Subtractive hybridization and optical mapping of the enterotoxigenic *Escherichia coli* H10407 chromosome: isolation of unique sequences and demonstration of significant similarity to the chromosome of *E. coli* K-12

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Enterotoxigenic *Escherichia coli* (ETEC) is a primary cause of diarrhoea in infants in developing countries and in travellers to endemic regions. While several virulence genes have been identified on ETEC plasmids, little is known about the ETEC chromosome, although it is expected to share significant homology in backbone sequences with *E. coli* K-12. In the absence of genomic sequence information, the subtractive hybridization method and the more recently described optical mapping technique were carried out to determine the degree of genomic variation between virulent ETEC strain H10407 and the non-pathogenic *E. coli* K-12 strain MG1655. In one round of PCR-based suppression subtractive hybridization, 153 fragments representing sequences unique to strain H10407 were identified. BLAST searches indicated that few unique sequences showed homology to known pathogenicity island genes identified in related *E. coli* pathogens. A total of 65 fragments contained sequences that were either linked to hypothetical proteins or showed no homology to any known sequence in the database. The remaining sequences were either phage or prophage related or displayed homology to classifiable genes that function in various aspects of bacterial metabolism. The 153 unique sequences showed variable distribution across different ETEC strains including ETEC strain B7A, which is attenuated in virulence and lacked several H10407-specific sequences. Restriction-enzyme-based optical maps of strain H10407 were compared to *in silico* restriction maps of strain MG1655 and related *E. coli* pathogens. The 5.1 Mb ETEC chromosome was ~500 kb greater in length than the chromosome of *E. coli* K-12, collinear with it and indicated several discrete regions where insertions and/or deletions had occurred relative to the chromosome of strain MG1655. No major inversions, transpositions or gross rearrangements were observed on the ETEC chromosome. Based on comparisons with known genomic sequences and related optical-map-based restriction site similarity, the sequence of the H10407 chromosome is expected to demonstrate ~96% identity with that of *E. coli* K-12.

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Abbreviations: CF, colonization factor; CFA, colonization factor antigen; EHEC, enterohaemorrhagic *Escherichia coli*; ETEC, enterotoxigenic *E. coli*; LEE, locus of enterocyte effacement; LT, heat-labile enterotoxin; PAI, pathogenicity island; PCF, putative colonization factor; SSH, suppression subtraction hybridization; ST, heat-stable enterotoxin; UPEC, uropathogenic *E. coli*.

The GenBank/EMBL/DDBJ accession numbers for the 153 sequences unique to the H10407 chromosome reported in this paper are listed in Table 2. The GenBank/EMBL/DDBJ accession number for strain H10407 *eaeH* gene is DQ109813.

A fuller version of Table 2 is available as supplementary data with the online version of this paper.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) causes watery diarrhoea in humans and animals and is responsible for 400 000 to 800 000 deaths per year (Gilligan, 1999). Approximately 280 million episodes occur annually among children under age 5 years in developing countries (Wenneras & Erling, 2004). ETEC is also the leading cause of diarrhoea among travellers from industrialized countries (Arduino & DuPont, 1993; Cartwright, 1993; Wolf *et al.*, 1993).

ETEC infection is acquired by consumption of contaminated food or water. The bacteria attach to and colonize the small intestine using a heterogeneous group of surface

colonization factor antigens (CFAs) (Knutton *et al.*, 1987). CFAs and putative colonization factors (PCFs), jointly referred to as colonization factors (CFs), mediate the attachment of bacteria to the intestinal mucosa, whereas heat-labile (LT) and heat-stable (ST) enterotoxins induce diarrhoea (Levine *et al.*, 1983). There are different serotypes of ETEC strains producing a variety of CFs (Wolf, 1997). ETEC strain H10407 (O78:H11:CFA/I:LT⁺:STib⁺), a clinical isolate from a patient in Bangladesh (Evans & Evans, 1973), is a prototypic virulent strain, which has been tested in oral challenge studies and shown to cause diarrhoea in humans (Tacket *et al.*, 1988). Like most ETEC, strain H10407 carries plasmids and these encode most of the virulence determinants described so far such as CFs, LT and ST (Evans *et al.*, 1975; Smith *et al.*, 1979; Yamamoto & Yokota, 1983). The complete annotated sequence of the ETEC plasmids is not yet available; it will undoubtedly reveal additional genes with functions associated with the determination of virulence. There is some evidence to suggest that the antibacterial immunity induced by ETEC is to a large extent CF-specific (Freedman *et al.*, 1998).

