Genomic comparison of the O-antigen biosynthesis gene clusters of *Escherichia coli* O55 strains belonging to three distinct lineages

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Typical enteropathogenic *Escherichia coli* (EPEC) O55:H7 is regarded as the closest relative of enterohaemorrhagic *E. coli* (EHEC) O157:H7. Both serotypes usually express the γ 1 intimin subclass and trigger actin polymerization by the Tir-TccP pathway. However, atypical O55:H7 strains capable of triggering actin polymerization via the Tir-Nck pathway have recently been identified. In this study, we investigated the genotypic differences and phylogenetic relationships between typical and atypical O55:H7 strains. We show that the atypical O55:H7 strains, which express the θ intimin subclass and lack both *tccP* and *tccP2*, belong to an *E. coli* lineage distinct from the typical O55:H7 and from the EPEC O55:H6, which also uses the Tir-Nck actin polymerization pathway. We conducted genomic comparisons of the chromosomal regions covering the O-antigen gene cluster and its flanking regions between the three O55 lineages by RFLP analysis of PCR products and DNA sequencing analysis of about 65 kb chromosomal regions. This unexpectedly revealed that horizontal transfer of large fragments (≥ 40 kb) encoding the O55-antigen gene cluster and part of the neighbouring colanic acid gene cluster was involved in the emergence of the three O55 *E. coli* lineages. The data provide new insights into the mechanisms involved in the generation of a wide variety of O-serotypes in Gram-negative bacteria.

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INTRODUCTION

The O-antigen constitutes the outermost part of the LPS present in the outer membrane of Gram-negative bacteria.

Abbreviations: A/E, attaching and effacing; CGH, comparative genomic hybridization; EAEC, enteroaggregative *Escherichia coli*; EAF, enteropathogenic *Escherichia coli* adherence factor; EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; HGT, horizontal gene transfer; IS, insertion sequence; LEE, locus of enterocyte effacement; NJ, neighbour-joining; T3SS, type III secretion system; SNP, single nucleotide polymorphism.

The GenBank/EMBL/DBJ accession numbers for the sequences reported in this paper are AB334576–AB334719 (housekeeping genes), AB334558–AB334567 (*eae*), AB355995–AB356000 (*tccP*), AB356001 (*tccP2*), AB334568–AB334575 (*fliC*) and AB353131–AB353133 (O55-antigen gene clusters and their flanking regions). The GEO accession number for the microarray data reported in this paper is GSE8889.

A supplementary figure and table are available with the online version of this paper.

The chemical composition and structure of O-antigen exhibit high levels of variation even in a single species. Thus, together with flagellin (H antigen) and capsule (K antigen), O-antigen is widely used as a surface marker for serotyping of various Gram-negative bacteria. In *Escherichia coli*, more than 170 O-antigens have so far been identified (Scheutz *et al.*, 2004). In general, most genes required for O-antigen biosynthesis are clustered on the *E. coli* chromosome between the colanic acid biosynthesis gene cluster and the histidine biosynthesis (*his*) operon (Reeves *et al.*, 1996). The organization of O-antigen biosynthesis gene clusters is O-antigen-specific. Sequence comparisons in many bacterial species and strains have shown that the variation between O-antigens has been generated by horizontal transfer of a part or the entire O-antigen gene clusters (Cunneen & Reeves, 2007; Curd *et al.*, 1998; Fegan *et al.*, 2006; Sugiyama *et al.*, 1998; Wang *et al.*, 2002; Xiang *et al.*, 1994). Thus, the serotype of a strain correlates to some extent with its phylogeny, and often provides valuable information on its pathotype.

Enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) are important enteric pathogens for humans. EPEC is the most common bacterial cause of prolonged diarrhoea in infants in unindustrialized countries (Donnenberg *et al.*, 1997; Nataro & Kaper, 1998), while EHEC, which causes diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome, is one of the most important intestinal pathogens in industrialized countries (Kaper, 1998; Nataro & Kaper, 1998). EPEC and EHEC colonize the intestinal epithelia via attaching and effacing (A/E) lesions (Nataro & Kaper, 1998), which are characterized by the degeneration of the epithelial cell brush border microvilli and the formation of actin-rich pedestals within the host enterocytes beneath the adherent bacteria (Nataro & Kaper, 1998). Most of the genes required to induce A/E lesions are located on the locus of enterocyte effacement (LEE) pathogenicity island (Elliott *et al.*, 1998; Mellies *et al.*, 1999), which encodes the outer-membrane adhesin intimin, a type III secretion system (T3SS), transcriptional regulators, and several T3SS effector proteins including the translocated intimin receptor (Tir).

