Shiga Toxin 2-Converting Bacteriophages Occupy *sbcB* Gene as a Primary Integration Site in Bovine-Origined *Escherichia coli* O157:H7 and Non-O157 from Thailand

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Abstract: Shiga toxin-producing *Escherichia coli* (STEC) is an important pathogen defined by the production of Shiga toxins which are encoded in *stx* phage's genome. *E. coli* O157:H7 diversity is affected mainly by prophages integration into the chromosome. Five integration sites have been documented to be frequently integrated by *stx* phages. In this study, 41 bovine-origined *E. coli* O157: H7 and five non-O157 strains isolated from Thailand in different time points ranging from 1998 to 2012, were investigated to observe the integration sites occupied by *stx* phages. Of 41 *E. coli* O157:H7 strains, 40 (97.56%) revealed the stx_2 phages integration into *sbcB* gene. Four of 41(9.76%) exhibited the double lysogenic state (*sbcB* and *yehV*). Moreover, 2 of 41 (4.88%) represented the being of triple lysogen (*sbcB*, *yehV*, and Z2577). Two of five *E. coli* non-O157 strains revealed the insertion in *sbcB* and Z2577 genes while three exhibited the intactness in all five integration sites examined. *wrbA* which have previously been reported to play a role as a primary integration site of *E. coli*, was not found to be occupied by any stx_2 phages. Hence, we firstly suggested that *sbcB* gene is responsible for a primary integration site of stx_2 phages in Thai *E. coli* O157:H7 lineage.

[Sukhumungoon P, Nakaguchi Y. **Shiga Toxin 2-Converting Bacteriophages Occupy *sbcB* Gene as a Primary Integration Site in Bovine-Origined *Escherichia coli* O157:H7 and Non-O157 from Thailand**. *Life Sci J* 2013;10 (4):2334-2340] (ISSN:1097-8135). http://www.lifesciencesite.com. 311

Keywords: stx_2-phage; integration site; O157:H7; *sbcB*; Thailand

1. Introduction

Shiga toxin (Stx) is a major virulence factor of Shiga toxin-producing *Escherichia coli* (STEC) (Mellmann *et al*., 2008). Pathogenic *E. coli* strains capable of producing Stx are able to cause hemorrhagic colitis (HC) (Riley *et al*., 1983) and hemolytic uremic syndrome (HUS) (Karmali *et al*., 1983). Stx is encoded on bacteriophage genome. After infection into the bacteria, they are able to integrate into the chromosome of the bacterial host. The acquisition of *stx* phages affects bacterial host in several aspects. One of them is the increase of pathogenicity of the host as phage carrying toxin gene from one bacterial species to others. Moreover, *stx* phages play a role in bacterial evolution since the different integration sites occupied by phages result in the diverged evolution of bacterial host (Serra-Moreno *et al*., 2007). The major factor affecting the diversification of *E. coli* O157:H7 is prophages (Ooka *et al*., 2009). In *E. coli* O157:H7 Sakai strain, its genome contains extra 1.4 Mb DNA sequences that are not found in the genome of *E. coli* K-12 and most of these sequences are prophages (Ooka *et al*., 2009). The integration of such phages can be occurred through transposition or site-specific recombination and integration sites for phages are found to be housekeeping genes or the region in the close proximity with tRNA genes (Schmidt, 2001). In *E. coli* O157, five integration sites have frequently been documented to be occupied by *stx*-phages, *wrbA* gene (codes for NADH: quinone oxidoreductase) (Patridge and Ferry, 2006), *yehV* gene (codes for transcriptional regulator) (Yokoyama *et al*., 2000), *sbcB* gene (codes for exonuclease I) (Ohnishi *et al*., 2002), *yecE* gene (the function is unknown) (Recktenwald and Schmidt, 2002), and Z2577 gene (codes for oxidoreductase) (Koch *et al*., 2003). When phage infects bacterial cell, one of those sites is responsible as a primary site for phage integration. If the primary site has already been occupied, then phage integrates into secondary target site (Serra-Moreno *et al*., 2007). Several studies reported the preference site for *stx* phages integration, *yehV* gene was shown to be a preferred site integrated by *stx* phages in *E. coli* O157:H7 isolated from Spain (Serra-Moreno *et al*., 2007). The study from Mellmann *et al*. (2008) demonstrated that stx_2-phages prefer to integrate into *yecE* in sorbitol-fermenting *E. coli* O157: NM (non-motile) isolated from patients.