Despite the clinical importance of ETEC, little is known about the ETEC chromosome. Among the few virulence-associated loci that have been identified are *tia* and *tib*, two separate loci that mediate adhesion and invasion of strain H10407 to cultured epithelial cells, and the *gsp* operon, which encodes a type II secretion pathway for regulating the secretion of LT (Elsinghorst & Kopecko, 1992; Elsinghorst & Weitz, 1994; Lindenthal & Elsinghorst, 1999; Tauschek *et al.*, 2002). The *tia* locus is not universally distributed, being present in about 40% of ETEC strains (Fleckenstein *et al.*, 1996). The *tib* locus encodes a glycoprotein TibA, which has been implicated in bacterial autoaggregation and biofilm formation (Sherlock *et al.*, 2005). DNA microarray-based genomic hybridization has been used to demonstrate the extent of *E. coli* K-12 genes that are missing in strain H10407 (Fukuiya *et al.*, 2004). The loss of K-12 genes may be related to adaptive genomic changes that facilitate acquisition of the virulent phenotype (Dobrindt *et al.*, 2003; Fukuiya *et al.*, 2004). Since the completion of the chromosomal sequencing of the laboratory *E. coli* K-12 strain MG1655 (Blattner *et al.*, 1997), several pathogenic *E. coli* genomes have been sequenced. These include genomes of enterohaemorrhagic *E. coli* (EHEC), *Shigella* and uropathogenic *E. coli* (UPEC) strains (Hayashi *et al.*, 2001; Jin *et al.*, 2002; Perna *et al.*, 2001; Wei *et al.*, 2003; Welch *et al.*, 2002). Comparison of these genomes with the MG1655 sequence has demonstrated that while these genomes share a common *E. coli* K-12 backbone sequence, there is a surprising level of diversity. The differences in overall gene content are attributable to horizontal gene transfer events which occur by the acquisition of various virulence-associated plasmids, bacteriophages and pathogenicity islands (PAIs) (Dobrindt & Reidl, 2000).

The complete annotated genome sequence of a virulent ETEC strain is not yet available. However, several

techniques, which are sequence-based, can be used to isolate pathogen-specific DNA sequences and also to obtain a broad view of the genome structure as it relates to strain MG1655. Suppression subtractive hybridization (SSH) has been successfully used to isolate DNA fragments that are present in one set of DNA sequences (tester DNA, in this case the ETEC chromosomal DNA) but not in another (driver DNA, in this case strain MG1655). The unique ETEC-specific chromosomal sequences can be compared with and annotated in relation to the sequenced enteropathogenic *E. coli* (EPEC), EHEC, UPEC and *Shigella* strains (Diatchenko *et al.*, 1996; Winstanley, 2002). Another novel technique to compare two related genomes is optical mapping, which generates high-resolution ordered restriction maps from single DNA molecules including whole bacterial chromosomes (Cai *et al.*, 1998; Lin *et al.*, 1999), and provides a powerful approach to comparative genomic analysis. Comparison of an optical map of a strain with *in silico* restriction maps of closely related sequenced strains reveals major genomic structural variations such as deletions, inversions, translocations, duplications and gross rearrangements (Zhou *et al.*, 2004a). When these two techniques were applied to the ETEC chromosome, the results demonstrated that the H10407 chromosome is highly homologous to that of strain MG1655, more so than any of the other pathogenic *E. coli* strains. It is hoped that these studies will complement the genome sequencing data of strain H10407 when it is completed.

METHODS

Bacterial strains, plasmids, culture conditions. Bacterial strains used in this study are listed in Table 1. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) broth, or on LB agar supplemented with appropriate antibiotics or substrates for screening at the following final concentrations: ampicillin, 100 µg ml⁻¹; IPTG, 0.2 mM; X-Gal, 80 µg ml⁻¹. Plasmids pHC79 and pCRII-TOPO (Invitrogen) were used to construct the cosmid library and the subtractive library, respectively.

Recombinant DNA techniques. Recombinant DNA methods including restriction-enzyme digestion, ligation reactions, agarose gel electrophoresis and plasmid analysis were performed following standard protocols (Sambrook & Russell, 2001) or the manufacturer's instructions. Plasmid and cosmid purification were performed with QIAprep Miniprep kit (Qiagen) and Qiagen Plasmid Midi kit, respectively. Elution of DNA fragments from agarose gels were performed with the QIAquick gel extraction kit (Qiagen).

Genomic DNA preparation and chromosomal DNA purification. Genomic DNA from strains H10407 and MG1655 was isolated by using the Genomic-Tip 100/G kit (Qiagen), according to the manufacturer's protocol. To separate H10407 chromosomal DNA from plasmids, the total genomic DNA was fractionated on 0.5% (w/v) agarose gel and the DNA eluted with the QIAquick gel extraction kit. To reduce the size, the chromosomal DNA was sheared by repeated passage through a 21-gauge needle 8–10 times.