LEE-positive strains can be classified into several subtypes based on sequence variation within LEE genes (Blanco *et al.*, 2006; Lacher *et al.*, 2006; McGraw *et al.*, 1999; Ogura *et al.*, 2007). Variation in the *eae* gene, which encodes intimin, has led to classification of more than 25 intimin subtypes (Lacher *et al.*, 2006). Based on the sequence variation in Tir, the protein can be classified into two subtypes (DeVinney *et al.*, 1999): Tir Y-P, which contains a tyrosine residue (Y474 in EPEC E2348/69) that is phosphorylated by host tyrosine kinases (Kenny, 1999) and uses Nck to trigger actin polymerization (Gruenheid *et al.*, 2001), and

Tir-S, which lacks a Y474 equivalent and typically triggers actin polymerization by the TccP pathway (Garmendia *et al.*, 2004). The former is represented by the *tir* gene of EPEC O127:H6 strain E2348/69 (thus referred to as *tir*_{E2348/68}) and the latter by that of EHEC O157:H7 strain Sakai (*tir*_{Sakai}) (Ogura *et al.*, 2007). Previous studies have shown that *tir*_{E2348/68} is widely distributed in various EPEC and EHEC lineages (Ogura *et al.*, 2007; Ooka *et al.*, 2007), while *tir*_{Sakai} was found mainly in EHEC O157:H7 and its close relatives O55:H7 strains (Garmendia *et al.*, 2005).

Most O55:H7 strains are classified as EPEC (Pupo *et al.*, 1997; Whittam *et al.*, 1993), and the O157:H7 lineage is known to have emerged from an O55:H7-like EPEC ancestor by shifting the O-antigen from O55 to O157 and acquiring Shiga toxin (Stx1 and/or Stx2)-converting phages and a large virulence plasmid (pO157) (Wick *et al.*, 2005). Therefore, O55:H7 and O157:H7 strains have a very similar or nearly identical genetic backbone. In fact, similarly to EHEC O157:H7, most O55:H7 strains possess an LEE island encoding γ 1-intimin and Tir_{Sakai} (Garmendia *et al.*, 2005). However, in a recent study (Garmendia *et al.*, 2005), a few O55:H7 strains were found to encode Tir_{E2348/68}. The aim of this study was to investigate the phylogenetic relationships and genotypic differences between typical O55:H7 strains expressing Tir_{Sakai}, atypical O55:H7 strains expressing Tir_{E2348/68} and EPEC O55:H6 strains, which are also known to express Tir_{E2348/68} (Ogura *et al.*, 2007).

METHODS

Bacterial strains and preparation of genomic DNA. The bacterial strains used in this study are listed in Table 1. The sequenced EHEC

Table 1. Characteristics of *E. coli* strains used in this study

Strain	Serotype	Country of origin	<i>tir</i> type	<i>eae</i> type	<i>tccP</i> (bp)	<i>tccP2</i> (bp)	EAF plasmid	<i>bfpA</i>	<i>stx1</i>	<i>stx2</i>	<i>irp2</i>	Reference or source*
TB182A	O55:H7	USA	<i>tir</i> _{Sakai}	γ 1†	+	(1,014)†	—	—	—	—	—	Bokete <i>et al.</i> (1997)
DEC5d	O55:H7	Sri Lanka	<i>tir</i> _{Sakai}	γ 1†	+	(873)†	—	—	—	—	—	Reid <i>et al.</i> (2000)
WC211	O55:H7	Thailand	<i>tir</i> _{Sakai}	γ 1†	+	(1437)†	—	—	—	—	—	Stuart Knutton
ICC57	O55:H7	Chile	<i>tir</i> _{Sakai}	γ 1†	+	(873)†	—	—	—	—	—	Luis Trabulsi
5905	O55:H7	USA	<i>tir</i> _{Sakai}	γ 1†	+	(873)†	+	(1176)†	—	—	+	Reid <i>et al.</i> (2000)
12646	O55:H7	Japan	<i>tir</i> _{Sakai}	γ 1†	+	(873)†	—	—	—	+	—	NIID
WC416	O55:H7	Thailand	<i>tir</i> _{E2348/69}	$\theta\gamma$ †	—	—	—	—	—	—	+	Stuart Knutton
ICC58	O55:H7	Brazil	<i>tir</i> _{E2348/69}	θ †	—	—	—	—	—	—	+	Luis Trabulsi
ICC219	O55:H6	Brazil	<i>tir</i> _{E2348/69}	α †	—	—	+	+	—	—	—	Luis Trabulsi
ICC221	O55:H6	Brazil	<i>tir</i> _{E2348/69}	α †	—	—	+	+	—	—	—	Luis Trabulsi
ICC222	O55:H6	Brazil	<i>tir</i> _{E2348/69}	α †	—	—	+	+	—	—	—	Luis Trabulsi
Sakai	O157:H7	Japan	<i>tir</i> _{Sakai}	γ 1	+	(1014)	+	(1034)‡	—	—	+	Hayashi <i>et al.</i> (2001)

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†These sequences were determined in this study.

‡Pseudogene.

O157:H7 strain RIMD 0509952 (referred to as O157 Sakai) (Hayashi *et al.*, 2001) was used as a reference strain in long PCR and microarray experiments. The bacterial strains were grown to stationary phase at 37 °C in Luria–Bertani broth. Genomic DNA was purified using the Genomic-tip 100/G and Genomic DNA buffer set (both from Qiagen) according to the manufacturer's instructions.

Subtyping of *tir* genes and detection of pathotype-associated genes. Subtyping of *tir* genes into *tir*_{E2348/68} or *tir*_{Sakai} was done by PCR using allele-specific primers as described previously (Ogura *et al.*, 2007). Detection of the nine pathotype-associated genes listed in Table 2 was also done by PCR. All PCRs were performed according to the protocols described previously (see references in Table 2).