It was noted that stx_2 phages seem to use different insertion sites in different host strains (Serra-Moreno *et al*., 2007). This encourages us to investigate the insertion sites of stx_2 phages in *E. coli* O157:H7 strains obtained in Thailand. Additionally, as mentioned above, the acquisition of *stx*-phages may influence not only the bacterial evolution but also the bacterial pathogenicity. Bovine-origined *E. coli*

O157:H7 isolated in Thailand, have previously been shown to be less virulence because of certain factors, the possession of Q_{21} gene type of *stx*-phage and the defect in stx_2 promoter (Koitabashi *et al*., 2006). Thus, in this study, we attempted to evaluate the insertion sites frequently occupied by *stx* phages in numerous *E. coli* O157:H7 and non-O157 strains isolated from beef and bovine feces in Thailand and we note that the preferred integration site of *stx* phages was probably associated with the pathogenicity of *E. coli* O157:H7 strains isolated in this area.

2. Material and Methods

Bacterial Strains

Forty-one *E. coli* O157:H7 and five non-O157 strains collected from beef and bovine feces in four time points,1998 to 2012, were included in the analysis (Table 2). These *E. coli* O157:H7 and non-O157 strains were obtained by immunomagnetic separation technique. All but one O157:H7 strain contained at least one type of *stx* genes. *E. coli* O157:H7 strain M2 was shown to be stx_1^-, stx_2^-, eae^+ genotype.

Molecular Characterization of the Strains

All virulence genes, stx_1, stx_2, and *eae* including *rfb*O157 and *fliC*H7 genes, were investigated by PCR using the oligonucleotide primers shown in Table 1. The production of Stx was screened by toxin-non-producing (TNP)-PCR, previously described by Koitabashi *et al*. (2006). Confirmation of Stx production was evaluated by reverse passive latex agglutination (RPLA) assay (VTEC-RPLA kit, Denka Seiken Co., Ltd, Japan).

PCR Amplification of the Insertion Locus

A single PCR was performed for amplification of each insertion locus using specific primers (Table 1). Briefly, single colony of each strain was grown in 3 ml Luria-Bertani (LB) broth (Difco, USA) for 16-18 h with 150 rpm shaking at 37°C. One milliliter of culture broth was boiled for 10 min and then immediately immersed on ice for 5 min prior to be centrifuged at 11,000 x *g* for 5 min. The boiled supernatants were decimal diluted using sterile deionized water for the preparation of PCR templates. PCR was performed using Go*Taq* Flexi® system (Promega, USA). The amplicons were analyzed by 1% agarose gel electrophoresis (Invitrogen, USA). The gel was stained with ethidium bromide before image capture using Gel documentation system (Syngene, USA). If the PCR exhibited no amplicon, it was attributed that a prophage was occupied in that particular locus (PCR amplification was not allowed to be amplified because of the large prophage genome) (Figure 1).

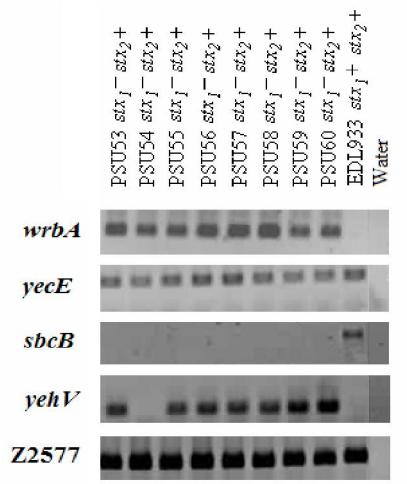

Figure 1. Representatives of *E. coli* O157:H7 and non-O157 illustrated the *stx* phages integration in 5 target genes.