Subtractive hybridization and screening. SSH was carried out using the PCR-Select Bacterial Genomic Subtraction Kit (Clontech) and essentially following the manufacturer's instructions. H10407 chromosomal DNA was used as the tester and MG1655 DNA was used as the driver DNA. During SSH, a high annealing temperature

Table 1. Bacterial strains used in this study

Strains	Genotype, serotype or phenotype	Source or ref.
HB101	F ⁻ $\Delta(gpt-proA)62$ <i>leuB6 glnV44 ara-14 galK2 lacY1</i> $\Delta(mcrC-mrr)$ <i>rpsL20 (Str^R)xyl-5 mtl-1 recA13</i>	WRAIR*
TOP10F'	F' $\{lacI^qTn10(Tet^R)\}$ <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi80lacZ\Delta M15 \Delta lacX74$ <i>recA1 deoR araD139</i> $\Delta(ara-leu)7697galU galK rpsL(Str^R)$ <i>endA1 nupG</i>	Invitrogen
<i>E. coli</i> O157:H7 EDL933	O157:H7	WRAIR
<i>E. coli</i> K-12 MG1655		WRAIR
ETEC strains		
H10407	O78:K80:H11, LT, ST, CFA/I	WRAIR (Evans & Evans, 1973)
E20738A	O114:H21, LT, CS17	WRAIR (McConnell <i>et al.</i> , 1990)
B7A	O148:H28, LT, ST, CS6, PCFO148	WRAIR (Knutton <i>et al.</i> , 1987; Levine <i>et al.</i> , 1979)
M424C1	O6:H16, LT, ST, CS1, CS3	WRAIR (Reid <i>et al.</i> , 1993)
E2528-C1	O25:NM, LT	WRAIR (Levine <i>et al.</i> , 1979)
E9034A	O8:H9, LT, ST, CS3 (+longus)	WRAIR (Levine <i>et al.</i> , 1984)
E7473	O78:H12, ST, CFA/I	WRAIR (Smith <i>et al.</i> , 1979)
E23477A	O139:H28, LT, ST, CS1, CS3	WRAIR (Levine <i>et al.</i> , 1984)
WS2010A	O8:H9, ST, CS3	WRAIR
C91f	O6:H16, LT, ST, CS2	WRAIR (Smyth, 1982)
WS1833C	O8:H9, LT, ST, CS3	WRAIR
1392/75-2A	O6:H16, CS1, CS3	WRAIR

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of 63 °C was used to enrich for the recovery of H10407-specific unique sequences. The PCR products obtained from SSH, representing tester-specific sequences, were cloned into pCRII-TOPO (Invitrogen), transformed into *E. coli* strain TOP10F' and the transformation mixture plated on LB agar plates containing 0.2 mM IPTG, 80 µg X-Gal ml⁻¹ and 100 µg ampicillin ml⁻¹. A total of 480 white colonies were randomly selected for plasmid preparation and sequencing using vector-based primers flanking the cloning site on the vector.

Construction and screening of cosmid library. A partial *Sau3AI* digest of the H10407 genomic DNA was cloned into the cosmid vector pHC79. *In vitro* packaging was performed with Gigapack III Plus (Stratagene), according to the manufacturer's protocol. To screen the cosmid library for H10407-specific unique sequences, 1 × 10³ colonies were replicated onto a 0.2 µm nitrocellulose filter (Bio-Rad). Immobilized colonies were lysed by floating the filter on 10% (w/v) SDS for 5 min, dried and hybridized to labelled probes.

RNA preparation and cDNA synthesis. Total RNA from strain H10407 was prepared from exponential-phase cultures grown in LB medium at 37 °C using the Qiagen Midi Kit according to the manufacturer's instructions. To synthesize the first strand of cDNA, 20 µg RNA was annealed with random hexamers at 37 °C for 10 min, followed by incubation at 70 °C for 10 min and chilling on ice for 5 min. After the initial annealing step, cDNA synthesis was carried out using a kit containing Cyscript reverse transcriptase (Amersham). Second-strand cDNA synthesis was carried out with Klenow enzyme (Roche) at 37 °C for 1 h. The resulting double-stranded cDNA was purified using the QIAquick PCR Purification Kit (Qiagen) and eluted into 50 µl H₂O.

Preparation of DNA from different ETEC strains. Approximately 6 mg genomic DNA from 11 different ETEC strains was

partially digested with 80 units *EcoRI* at 37 °C overnight. The digested DNAs were labelled and used as probes against the H10407-specific sequences on a DNA dot blot.

Labelling of probes. Probes were labelled with biotin-14-dCTP using the BioPrime DNA Labelling System (Invitrogen). cDNA derived from 20 µg RNA, 250 ng digested genomic DNA or PCR-amplified H10407-specific sequences were used as probes. Unincorporated dNTPs and fragments <30 bp were removed using Spin-50 column (Biomax).

PCR amplification, dot blot hybridization and image analysis. The 504 bp *eae* gene fragment was amplified by PCR using primers *eae-F* (5'-GATATTAATTATCGACGATTTGGTC-3') and *eae-R* (5'-GCGCTCCGACCTGACCAATGCCAG-3') and genomic DNA of *E. coli* O157:H7 EDL933 as template. The PCR-amplified *eae* DNA was used as a negative control for the dot blot hybridizations since the *eae* gene is absent in the H10407 chromosome. Nested primers 1 and 2 provided in the CLONTECH PCR-Select Bacterial Genomic Subtraction Kit were used to PCR amplify the individual unique sequences in the H10407 subtractive library. The PCR products were used to prepare DNA dot blots and, in some cases, labelled and used as probes.