Sequencing of *eae*, *tccP*, *tccP2*, *fliC* and 15 housekeeping genes. The entire coding regions of the *eae*, *tccP*, *tccP2* and *fliC* genes, and internal regions of 15 housekeeping genes (*arcA*, *aroE*, *aspC*, *clpX*, *cyaA*, *dnaG*, *fadD*, *grpE*, *icdA*, *lysP*, *mdh*, *mtlD*, *mutS*, *rpoS* and *uidA*) were amplified and sequenced for each test strain. Primers used for PCR and sequencing analyses of the *eae*, *tccP*, *tccP2* and *fliC* genes are listed in Table 2. PCR amplification was performed with an EX taq PCR kit (Takara Bio) by 30 amplification cycles of denaturation for 30 s at 96 °C, annealing for 30 s at 55 °C and primer extension for 60 s at 72 °C. Primers and PCR conditions for the analyses of 15 housekeeping genes were set up according to the protocol provided by the multi-locus sequence typing database for pathogenic *E. coli* (EcMLST: <http://shigatox.net/mlst>).

Phylogenetic analysis. Multiple alignments of sequences were constructed by using the CLUSTAL W program (Thompson *et al.*, 1994) in the MEGA3 software (Kumar *et al.*, 2004), and then neighbour-joining (NJ) trees were generated by using Tamura–Nei model. A bootstrap test with 1000 replicates was used to estimate the confidence of the branching patterns of the trees.

Microarray analysis. The protocol of comparative genomic hybridization (CGH) analysis using an O157 Sakai oligoDNA microarray has been described previously (Ogura *et al.*, 2006). Briefly, probes were prepared for all protein-coding genes on the O157 Sakai genome (5447 genes in total). Probes were mostly 60-mer in length and two probes were prepared for each gene. Repeated genes with almost identical sequences (542 genes in total) were grouped into 151 repeated gene families, and each family was represented by a single probe. Three micrograms of genomic DNA from the reference strain (O157 Sakai) and each test strain were used to generate Cy5- and Cy3-labelled samples, respectively, and cohybridized on a single array. For each test strain, DNA labelling and hybridization were performed twice independently. Fluorescence intensities of the spots were collected using the ArrayVision 8.0 software (Imaging Research). After excluding low-quality signals with lower signal intensity and spotting abnormalities, the signal intensities of each spot were corrected by subtracting the local background, and subjected to the 'presence or absence' determination using the array-based genotyping software GACK (Kim *et al.*, 2002). Finally, the presence or absence of each gene was determined according to the judgments of each spot in two independent hybridizations as described previously (Ogura *et al.*, 2006). Hierarchical cluster analysis was performed using the Cluster 3.0 software (de Hoon *et al.*, 2004), and the result was visualized using the Java TreeView software (Saldanha, 2004).

Long PCR and RFLP analyses. The O55-antigen gene cluster and part of the *his* operon in the O55:H7 strain TB182A have been sequenced previously (Wang *et al.*, 2002). Based on the sequences of TB182A (accession no. AF461121) and O157 Sakai (BA000007), eight pairs of PCR primers were designed to amplify the 85.6 kb chromosomal region covering the O55-antigen gene cluster and its flanking regions (Table 2, Fig. 2). Each segment to be amplified

(7.8–19.0 kb) overlapped its neighbours by 21–350 bp. Some primers used were previously designed for the whole-genome PCR scanning analysis of O157 strains (Ohnishi *et al.*, 2002). The protocol for long PCR amplification was also described previously (Ohnishi *et al.*, 2002). For the RFLP analysis of long PCR products, PCR products were purified with a QIAquick PCR purification kit (Qiagen), digested with *Hae*III (Takara Bio), and analysed by agarose gel electrophoresis.

Sequence analysis of O55-antigen gene clusters and their flanking regions. Each long PCR product used for the PCR-RFLP analysis was sheared by sonication and their random shotgun libraries with 1.2–1.7 kb inserts were constructed using the pTS1 vector (O'Connell *et al.*, 1993). Each clone was sequenced with universal primers T7 and T3 using an ABI PRISM 3100 automated sequencer (Applied Biosystems). Sequence data were assembled by the Sequencher software, ver. 4.2.2 (Gene Codes). The open reading frame finder program in the *in silico* MolecularCloning Genomic Edition, version 1.4.6 (*in silico* biology, Kanagawa, Japan) was used for the initial gene finding and annotation. Final annotation was manually done according to the results of database searches using the BLASTP program (Altschul *et al.*, 1997). Sequence alignments and comparisons were performed by the Sequencher and the CLUSTAL W programs.