3. Results and Discussion

The genotypic characteristics of all *E. coli* O157:H7 and non-O157 strains in this study were displayed in Table 2. All informations from a total of 41 *E. coli* O157:H7 and five non-O157 strains were analyzed. Forty six O157:H7 and non-O157 strains were examined for the production of Stx by TNP-PCR and RPLA. The results from these two assays were corresponded. The Stx2 titer of all O157:H7 was ≤ 4. However, the Stx2 titer of non-O157 was 16 (PSU1 and PSU17) (data not shown). Stx2 titer of *E. coli* non-O157 strain M7 was below the detection limit (Sukhumungoon *et al*., 2011a). Particularly, PSU5023 produced Stx1 titer equaled to 2,048 (Sukhumungoon *et al*., 2011b).

In this study, 41 *E. coli* O157:H7 and five non-O157 strains isolated from Thailand, including *E. coli* O157:H7 strain EDL933 and *E. coli* strain K-12, were examined for five integration sites reported to be frequently occupied by *stx*-phages (Table 2). In the number of *E. coli* O157:H7 tested, the results demonstrated that *sbcB* gene was occupied by prophages in 97.56% (40 of 41) of the *E. coli* O157:H7 strains (Table 3). This result was not corresponded to the work from Muniesa *et al*. (2004). They examined *E. coli* O157:H7 and other STEC from cattle, beef and other ruminant, demonstrating that *yehV* gene was the most common site to be occupied. It may suggest that the primary integration site for stx_2 phages to enter the lysogenic state in Thai O157:H7 lineage, is *sbcB* gene. Serra-Moreno et al. (2007) reported the integration sites and frequency of each site occupied in *E. coli* non-O157. Z2577 was the most frequent site to be occupied by phages (38% of the strains tested) while *yehV* was followed as a second rank (28%).

Table1. Oligonucleotides used in this study

Oligonucleotide	Sequences (5'to 3')	Gene	Amplicon size (bp)	Reference
EVT-1 EVT-2	CAACACTGGATGATCTCAG CCCCCTCAACTGCTAATA	*stx*$_1$	350	Sukhumungoon *et al*., 2011a
EVS-1 EVS-2	ATCAGTCGTCACTCACTGGT CCAGTTATCTGACATTCTG	*stx*$_2$	404	Sukhumungoon *et al*., 2011a
AE-19 AE-20	CAGGTCGTCGTGTCTGCTAAA TCAGCGTGGTTGGATCAACCT	*eae*	1,087	Gannon *et al*., 1993
O157-F O157-R	CGTGATGATGTTGAGTTG AGATTGGTTGGCATTACTG	*rfb*O157	420	Maurer *et al*., 1999
FlicH7-F FlicH7-R	GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC	*fliC*H7	625	Gannon *et al*., 1997
TNP-f1 TNP-r1	CCATGAGCAAATGATGATTG TTTAGTTCTCTTATGCCCAC	TNP-A	458	Koitabashi *et al*., 2006
TNP-f2 TNP-r2	CTAAATTCATGGAGAGCGTG TTAACGTCAGGCACAAAGAG	TNP-B	694	Koitabashi *et al*., 2006
TNP-f3 TNP-r2	AACCGGAAACGTGTAGAG TTAACGTCAGCACAAAGAG	TNP-C	268	Koitabashi *et al*., 2006
TNP-f4 TNP-r3	GAACATATCAAAATCAGGC GGGAATAGGATACCGAAG	TNP-D	549	Koitabashi *et al*., 2006
*wrb*A1 *wrb*A2	ATGGCTAAAGTTCTGGTG CTCCTGTTGAAGATTAGC	*wrbA*	600	Toth *et al*., 2003
EC10 EC11	GCCAGCGCCGAGCAGCACAATA GGCAGGCAGTTGCAGCCAGTAT	*yecE*	400	DeGreve *et al*., 2002
*sbc*B1 *sbc*B2	CATGATCTGTTGCCACTCG AGGTCTGTCCGTTTCCACTC	*sbcB*	1,800	Ohnishi *et al*., 2002
Primer A Primer B	AAGTGGCGTTGCTTTGTGAT AACAGATGTGTGGTGAGTGTCTG	*yehV*	340	Shaikh and Tarr, 2003
Z2577F Z2577R	AACCCCATTGATGCTCAGGCTC TTCCCATTTTACACTTCCTCCG	Z2577	909	Koch *et al*., 2003