To prepare the DNA dot blot, PCR products were heated at 98 °C for 5 min and chilled on ice. Then 1 µl denatured PCR product was transferred to a 0.2 µm nitrocellulose filter (Bio-Rad). The DNA on the filter was denatured and neutralized using routine methods and cross-linked to the membrane using a Gene Linker UV Chamber (Bio-Rad). Prehybridization was carried out in 6 × SSC, 5 × Denhardt's reagent, 50% (v/v) formamide, 100 µg denatured salmon sperm DNA ml⁻¹, at room temperature for 1 h. Biotin-labelled probe (50 ng ml⁻¹) was added, and hybridization was carried out at room temperature overnight. The filter was washed under stringent conditions [2 × SSC, 1% (w/v) SDS at 65 °C for 20 min, followed by 0.2 × SSC, 0.1% (w/v)

SDS at 65 °C for 20 min]. The signal was developed by treatment with streptavidin-alkaline phosphatase (Perkin Elmer Life Science) and Lumi-Phos WB chemiluminescent substrate (Pierce), followed by maximal exposure to CL-X-Posure Film (Pierce) overnight. The X-ray image was scanned (ScanMaker 5700, Microtec), and the intensity of each dot was measured on a Macintosh computer using the NIH Image program (developed at the U S National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

DNA sequencing, primer walking and data analysis. DNA sequences were determined by the dideoxy-chain-termination method using the Big Dye Terminator v3.1 DNA sequencing kit (Applied Biosystems) on an automated sequencer, ABI Prism 3100 (Applied Biosystems). The sequence of the *eaeH* gene was completed by primer-walking on selected cosmids. DNA sequence data were analysed with the Sequencher program (version 4.2 for Macintosh, Gene Codes Corp.) to remove the vector sequence and the redundant sequences. The H10407-specific sequences were those determined to show no significant alignment to the *E. coli* K-12 strain MG1655 (NC_000913). Database searches for homologous genes and proteins were performed by using the National Center for Biotechnology Information (NCBI) BLAST server.

Optical mapping of the H10407 chromosome. The optical maps of strain H10407 with *Bam*HI, *Bsi*WI and *Eag*I restriction enzymes were constructed commercially by OpGen. In this method, purified chromosomal DNA is deposited onto an optical mapping surface such as a derivatized glass coverslip, using a microfluidic device. The DNA is encased in a thin layer of acrylamide and incubated with restriction enzymes in a humidified chamber at 37 °C for 60–120 min. The digested DNA is labelled with fluorescent YOYO-1 and the individual molecules imaged with fluorescence microscopy. Digital images are collected by a fully automated image-acquisition system and image files are processed to create single-molecule optical maps using OpGen's software programs. Individual molecule restriction maps were overlapped by optical map assembler software. Briefly, this assembler works by comparing single-molecule restriction maps and estimating the probability that these two molecules arose from overlapping genomic locations given a description of the likelihood of possible errors resulting from incomplete digestion of the molecule, spurious cuts of the molecule, and errors in the sizing estimates of the resulting fragments. Through repeated overlapping of molecules, the assembler reconstructs the ordered restriction map of the genome. This technique has been previously applied to map other bacterial genomes (Lai *et al.*, 1999; Lim *et al.*, 2001; Lin *et al.*, 1999). The map alignments between two different strains were generated with a dynamic programming algorithm based upon previous methods (Myers & Huang, 1992; Tang & Waterman, 2001; Waterman *et al.*, 1984). This method finds the optimal alignment of two restriction maps according to a scoring model that incorporates fragment sizing errors, false and missing cuts, and missing small fragments. For a given alignment, the score is proportional to the log of the length of the alignment, penalized by the differences between the two maps, such that longer, better-matching alignments will have higher scores. The regions of gaps, insertions and deletions were obtained by comparing the ETEC strain H10407 map to the *in silico* restriction map of *E. coli* K-12 strain MG1655 using OpGen's MapViewer software, similar to previously reported work (Reslewic *et al.*, 2005).

RESULTS

Subtractive hybridization of strain H10407 chromosomal DNA with strain MG1655

SSH was carried out between the gel-purified H10407 chromosomal DNA, referred to as the tester DNA, and

chromosomal DNA from strain MG1655, referred to as the driver or reference DNA. The tester-specific H10407 subtracted DNA fraction was obtained after two rounds of sequential hybridizations with driver DNA in excess. PCR amplification using 23S RNA-specific sequence primers demonstrated that the abundance of conserved sequences in the H10407 subtracted DNA fraction was reduced dramatically (data not shown). The H10407-specific sequences were cloned and plasmid digests of 364 of 480 randomly selected recombinant clones contained insertions of different sizes. DNA sequencing indicated that 194 clones were distinct and 153 of the 194 distinct clones (79 %) were unique to strain H10407 as determined by lack of significant homology (expected value $> 1e-40$) to strain MG1655. The sizes of the 153 fragments ranged from 78 bp to 1070 bp (mean 391 bp). The combined size of the sequenced fragments that were contained in the 153 clones was ~ 60 kb and these sequences demonstrated a mean G + C content of 51 mol%, although individual sequences varied in G + C content (see the supplementary table available with the online version of the paper). Note that the sequences in the unique clones represented only portions of individual genes or genetic elements and were by no means complete gene sequences.