RESULTS

Subtyping of Tir, TccP/P2 and intimin

Of the eight O55:H7 strains examined (Table 1), six (TB182A, DEC5d, WC211, ICC57, 5905 and 12646) possessed *tir*_{Sakai}, while two (WC416 and ICC58) possessed *tir*_{E2348/68} (Garmendia *et al.*, 2005). All the O55:H7 strains with *Tir*_{Sakai} were *tccP* positive (strain 5905 was also *tccP2* positive) and possessed the *eae* gene (2805 bp) encoding γ 1 subclass intimin (Int- γ 1), which was almost identical to the *eae* gene of O157 Sakai, with at most three nucleotide differences. In contrast, the two O55:H7 strains with *Tir*_{E2348/68} were *tccP* and *tccP2* negative and possessed an *eae* gene (2808 bp) encoding the θ subclass intimin (Int- θ), which was almost identical to the *eae* gene of EHEC O111:H8 strain DEC8b (accession no. AF449420), with a single nucleotide difference. Although the intimin of strain ICC58 had been classified as Int- γ in several previous studies where PCR-based intimin-typing systems were employed, the sequence analysis clearly indicated that strain ICC58 encodes Int- θ . On the basis of their virulence gene combination, the eight O55:H7 strains examined here were divided into two subgroups referred to as O55:H7/Int- γ 1 and O55:H7/Int- θ , respectively. The three O55:H6 strains with *Tir*_{E2348/68} were *tccP* and *tccP2* negative, and possessed the *eae* gene (2820 bp) encoding the α subclass intimin (Int- α), which was almost identical to the *eae* gene of EPEC O127:H6 strain E2348/69 (accession no. AF022236), with three nucleotide differences.

Other virulence gene profiles

The presence of other virulence genes known to be associated with EPEC, EHEC or enteroaggregative *E. coli*

Table 2. Primers used in this study

Locus	Primer	Sequence	Reference
PCR and sequencing:			
N-terminal part of <i>eae</i>	cesT-F9	TCAGGGAATAACATTAGAAA	Hyma <i>et al.</i> (2005)
	eae-R3	TCTTGTGCGCTTTGGCTT	
C-terminal part of <i>eae</i>	eae-F1	ACTCCGATTCTCTGGTGAC	Hyma <i>et al.</i> (2005)
	escD-R1	GTATCAACATCTCCCGCCCA	
<i>tccP</i>	tccP-SF	GATGAGTATTGCATCGAGTGTC	Ogura <i>et al.</i> (2007)
	tccP-SR	CGGTAAGTGTGAGGTCAGAGC	
<i>tccP2</i>	tccP2-Sfa	AAACGGATAAATAAGACTATCC	Ogura <i>et al.</i> (2007)
	tccP2-SRa	AATAACCGGTAACTGTCAGGTC	
<i>fliC</i>	1575	GGGTGGAAACCAATACG	Wang <i>et al.</i> (2000)
	1576	GCGCATCAGGCAATTTGG	
(Internal sequencing)			
N-terminal part of <i>eae</i>	SK1	CCCGAATTCGGCACAAGCATAAGC	Karch <i>et al.</i> (1993)
C-terminal part of <i>eae</i> (Int- α)	LP2	CCCGAATTCCTTATTTACACAAGTGGC	Schmidt <i>et al.</i> (1993)
C-terminal part of <i>eae</i> (Int- γ)	LP3	CCCGAATTCCTTATCTACACAAACCGC	Schmidt <i>et al.</i> (1993)
<i>fliC</i>	1806	GCTGCAACGGTAAGTGAT	Wang <i>et al.</i> (2000)
Subtyping of <i>tir</i> genes:			
E2348/69-like <i>tir</i>	tirY474 F	CATATTTATGATGAGGTCGCTC	Ogura <i>et al.</i> (2007)
	tir-R	TAAAAGTTCAGATCTTGATGACAT	
Sakai-like <i>tir</i>	tirS478 F	TCTGTTCAGAATATGGGGAATA	Ogura <i>et al.</i> (2007)
	tir-R	TAAAAGTTCAGATCTTGATGACAT	
Detection of pathotype-associated genes:			
<i>tccP</i>	tccP-F	ATGATTAACAATGTTTCTTCACTT	Ogura <i>et al.</i> (2007)
	univ-tccP-R	TCACGAGCGCTTAGATGTATTAAT	
<i>tccP2</i>	tccP2 F	ATGATAAATAGCATTAAATCTTT	Ogura <i>et al.</i> (2007)
	univ-tccP-R	TCACGAGCGCTTAGATGTATTAAT	
EAF plasmid specific region	EAF1	CAGGGTAAAAGAAAGATGATAA	Franke <i>et al.</i> (1994)
	EAF25	TATGGGGACCATGTATTATCA	
<i>bfpA</i>	EP1	AATGGTGCTTGCGCTTGCTGC	Gunzburg <i>et al.</i> (1995)
	EP2	GCCGCTTTATCCAACCTGGTA	
<i>stx1</i>	LP30	CAGTTAATGTGGTGGCGAAGG	Cebula <i>et al.</i> (1995)
	LP31	CACCAGACAATGTAACCGCTG	
<i>stx2</i>	LP43	ATCCTATTCGGGGAGTTTACG	Cebula <i>et al.</i> (1995)
	LP44	GCGTCATCGTATACACAGGAGC	
<i>aggR</i>	aggR(F)	CTAATTGTACAATCGATGTA	Nataro <i>et al.</i> (1994)
	aggR(R)	ATGAAGTAATTCTTGAAT	
<i>astA</i>	EAST11a	CCATCAACACAGTATATCCGA	Yamamoto & Nakazawa (1997)
	EAST11b	GGTCGCGAGTGACGGCTTTGT	
<i>irp2</i>	irp2(FP)	AAGGATTCGCTGTTACCGGAC	Schubert <i>et al.</i> (1998)
	irp2(RP)	TCGTCGGGCAGCGTTTCTTCT	
Long PCR			
Fragment size (bp)*			
Segment 1	8534	208FrF	GATAAACTCGGGCTCGCCGTG
		O55re-3R	AGCAGGGCGATGACTTCATCCA
Segment 2	9023 [9027†]	O55re-2F	CCACCTCTTATCCTCACCTGCT
		O55re-2R	TCCGGTACTGGCTATGTAGGCT
Segment 3	18 482 [18 960†]	O55re-1F	CATAGTCGGTTGGAGTGCGGAT
		O55re-1R	TTGCCGGAACGGAGAGAGTAGA
Segment 4	10 336	O55re-0F	GCCGTTTCAAGTAGTCGGGTTC
		O55re-0R	TCTGTCTGCCCAAACACAACAC
Segment 5	13 745	O55re-AF	TGGATAGAGTTTCGACTGCGCC
		213R	CTTTCCTTCCAGCCGTTTCGTT
Segment 6	8813	213F	TCCGCTCGTTTACCTTTAGCGC
		214R	TAAGGGTAAAACGACTGGCCCG