In this study, five *E. coli* non-O157 strains were also monitored for prophages insertion sites occupancy. *E. coli* non-O157 strain PSU1 was found to be occupied at Z2577 gene by a prophage. While, non-O157 strain PSU17 revealed that *sbcB* gene was occupied (Table 3). The remaining strains, M7, PSU5023, and PSU5030, revealed the intactness in all five genes examined. Although the results of integration site occupancy in *E. coli* non-O157 in Thailand were obtained, they cannot be concretely attributed that Z2577 and *sbcB* genes are always responsible for prophages integration because the number of the strains examined were not high. In addition to the intactness of five integration sites of three non-O157 strains (M7, PSU5023 and PSU5030), in which they appeared *stx*$^+$, this suggested other genes which have the potentiality to be responsible for *stx*-phages occupancy. Alternatively, they may not be a lysogenic. It was noted that *E. coli* K-12 displayed a prophage insertion in *sbcB* gene (Table 3). This was not surprising because *E. coli* strain K-12 has previously been reported to possess 11 prophages and prophage-like elements (Ohnishi *et al*., 2001).

Owing to the possession of several target sites for the phages integration in *E. coli* O157, double lysogen can be frequently found (LeJeune *et al*., 2004; Serra-Moreno *et al*., 2007; Sukhumungoon *et al*., 2011b). Serra-Moreno et al. (2007) showed that 40 of 168 STEC strains (23.81%) examined displayed more than 1 site occupied by prophages and in these 40 STEC, 21 belong to O157:H7 serogroup. Based on our results, we found a similar phenomenon. Four *E. coli* O157:H7 strains showed the characteristic of double lysogen, strains M10, M17, PSU54, and Thai-12, while two were found to be the triple lysogens (Thai-1 and Thai-13) (Table 3). The presence of triple prophages in this study was also not surprising because several studies have documented that STEC were the carriers of multiple prophages (Allison *et al*., 2003; Garcia-Aljaro *et al*., 2005). Contrary, one *E. coli* O157:H7 strain M2 showed undetectable insertion site occupied. This result was consistent with the genotypic characteristics of this bacterial strain, *stx*$_1^-$, *stx*$_2^-$. Thus, this may resulted from the lack of *stx* phages integrated in all five sites tested in this study (Table 3).

Table 2. *E. coli* O157:H7 and non-O157 strains used in this study

Strain	Origin	Year of isolation	Molecular trait						Reference
			stx1	*stx2*	*eae*	*rfbO157*	*fliC*H7	[a]TNP-PCR	
PSU1	Beef	2012	+	+	-	-	-	-	This study
PSU2	Beef	2012	-	+	+	+	+	+	
PSU3	Beef	2012	-	+	+	+	+	+	
PSU4	Beef	2012	-	+	+	+	+	+	
PSU5	Beef	2012	-	+	+	+	+	+	
PSU6	Beef	2012	-	+	+	+	+	+	
PSU17	Beef	2012	-	+	-	-	-	-	
M1	Beef	2008	-	+	+	+	+	+	Sukhumungoon *et al*., 2011a
M2	Beef	2008	-	-	+	+	+	+	
M3	Beef	2008	-	+	+	+	+	+	
M4	Beef	2008	-	+	+	+	+	+	
M5	Beef	2008	-	+	+	+	+	+	
M6	Beef	2008	-	+	+	+	+	+	
M7	Beef	2008	+	+	-	-	-	-	
M8	Beef	2008	-	+	+	+	+	+	
M9	Beef	2008	-	+	+	+	+	+	
M10	Beef	2008	-	+	+	+	+	+	
M11	Beef	2008	-	+	+	+	+	+	
M12	Beef	2008	-	+	+	+	+	+	
M13	Beef	2008	-	+	+	+	+	+	
M14	Beef	2008	-	+	+	+	+	+	
M15	Beef	2008	-	+	+	+	+	+	
M16	Beef	2008	-	+	+	+	+	+	
M17	Beef	2008	-	+	+	+	+	+	
M18	Beef	2008	-	+	+	+	+	+	
M19	Beef	2008	-	+	+	+	+	+	
M20	Beef	2008	-	+	+	+	+	+	
M21	Beef	2008	-	+	+	+	+	+	
PSU5023	Beef	2011	+	-	-	-	-	-	Sukhumungoon *et al*., 2011b
PSU5026	Beef	2011	-	+	[b]ND	+	+	+	
PSU5027	Beef	2011	-	+	ND	+	+	+	
PSU5028	Beef	2011	-	+	ND	+	+	+	
PSU5029	Beef	2011	-	+	ND	+	+	+	
PSU5030	Beef	2011	-	+	ND	-	-	-	
PSU53	Beef	2012	-	+	+	+	+	+	This study
PSU54	Beef	2012	-	+	+	+	+	+	
PSU55	Beef	2012	-	+	+	+	+	+	
PSU56	Beef	2012	-	+	+	+	+	+	
PSU57	Beef	2012	-	+	+	+	+	+	
PSU58	Beef	2012	-	+	+	+	+	+	
PSU59	Beef	2012	-	+	-	+	+	+	
PSU60	Beef	2012	-	+	+	+	+	+	
Thai-1	Beef	1998	+	+	+	+	+	+	Vuddhakul *et al*., 2000
Thai-2	Beef	1998	-	+	+	+	+	ND	
Thai-12	Beef	1998	-	+	+	+	+	+	
Thai-13	Bovine fece	1998	+	+	+	+	+	+	
EDL933	Clinical	1982	+	+	+	+	+	ND	O'Brien *et al*., 1983
K-12	Clinical	1922	-	-	-	-	-	ND	Lederberg, 1951