The H10407 chromosome lacks genes observed in known PAIs

Based on BLASTN search, the 153 H10407-specific DNA sequences were grouped into different categories as shown in Table 2. Although several hypothetical and unknown sequences were identified, most known virulence-associated genes on PAIs, previously identified in *E. coli* pathogens such as ETEC, EPEC, *Shigella* and UPEC strains, do not appear to have been recovered in the H10407 subtractive library (Schmidt & Hensel, 2004).

Group I: sequences that are part of genes previously described in H10407. Previously described chromosomal sequences corresponding to the *gsp* operon and the *tia* and *tib* loci were recovered in the H10407-specific SSH library, validating the use of the SSH technique. The *gsp* operon encodes a type II secretion system for LT secretion (Tauschek *et al.*, 2002). The combined sizes of the genes to which some homology was seen with the unique sequences comprised two-thirds of the 8.5 kb *gsp* operon. This particular operon is lacking in *E. coli* K-12. In addition, fragments with sequences homologous to the *eatA* gene, encoded on the 92 kb plasmid (Patel *et al.*, 2004), were also recovered in the H10407 SSH library. The recovery of these fragments indicates a low level of plasmid contamination in the chromosomal DNA preparations, which probably occurred during extraction of the chromosomal DNA from agarose gels. However, other known plasmid genes such as the CFA/I operon, ST and LT were not recovered in the subtractive library. Seven of nine ETEC fragment sequences that fall into the group I category are of chromosomal origin. Thus, as expected,

Table 2. Analysis of ETEC H10407-specific DNA fragments obtained from the subtractive library

A fuller version of this table is available as supplementary data with the online version of the paper.

Frag. no.	Size (bp)	E*	B7A†	Genes/proteins of best BLAST hit‡	E-value§	Accession no.
I. Genes described in ETEC H10407 (9)						
284	627	E	P	(Rll:1–296) <i>gspD</i> , hypothetical type II secretion protein, ETEC H10407	e–162	CZ548273
213	294	E	P	<i>gspD</i> , hypothetical type II secretion protein, ETEC H10407	e–164	CZ548270
449	512	E	P	<i>gspF</i> , hypothetical type II secretion protein, ETEC H10407	0	CZ548277
278	337	E	P	(R:1–249) <i>gspG</i> , hypothetical type II secretion protein, ETEC H10407	e–125	CZ548272
633	211	E		<i>tia</i> , ETEC H10407	e–107	CZ548280
511	648	E		(R:9–257) <i>tibA</i> , ETEC H10407	e–145	CZ548279
301	483	E		<i>tibA</i> , ETEC H10407	0	CZ548274
217	410	E		<i>eatA</i> , secreted autotransporter, ETEC H10407 plasmid pCS1	0	CZ548271
332	449	E		<i>eatA</i> , secreted autotransporter, ETEC H10407 plasmid pCS1	6e–54	CZ548275
II. Genes with known functions (34)						
609	173	E	P	<i>agaA</i> , <i>N</i> -acetylgalactosamine-6-phosphate deacetylase, <i>E. coli</i> C	2e–89	CZ305760
222	273		P	<i>agaE</i> , <i>E. coli</i> C	e–143	CZ305641
293	213	E		<i>altD</i> , D-arabitol dehydrogenase, <i>E. coli</i> C	e–115	CZ305666
259	318	E	P	<i>atlK</i> , xylulose kinase, <i>E. coli</i> C	e–171	CZ305658
660	531			<i>rtlD</i> , ribitol dehydrogenase; <i>rtlK</i> , ribitol kinase, <i>E. coli</i> C	0	CZ305775
672	288	E	P	<i>deoQ</i> , putative transcriptional regulator, <i>E. coli</i> strain AL862	e–140	CZ305780
327	431	E	P	(R:136–431) putative enzyme for fatty acid biosynthesis, <i>E. coli</i> CFT073	e–162	CZ305676
595	577	E		(R:184–577) putative beta-ketoacyl-ACP synthase, <i>E. coli</i> CFT073	0	CZ305755
424	189			Putative acetyl-CoA:acetoacetyl-CoA transferase, <i>E. coli</i> CFT073	2e–16	CZ305704
495	553			(R:1–384) putative 3-oxoacyl-[ACP] synthase, <i>E. coli</i> O157:H7 EDL933	0	CZ305731
584	203			Putative beta-ketoacyl-ACP synthase, <i>E. coli</i> O157:H7 EDL933	e–102	CZ305750
447	347	E	P	<i>traD</i> , bacterial conjugation membrane protein, plasmid R100	e–155	CZ305714
239	421	E	P	<i>traI</i> , <i>E. coli</i> plasmid pC15-1	0	CZ305649
452	444	E	P	<i>traK</i> for pilus biogenesis, plasmid R100	0	CZ305715
459	464	E	P	<i>traL</i> , <i>E. coli</i> plasmid p1658/9	e–146	CZ305717
483	853	E	P	(R:1–369) <i>silA</i> , putative inner-membrane proton/cation antiporter, <i>Salmonella typhimurium</i> plasmid pMG101	0	CZ305728
667	961	E	P	(R:1–570) <i>silC</i> , putative outer-membrane protein, <i>Klebsiella pneumoniae</i> plasmid pLVPK	0	CZ305778
360	342			<i>silC</i> , putative outer-membrane protein, <i>Sal. typhimurium</i> plasmid pMG101	0	CZ305687
411	129	E		<i>silC</i> , putative outer-membrane protein, <i>Sal. typhimurium</i> plasmid pMG101	5e–46	CZ305700
398	144	E		<i>fliC</i> , flagellin, <i>E. coli</i> strain F199	1e–47	CZ305697