Table 2. cont.

Locus		Primer	Sequence	Reference
Segment 7	7780	214F	CCGGGCCAGTCGTTTACCCT	Ohnishi <i>et al.</i> (2002)
		215FrR	TGCTTGAGACCAACGCGACCAT	This study
Segment 8	9965	215F	ACCGAAGGCACCATTAAAGGCTG	Ohnishi <i>et al.</i> (2002)
		216R	ATTCCCCGGCACAGCGTCAGG	

*Expected sizes of PCR products in O157 Sakai.

†Expected sizes of PCR products in O55:H7 strain TB182A.

(EAEC) was examined in each of the O55 strains by PCR (Table 1). The O55:H7/Int- θ and O55:H7/Int- γ 1 strains did not possess the EPEC adherence factor (EAF) plasmid or the *bfpA* gene encoding a subunit of bundle-forming pili. Therefore, they were categorized as atypical EPEC (Trabulsi *et al.*, 2002), except for O55:H7/Int- γ 1 strains 5905 and 12646, which were *stx2* or *stx1* positive and thus categorized as EHEC. The O55:H6 strains were typical EPEC as they harbour the EAF plasmid and carry the *bfpA* gene (Trabulsi *et al.*, 2002). Of note is the fact that the O55:H7/Int- θ strains were found to contain the *irp2* gene, which encodes an iron-repressible high-molecular-weight protein HMWP2, one of the virulence gene markers of EAEC (Schubert *et al.*, 1998). However, other EAEC markers, *aggR* (encoding a regulator for the expression of aggregative adherence fimbriae) and *astA* (encoding heat-stable enterotoxin EAST1), were both negative in the O55:H7/Int- θ strains (data not shown).

Comparison of *fliC*_{H7} sequences

The *fliC* genes encoding H7 antigen are known to exhibit sequence variations, and 10 distinct subtypes (H7-1 to H7-10) have been described (Wang *et al.*, 2000). All the *fliC*_{H7} sequences of the O55:H7/Int- γ 1 strains were identical to the H7-5 sequence of O157:H7 strains. In contrast, those of the O55:H7/Int- θ strains were almost identical to the H7-7 sequence of an O19ab:H7 strain, with one or two nucleotide differences. Eight nucleotide differences exist between the DNA sequences of H7-5 and H7-7 (Wang *et al.*, 2000).

Phylogenetic relationship of O55 strains

We analysed the phylogenetic relationship of the 11 O55 strains (six O55:H7/Int- γ 1, two O55:H7/Int- θ and three O55:H6/Int- α) based on the concatenated nucleotide sequences (7395 bp) of 15 housekeeping genes. As shown in Fig. 1, the O55 strains formed three distinct clusters. All the O55:H7/Int- γ 1 strains were tightly associated with the EHEC O157:H7 lineage, while the two O55:H7/Int- θ strains and the three O55:H6/Int- α strains formed distinct clusters. The nucleotide sequence identity of the housekeeping genes within each O55 lineage was >99.9%, but those between the three lineages ranged from 97.2 to 97.6%.

Differences in gene content between the three O55 lineages

To examine the genotypic differences among O55 strains, we analysed the gene contents of eight O55 strains from the three lineages (three O55:H7/Int- γ 1, two O55:H7/Int- θ and three O55:H6/Int- α strains) by using the O157 Sakai oligoDNA microarray (Fig. 2). Most O157 Sakai genes are represented by two probes on this array (Ogura *et al.*, 2006). The probes were divided into two groups by their homologies to the K-12 genome sequences: probes sharing $\geq 90\%$ identity and probes sharing <90% identity. Genes were then classified into ‘conserved in K-12’ genes (both probes share $\geq 90\%$ identity), ‘partly conserved in K-12’ genes (one probe shares $\geq 90\%$ identity but the other <90% identity), or ‘Sakai-specific’ genes (both probes share <90% identity). Genes with more than one copy in the O157 Sakai genome (referred to as repeated genes) were analysed separately from singleton genes (see Supplementary Table S1, available with the online version of this paper).

