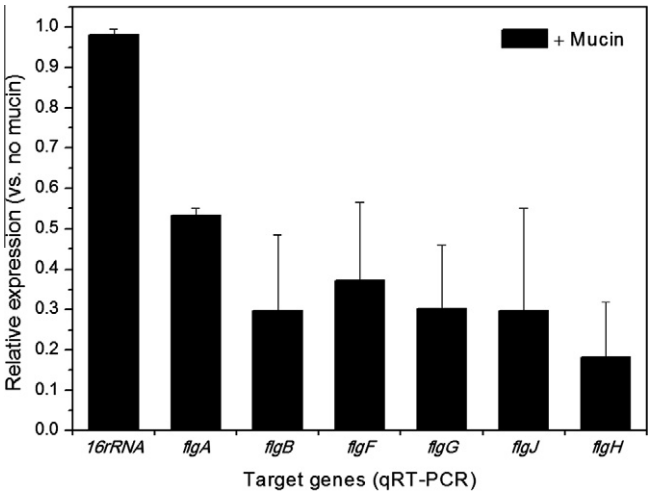

Fig. 2. Transcriptional repression of *E. coli* O157:H7 flagella genes by mucin. The transcriptional expression of the *flg* operon-associated genes was measured by qRT-PCR.

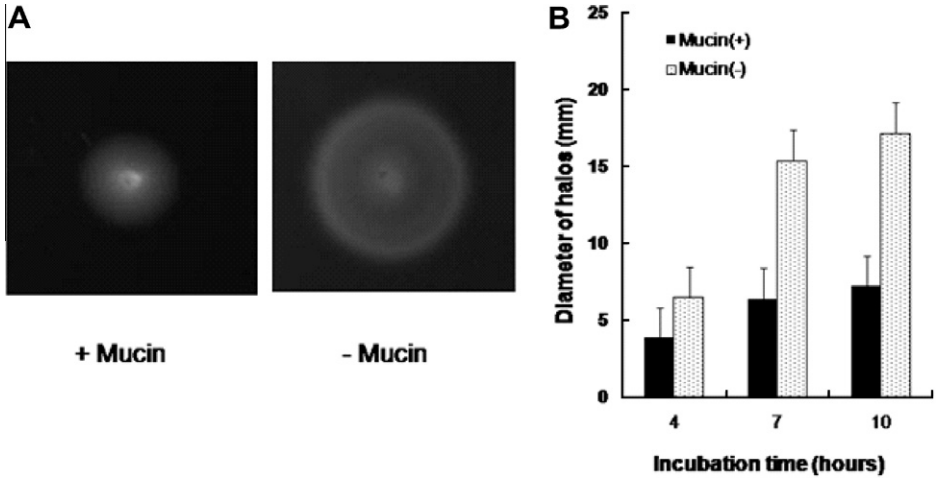

Fig. 3. Swarm motility of *E. coli* O157:H7. (A) *E. coli* O157:H7 EDL933 strain was grown at 37 °C for 24 h on 0.3% Trypton agar plates with or without mucin. (B) The diameters of the motility halos were monitored at the indicated time points. The data were obtained from three independent experiments and represented as the means ± standard errors.

(i.e. *flgABFGH* and *flgJ*) by the presence of mucin (Fig. 2). Consistently, the bacterial swarm motility was significantly reduced when EHEC O157:H7 EDL933 was grown in the presence of mucin (Fig. 3). These results suggest that EHEC motility is negatively regulated in the presence of mucin, possibly due to the transcriptional repression of the flagellation-related genes. Our findings indicate that mucin may directly or indirectly affect the *flg* gene expression (encoding the components of the flagella basal body). In general, it is known that flagella can serve as adhesive appendages in the initial phases of colonization. For example, the motility and the attendant presence of flagella are required for host colonization and induction of inflammation [10]. We cannot explain why bacterial flagellation is blocked by the mucin components in EHEC O157:H7. Since bacterial flagellins are well defined as a strong immune inducer, however, we speculate that such a reduced flagellation may provide an advantage to EHEC during the host GI infection via limiting the host immune response.

In conclusion, our results provide some insights on the interaction between EHEC O157:H7 and the mucin components during the GI infection, especially on the bacterial response to the mucin for the first time in our knowledge. Further study will be needed for elucidating biological significance of the observed flagella repression by the mucin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.041.

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