Table 2. cont.

Frag. no.	Size (bp)	E*	B7A†	Genes/proteins of best BLAST hit‡	E-value§	Accession no.
629	153	E	P	<i>fliC</i> , flagellin, <i>E. coli</i> strain F199	2e-67	CZ305766
345	246	E		<i>fliD</i> , filament capping protein, <i>Sal. typhimurium</i> LT2	3e-33	CZ305682
309	503	E		(R:2-229) SapA-like putative structure protein, <i>E. coli</i> CFT073	9e-29	CZ305670
361	442			(R:229-444) putative membrane protein, enterobacteria phage epsilon15	1e-83	CZ305688
551	665	E		(R:325-655) putative autotransporter, <i>S. flexneri</i> 2a SRL PAI	e-177	CZ305744
238	418	E	P	Putative autotransporter, <i>S. flexneri</i> 2a SRL PAI	0	CZ305648
219	636	E	P	(R:390-636) adhesin AIDA-I precursor, <i>E. coli</i> O157:H7	e-126	CZ305638
363	636	E	P	<i>eaeH</i> , putative adhesin, <i>E. coli</i> O157:H7 EDL933	0	CZ305689
673	493	E	P	Putative derepression protein, <i>E. coli</i> O157:H7	0	CZ305781
671	148		P	Putative outer-membrane usher protein, <i>E. coli</i> O157:H7	9e-51	CZ305779
312	276	E	P	Putative peptidase, <i>Serratia marcescens</i> plasmid R478	e-148	CZ305671
392	413			Putative peptide synthetase, <i>E. coli</i> CFT073	4e-98	CZ305696
489	256			(R:108-256) haemolysin expression modulating protein, <i>E. coli</i> CFT073	5e-75	CZ305730
223	288			(R:189-288) haemolysin expression modulating protein, <i>E. coli</i> CFT073	9e-46	CZ305642
III. Genes related to phages/prophages (45)						
IV. Genes related to hypothetical proteins (27)						
V. Genes with no significant hits to GenBank (38)						

**In vitro* expression data were obtained using a dot blot of PCR-amplified DNA fragments hybridized to biotin-labelled cDNA transcribed from strain H10407 total RNA. E designates a sequence that showed two- to sevenfold greater signal intensity of hybridization compared to a negative control on the dot blot.

†The ETEC strain B7A (along with 10 other strains; see supplementary data) was tested in DNA dot blot hybridization for the presence of H10407-specific DNA fragments. P designates a sequence that showed more than twofold greater signal intensity over the negative control.

‡Genes/proteins were determined by BLAST search against GenBank with the NCBI BLASTN program.

§E-value indicates the probability of the match.

||Only the portions of DNA fragment that showed the best hits are listed. The segment positions are shown in parentheses.

the majority of the H10407-specific sequences obtained after SSH were derived from the chromosome.

Group II: sequences that are part of genes with previously described functions. Thirty-four H10407 unique sequences show homology to genes that encode known functions. Six of the fragments showed sequence similarity to gene sequences involved in carbohydrate metabolism (*agaA*, *agaE*, *altD*, *atlK*, *rtlK* and *deoQ*), while others had homology to gene sequences involved in fatty acid biosynthesis (Table 2). Many of the H10407-specific sequences in this category showed significant similarity to homologous sequences in UPEC strain CFT073, EHEC strain EDL933, to *E. coli* C and to *Shigella* (Table 2). The H10407

sil sequences (*silA*, *silC*) showed homology to the Ag⁺-resistant gene cluster (*sil*) on *Salmonella* plasmid pMG101. The *sil* operon encodes resistance to the highly toxic metal silver, which is often used as an antimicrobial agent (Gupta *et al.*, 2001). Other sequences isolated in the SSH library included those with homology to the flagellin genes, suggesting that the flagella assembly sequences in strain H10407 are not identical to those in *E. coli* K-12 (Table 2). H10407-specific DNA segments showed sequence homology to putative virulence-associated genes encoding SapA, a putative autotransporter, adhesins such as AIDA-I precursor and EaeH, peptidase, usher proteins and haemolysin expression modulating protein, which have been identified in other pathogenic *E. coli* genomes (Table 2).