Analysis of singleton genes and a hierarchical clustering tree are shown in Fig. 2 (for details, see Table S1). The O55 strains examined were divided into three clusters according

Fig. 1. Phylogenetic analysis of O55 strains based on the sequences of 15 housekeeping genes. The phylogenetic tree was constructed based on the concatenated sequences of 15 housekeeping genes by using the NJ algorithm. Bootstrap analysis was performed with 1000 replicates. The sequence data of *E. coli* K-12 strain W3110 and uropathogenic *E. coli* (UPEC) strain CFT073 were taken from the GenBank database (accession nos AP009048 and AE014075, respectively).

Fig. 2. Summary of the CGH analyses of O55 strains using an O157 Sakai oligoDNA microarray. The hierarchical clustering tree was obtained from the CGH data of 4905 singleton genes. The O157 Sakai singleton genes were categorized into three groups, 'conserved in K-12', 'partly conserved in K-12' and 'Sakai-specific' genes, and gene conservation (%) in each category was analysed for each test strain. For the data of repeated genes, see Supplementary Table S1.

to their gene contents. The clustering pattern was consistent with their phylogenies; the O55:H7/Int- γ 1 group was closely related to O157 Sakai.

Among the 4905 O157 Sakai singleton genes, 'Conserved in K-12' genes (3651 genes) were highly conserved in the O55:H7/Int- γ 1 strains (98.3–99.1%) and also in other O55 strains, although to slightly lesser extents (88.3–95.9% but most >90%). 'Partly conserved in K-12' genes (101 genes) were also highly conserved in the O55:H7/Int- γ 1 strains (94.1–96.0%), but intermediately in the other strains (51.5–75.2%). 'Sakai-specific' genes (1153 genes) exhibited moderate conservation in the O55:H7/Int- γ 1 strains (55.4–61.5%), but were very poorly conserved in the O55:H7/Int- θ (22.1–25.0%) and O55:H6 (25.9–35.9%) strains. 'Sakai-specific' genes that were found to be missing in the O55:H7/Int- γ 1 strains were mainly on prophage and prophage-like elements (see Supplementary Fig. S1, available with the online version of this paper). In the other O55 strains, the missing genes were dispersed on the genome. These results indicated that O55:H7/Int- θ strains were very different from the O55:H7/Int- γ 1 group also with respect to their gene repertoire.

PCR-RFLP analysis of O55-antigen gene clusters and their flanking regions

The results described above clearly indicated that O55:H7/Int- γ 1, O55:H7/Int- θ and O55:H6 strains belonged to distinct *E. coli* lineages, but expressed the same O55 antigen. In order to understand the genetic mechanism

underlying the emergence of O55 strains in the three different *E. coli* lineages, we compared the O-antigen gene clusters of the three O55 lineages. We first performed a PCR-RFLP analysis of eight chromosomal segments covering the 85.6 kb region where the O-antigen gene cluster is located (Fig. 3). Segment 3 encoding most of the O55 gene cluster and segment 4 encoding half of the colanic acid biosynthesis gene cluster exhibited an identical RFLP pattern in all the O55 strains. In contrast, segments 1, 2, 7 and 8 exhibited different patterns between the three lineages, but an almost identical pattern within a lineage, with only few minor variations. Segments 5 and 6 exhibited an identical pattern in all the O55:H7/Int- γ 1 and O55:H6 strains with one exception (segment 6 of WC211), but those of O55:H7/Int- θ strains were slightly different from the others. These results suggest that the three O55 lineages share an identical or nearly identical sequence of the O55 and part of the colanic acid biosynthesis gene clusters, and that the left and right junctions between the lineage-specific and shared sequences are located in segment 2 and in segments 5 or 7, respectively.

Sequence analysis of the O55-antigen gene clusters and their flanking regions

To gain more information about the genetic similarity of the three O55-antigen gene clusters and junction points between the lineage-specific and shared sequences, we sequenced the 65 kb chromosome regions encompassing segments 2 to 7 of a representative strain of each lineage (TB182A for O55:H7/Int- γ 1, WC416 for O55:H7/Int- θ and ICC219 for O55:H6). The sequence of the O55 gene cluster of TB182A was identical to that previously reported (Wang *et al.*, 2002).

Overall gene organization. The overall gene organization in the sequenced region was almost identical between the three strains, with a few exceptions in WC416 (Fig. 4), which lacked a 745 bp fragment encoding two small genes (corresponding to ECs2869 and 2870 of O157 Sakai), but contained a 684 bp fragment encoding the *yegJ* gene. The gene organization of the *yegH*–*yegL* region of WC416 was thus identical to that of *E. coli* K-12 strain WC3110 (data not shown). This may be linked to the relatively close phylogenetic relationship between H7/Int- θ strains and K-12 (Fig. 1).

Pairwise sequence comparison. Sequence similarities between the three strains were analysed by pairwise comparisons. Consistent with the results of PCR-RFLP analyses, nearly identical sequences were found in the O55 and part of the colanic acid biosynthesis gene clusters, which were located between lineage-specific sequences in each strain (Fig. 4).

Comparison of TB182A and WC416 defined the left and right junctions of the lineage-specific and shared sequences within *hisI* and *wzc*, respectively. The 42.3 kb region from

Fig. 3. PCR-RFLP analyses of the O55-antigen gene clusters and their flanking regions. (A) The upper panel is a schematic representation of the O-antigen gene clusters and their flanking regions in EHEC O157 Sakai and O55:H7 strain TB182A. Segments 1–8, each amplified by long PCR, are indicated by heavy lines. The lower panel shows the summary of PCR-RFLP of the 11 O55 strains. Segments exhibiting different RFLP patterns are indicated by boxes with different shading and patterns. (B) PCR-RFLP patterns of each segment. PCR products were digested with *Hae*III. Lanes M, AmpliSize Molecular Ruler (50–2000 bp ladder) (Bio-Rad); lanes S, O157 Sakai.