[a]TNP-PCR, Toxin-non-producing PCR
[b]ND, No data

Table 3. Insertion sites occupancy in *E. coli* O157:H7 and non-O157 strains isolated from Thailand

Strains tested	No. of strains									
	Carrying stx_1	Insertion loci					[d]Two insertion sites occupied	[e]Three insertion sites occupied	No detectable insertion site occupied	[a]Total
		wrbA	*yecE*	*sbcB*	*yehV*	Z2577				
O157:H7	2	0	0	[b]40	6	3	4	2	1	41
[c]Non-O157	3	0	0	1	0	1	0	0	3	5
O157:H7 EDL933	1	1	0	0	1	0	1	0	0	1
K-12 (DH5α)	0	0	0	1	0	0	0	0	0	1

[a] Total of tested strains
[b]34 of 40 *E. coli* O157:H7 strains exhibited only *sbcB* occupancy
[c]PSU1 was occupied by prophage at Z2577; PSU17 was occupied by prophage at *sbcB*.
[d]Strains M10, M17, PSU54, and Thai-12, integrated in *sbcB* and *yehV*
[e]Strains Thai-1 and Thai-13, integrated in *sbcB*, *yehV*, and Z2577

The integration by stx_2 phages depends on the host strains and the locus availability (Serra-Moreno *et al*., 2007). The sequence of phage attachment site and the properties of integrase gene, were also reported to play a pivotal role in integration processes (Rutkai *et al*., 2006). In this study, the cause that almost stx_2 phages integrated into *sbcB* gene of *E. coli* O157:H7 was not resulted from the *wrbA* and *yehV* unavailability because of the intactness in these genes. It was thought that stx_2 phages probably possess the specific attachment site to *sbcB* gene in Thai *E. coli* O157:H7 lineage. Additionally, according to the cumulative informations about genotype of *E. coli* O157:H7 collected in past fifteen-year round time, the phages distributing in Thai area were closely related.

In conclusion, we hypothesized that *sbcB* gene plays a role as the primary site for stx_2 phage integration in Thai *E. coli* O157:H7 lineage. Moreover, although certain factors were reported to affect the pathogenicity of *E. coli* O157:H7 in Thailand, it is noteworthy that the integration of stx_2 phage in these *E. coli* O157:H7 may be one of the potential factors to decrease the pathogenicity of the bacterial host. *sbcB* gene codes for 3'to 5' exonuclease, Exo I, which suppresses the illegitimate recombination by cleaving single-stranded DNA overhang at 3' end (Yamaguchi *et al*., 2000) and leads to the decrease in DNA rearrangement such as deletion, insertion or chromosomal translocation. The integration of prophages in this gene might affect the genetic stability and evolution of *E. coli* O157:H7 and, plausibly, resulting in low virulence in the lineage of *E. coli* O157:H7 isolated from Thailand.

Acknowledgements:

This work was supported in part by the fund from Faculty of Science, Prince of Songkla University. We thank Prof. Dr. Varaporn Vuddhakul for valuable suggestion on our article.

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20/11/2013