Group III: sequences with homology to phage/prophage genes. At least 45 DNA fragments had sequences that showed homology to prophage sequences of known PAIs from different pathogenic *E. coli* strains (Table 2). Insertion elements, integrases and transposases were the major contributing sequences to this group, suggesting the presence of H10407-specific mobile elements.

Group IV: sequences with homology to genes encoding hypothetical proteins. Twenty-seven fragments had sequences that showed homology to genes encoding conserved hypothetical proteins without a predicted function. These hypothetical proteins have been previously identified during genome sequencing of UPEC, EHEC and *Shigella flexneri* 2a.

Group V: sequences that do not show homology to any database sequence. This category of 38 fragments constitutes unknown sequences that did not match any sequence described in the database.

Expression profile of H10407 subtractive library fragments

To determine whether the sequences contained in the SSH library represented portions of transcriptionally active genes, total RNA was extracted from exponential-phase cultures of strain H10407 grown at 37 °C and hybridized to a DNA dot blot containing PCR-amplified DNA of the 153 H10407-specific fragments. DNA sequences which showed two- to sevenfold greater intensity of hybridization over negative control DNA are listed in Table 2 (denoted as E). The PCR-amplified *eae* DNA was used as a negative control for the dot blot hybridizations since the *eae* gene is absent in the H10407 chromosome. As expected, all the group I sequences were expressed, in agreement with previous studies (Fleckenstein *et al.*, 1996; Lindenthal & Elsinghorst, 1999; Patel *et al.*, 2004; Tauschek *et al.*, 2002). Twenty of 27 sequences in group IV and 24 of 38 sequences belonging to group V scored positive on the RNA blots, demonstrating that the group of hypothetical and unknown sequences are part of transcriptionally active genes and should be further characterized. In general, 71 % (109/153) of the H10407-specific fragments were positive on the RNA dot blots. Furthermore, sequences that did not score positive in these blots may be transcriptionally active under other growth conditions, so that the percentage of transcribed genes in the H10407 strain SSH library may be higher.

Prevalence of H10407-specific sequences among other ETEC strains

Genomic DNA from 11 clinical isolates of ETEC was labelled with biotin and hybridized to the DNA dot blot containing the H10407-specific unique sequences. Spots whose intensities were more than twofold higher than the negative control (PCR-amplified *eae* gene, absent in H10407) were scored as positive (denoted as P in Table 2). These represent sequences that are most likely present in a particular strain.

Hybridization results revealed that the presence of these unique sequences varied, most being present in some but not all strains and indicating the diversity within ETEC. The sequence corresponding to the *eaeH* gene was the only one in the group II category (Table 2) that hybridized to all the ETEC strains tested, indicating that this sequence is conserved. The *eaeH* gene encodes an intimin-like protein whose virulence function has not been characterized. The previously described *gsp* gene sequences were detected in all 11 ETEC strains, suggesting that this is also a conserved operon, although some portions of individual genes in the *gsp* operon may not be identical across all ETEC strains (Table 2). The *tibA* sequence was seen in 4 of 10 ETEC strains tested, confirming previous observations (Fleckenstein *et al.*, 1996). Interestingly, the plasmid-borne *eata* gene that was isolated in the SSH library is present in most of the ETEC strains, but not detected in strain B7A, confirming earlier studies (Patel *et al.*, 2004).

Strain H10407 does not encode the locus of enterocyte effacement (LEE) but contains putative adhesin gene *eaeH*

Since the sequence corresponding to the *eaeH* gene appeared to be conserved in ETEC, the H10407 *eaeH* gene was completely sequenced using an H10407 cosmid library that was probed with fragment no. 363 (Table 2). The full-length H10407 *eaeH* gene encodes 1418 amino acids. This gene has been previously described in other sequenced pathogenic *E. coli* strains (CFT073, O157:H7, O157:H EDL933). In strain MG1655, however, the *eaeH* gene appears as a truncated copy encoding only 293 amino acids. The LEE PAI in EPEC and EHEC strains contains the *eae* gene (separate and distinct from *eaeH*), which encodes intimin, a virulence-associated protein shown to be critical for mediating intimate attachment to the host cell (Jerse *et al.*, 1990; Perna *et al.*, 1998). The LEE PAI is flanked by prophage genes and sequences homologous to these flanking prophage genes were recovered in the H10407-specific SSH library. However, none of the LEE PAI-encoded virulence-associated genes were recovered in the H10407 subtractive library and a labelled *eae* gene, used as a probe, did not hybridize to strain H10407. In this respect, strain H10407 is similar to UPEC strain CFT073 (Welch *et al.*, 2002), which also lacks the major part of LEE.