Fig. 4. Pairwise sequence comparisons of the O55 gene clusters and their flanking regions. The genetic organization of the O55-antigen gene clusters and their flanking regions in O55 strains TB182A, WC416 and ICC219 is shown; black regions (ECs2869–70 region in WC416 and *hisI*–*wzz* intergenic region in ICC219) indicate gaps. Locations of SNPs, indicated by vertical lines (upper panel), and levels of % DNA sequence identity calculated with a 100 bp sliding window and a step size of 10 bp (lower panel) are shown for each pairwise comparison. Small indels (<9 bp) are indicated by arrowheads. The G+C content of TB182A is shown at the bottom (calculated with a 1 kb sliding window and a step size of 100 bp).

hisI to *wzc* had only 29 single nucleotide polymorphisms (SNPs) and a 6 bp indel. The nucleotide sequence identities of lineage-specific sequences were 96.8 % on the left hand and 97.7 % on the right hand, showing a similar level of sequence diversity as that observed in the 15 housekeeping genes (97.5 %). Between WC416 and ICC219, the left and right junctions were localized within *ugd* and *wzc*, respectively. The 40.0 kb region from *ugd* to *wzc* had only 33 SNPs and a 1 bp and a 6 bp indel. The identities of lineage-specific sequences were 95.2 % (left) and 97.2 % (right). Similarly, between ICC219 and TB182A, the left and right junctions were within *ugd* and *yegE*, the 49.6 kb region from *ugd* to *yegE* had 27 SNPs and a 1 bp indel, and the identities of lineage-specific sequences were 94.9 % (left) and 96.0 % (right). It is noteworthy that the *wzz* gene and its upstream region of O55:H6 strain ICC219 had a highly divergent sequence compared with the others.

Evolutionary relationship of flanking regions. To evaluate the evolutionary relationships of the three O55 lineage specific sequences flanking the O55-antigen and colanic acid biosynthesis gene clusters, concatenated sequences of the *hisGDCBHA* genes (left hand), and of the *alkA* and *yegDIKL* genes (right hand) (5667 and 5583 bp, respectively) were compared. Both sequences generated phylogenetic trees similar to that of housekeeping genes (Fig. 5). This result indicated that both flanking regions were inherited in each lineage through their evolution. The phylogenetic relationship of the colonic acid biosynthesis genes and their homologues in other *E. coli* strains was also analysed by using the concatenated sequences of the *wcaA–F* genes (3897 bp) and the *wcaK–M* genes (5061 bp). In both cases, the sequences from three O55 lineages form a single cluster, but showed no close relationship to any other *E. coli* sequences currently available (data not shown).

DISCUSSION

In this study, we analysed the phylogenetic and genotypic differences between typical EPEC O55:H7 strains possessing *tir*_{Sakai} and atypical EPEC O55:H7 strains possessing *tir*_{E2348/68}. The data clearly indicated that atypical O55:H7 strains belong to an *E. coli* lineage distinct from typical strains that are close relatives of EHEC O157:H7. While the typical O55:H7 strains were categorized as atypical EPEC and encoded Int- γ 1 (O55:H7/Int- γ 1) and TccP, atypical O55:H7 strains were also categorized as atypical EPEC but encoded Int- θ (O55:H7/Int- θ) and lacked TccP or TccP2. Although Int- θ was originally identified in *E. coli* O111 strains (Tarr & Whittam, 2002), it has been found in other *E. coli* serotypes including O5:H11, O35:H25, O76:H7, O84:H25 and O103:H2 (Ramachandran *et al.*, 2003). Interestingly, an O55:H7 strain with Int- θ (named LTO55-43) has been described before (Lacher *et al.*, 2006) and the strain information is available at the STEC centre Isolate Database (<http://www.shigatox.net/cgi-bin/stec/index>). Extracting data from this resource has shown that the sequences of 15 housekeeping genes of this strain are identical to those of the two O55:H7/Int- θ strains we analysed in this study. Strains ICC58 and LTO55-43 were isolated in Brazil but WC416 in Thailand (Table 1), suggesting the global distribution of the O55:H7/Int- θ -clonal group.

An intriguing genotype characteristic of the O55:H7/Int- θ strains was the presence of the *irp2* gene, which is located in an operon specifying the yersiniabactin-mediated iron-uptake system. This operon was originally identified on the high-pathogenicity island of *Yersinia pestis* (Fetherston *et al.*, 1995). Schubert *et al.* (1998) reported that 93 % of 60 EAEC isolates were *irp2* positive. The authors reported that the gene was detected in a few EPEC but not in EHEC strains. However, more recently, Girardeau *et al.* (2005) reported that 34 % of the strains belonging to the EHEC 2

Fig. 5. Phylogenetic analyses of the O55 gene cluster-flanking regions and housekeeping genes. The phylogenetic trees were constructed based on the sequences of *hisGDCBHA* genes (A), *alkA* and *yegDIKL* genes (B), and 15 housekeeping genes (C) by using the NJ algorithm. Bootstrap analysis was performed with 1000 replicates. Analyses using the UPGMA algorithm also generated similar phylogenetic trees (data not shown).

clonal group also harboured this island. The *irp2* gene could be used as an additional genetic marker to distinguish O55:H7/Int-0 strains from O55:H7/Int- γ 1 strains.

Horizontal gene transfer (HGT) is widely regarded as a major genetic mechanism to generate variations in O-serotypes between and within bacterial species, although the mechanism is not well understood. Most evidence for the horizontal transfer of O-antigen gene clusters has been obtained by comparing their sequences between closely related strains with different serotypes or phylogenetically unrelated strains with the same serotype. An excellent example of the former approach is provided by analyses of *Vibrio cholerae* O1 and O139 Bengal. Several studies have shown that a *V. cholerae* O1 progenitor had acquired a new O-antigen gene cluster and emerged as O139 Bengal (Mooi & Bik, 1997; Stroehrer & Manning, 1997; Stroehrer *et al.*, 1998). In this O-antigen shift, a 22 kb region for the O1-antigen gene cluster was replaced by a 35 kb fragment encoding the O139-antigen gene cluster. The O-antigen shift from EPEC O55:H7 to EHEC O157:H7 appears to be the result of recombination within the *galF* gene (Wang *et al.*, 2002), but more details remained to be clarified.

The latter type of studies of O-antigen evolution mainly involved sequence comparisons of O-antigen gene clusters between strains with a common antigenicity but belonging to different species. The data from a comparison of *E. coli* O9a and *Klebsiella* O3 suggested that about two-thirds of the O-antigen gene cluster of *E. coli* O9a was derived from *Klebsiella* O3 (Sugiyama *et al.*, 1998). It has also been shown that the gene organization of the O-antigen gene cluster of *E. coli* O98 and *Yersinia kristensenii* O11 is identical and the gene cluster of *Y. kristensenii* is flanked by remnants of insertion sequences (ISs), thus suggesting the IS-mediated transfer of the O-antigen gene cluster from *E. coli* O98 to *Y. kristensenii* O11 (Cunneen & Reeves, 2007). These and other studies have provided substantial evidence for the dissemination of O-antigen biosynthesis genes between species through HGT, but horizontally transferred sequences and/or their junctions have rarely been well defined.

The present sequence comparison of the O55-antigen gene clusters and their flanking regions between the three different *E. coli* lineages provided strong evidence that the O-antigen shifts have taken place via the horizontal transfer of large fragments (>40 kb) encoding not only the O-antigen gene cluster but also neighbouring colanic acid biosynthesis genes (Fig. 4). The possible recombination points on the chromosomes have also been identified (Fig. 4). Bacteriophage-mediated general transduction or plasmid-mediated conjugation appears to have been involved in the horizontal transfer of such large chromosomal fragments.

We cannot determine whether these fragments were transferred directly or indirectly to the three O55 lineages from a common ancestor. But, based on the sequence variations of 15 housekeeping genes, it can be estimated

that three lineages were separated from a common ancestor 4.7–5.7 $\times 10^6$ years ago if a uniform rate of synonymous substitution proposed for *E. coli* and *Salmonella enterica* (4.7×10^{-9} per site per year) is applied (Lawrence & Ochman, 1998). In contrast, the sequence variations of the 40 kb region shared by the different lineages imply that the three O55 gene clusters were separated 0.9–1.4 $\times 10^5$ years ago. Thus, each lineage acquired the O55 gene cluster relatively recently.

Of interest is the fact that possible recombination points are not the same between the three lineages. No repetitive sequences were found around the recombination points, such as IS elements and Chi octamer sequences (GCTGGTGG), which may be involved in the transfer and/or recombination of incoming foreign DNA (Dixon & Kowalczykowski, 1993). Although an H-repeat is present in the O55 gene cluster, it is not located at the junctions (Fig. 4). Thus, in either lineage, the replacement of the incoming fragment seems to have been achieved by homologous recombination between the allelic regions. To better understand the genetic mechanism(s) generating a wide variety of O-serotypes, we need to know more about whether transfer of such large fragments is involved in O-antigen shifts in other *E. coli* lineages and also in other bacterial species.

Although the O55-antigen biosynthesis genes of the three lineages were almost identical, the DNA sequence of the *wzz* gene of ICC219 was significantly divergent from its counterparts in the other two lineages. The encoded protein showed only 89.3 % amino-acid sequence identity to those of strains TB182A and WC416. The Wzz protein is involved in the chain length determination of O-antigen. Guo *et al.* (2005), who analysed two *E. coli* strains of serotype O86 that contained Wzz proteins with divergent sequences (90 % identical in amino-acid sequence), reported that the difference in Wzz proteins strongly affects the length of O-antigen chains. The length of the O-antigen chain affects various properties of Gram-negative bacteria, including sensitivities to the serum (Guo *et al.*, 2005), complement (Murray *et al.*, 2006) and bacteriophages (Iguchi *et al.*, 2007), and the function of T3SSs (West *et al.*, 2005). As such, ICC219 and the two O55 strains representing the other lineages (TB182A and WC416) provide good experimental organisms to examine how difference in O-antigen chain lengths might affect virulence potential.

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