Comparative optical mapping to identify the deletions, insertions and mismatched regions in the H10407 chromosome relative to *E. coli* K-12

To assess the overall genome structure of strain H10407, and compare it to the MG1655 sequence and other pathogenic *E. coli* genomes, optical maps of strain H10407 using restriction enzymes *Bam*HI, *Bsi*WI, and *Eag*I were constructed by OpGen. Based on the optical maps, the predicted size of the H10407 chromosome is 5143 kb, which is ~500 kb larger than the 4.6 Mb MG1655 sequence. In size, the H10407 chromosome resembles the chromosome of EHEC and UPEC strains.

The optical mapping system is largely automated and constructs ordered restriction maps of an entire microbial genome. When such an optical map is aligned with an *in silico* restriction map of a closely related sequenced genome, the comparisons between the two maps reveal inversions, insertions, deletions, duplications and translocations, while also providing orientation. The resolution of optical mapping is within 6–30 kb, which is the mean size of a restriction fragment (Zhou *et al.*, 2004b). When the map resolution approaches 5–7 kb, a significant proportion of restriction fragments in the map will be less than 1 kb and thus poorly represented in the final consensus map using the current DNA mounting procedures. Optical maps omit map information less than 500 bp in size and show sizing errors of ~2–5%.

The mean restriction fragment sizes of the optical maps were 10.6 kb by *Bam*HI, 9.2 kb by *Bsi*WI and 18 kb by *Eag*I. The three optical maps revealed some differences in the extent and location of map homology when they were compared to the *in silico* maps of *E. coli* K-12. The *Bam*HI- and *Bsi*WI-generated optical maps largely identified common regions of homology, while the *Eag*I-generated map showed reduced power to identify homology and was excluded from further analysis. When compared to the *in silico* map of strain MG1655, the H10407 chromosome appeared remarkably similar to the MG1655 chromosome except at a few discrete regions where deletions, insertions or mismatched regions of varying sizes were observed (Fig. 1a). Unlike the genome structure of EHEC, UPEC and *Shigella* strains, where the collinearity with the *E. coli* K-12 genome is broken by numerous inversions and translocations (Hayashi *et al.*, 2001; Jin *et al.*, 2002; Wei *et al.*, 2003; Perna *et al.*, 2001; Welch *et al.*, 2002), which contribute to significant dissimilarity in the restriction map profile, no such rearrangements were seen in the strain H10407 genomic optical map. Due to the nature of measuring map homology, small differences tend to be missed and not confidently defined. Only differences larger than 10 kb are schematically outlined in Fig. 1(a) and listed in Table 3.

The *Bam*HI-generated optical map indicates two regions on the strain H10407 optical map where deletions have occurred in relation to the map of *E. coli* K-12. These are designated D1 (~25 kb) and D2 (~21 kb) in Fig. 1(a) and Table 3. In the *Bsi*WI-generated map, the deletions appear as mismatched gaps, although the size differences of the gaps between the H10407 optical map and the K-12 map amounts to the loss of ~24 kb and ~21 kb DNA at D1 and D2 (Table 3). D1 corresponds to the region of prophage Rac and D2 corresponds to the region with the Qin loci in strain MG1655. The absence of these two prophages in strain H10407 has been previously confirmed by a DNA microarray study (Fukuya *et al.*, 2004).

The inserted sequences in the H10407 chromosome (I1–I7; Fig. 1a and Table 3) are local events and do not reflect translocated or duplicated regions of the backbone sequences. Bacterial genomes are quite dynamic and often acquire new genes or gene islands from phages or other species through

horizontal transfer. In several instances, these acquired genes are seen in more than one pathogenic strain within the same species as well as in different species, such as the LEE island genes seen in EPEC and EHEC strains, and the type III secretion system seen in many Gram-negative bacteria. Thus, annotation of the inserted regions in the H10407 chromosome was attempted by comparison of its optical map with the *in silico* maps of *E. coli* K-12 as well as those of other *E. coli* pathogens using relaxed settings for map homology. According to the sequence data of the *gsp* loci, inserted sequence region I5 overlaps the *pheV* tRNA gene sequence in *E. coli* K-12 and should contain the 11.7 kb GSP island in the ETEC chromosome (Tauschek *et al.*, 2002). The ~45 kb I6 region on the optical map coincides with the *selC* tRNA locus on the *E. coli* genome. Previous work has demonstrated that a 45 kb sequence is integrated at the *selC* tRNA locus in ETEC and contains the *tia* and *leoA* genes, as well as several hypothetical and unknown ORFs (Fleckenstein *et al.*, 2000). This means that the I6 region on the optical map corresponds to the inserted *tia*-*leoA* sequence. We could not confidently predict the content of the other insertions since either there was no match to reference strains at map level, or the match by one enzyme map could not be validated by another enzyme map. We anticipate that many of the H10407-specific sequences isolated by SSH will be located at these inserted sequence regions as well as at the mismatched gap regions identified on the H10407 optical map.

Four regions on the H10407 chromosome were identified as mismatched gaps relative to *E. coli* K-12 (G1~G4 in Fig. 1a and Table 3). G1 and G4 are located at the ends of the linearized chromosome and probably reflect a single mismatched gap (G1+G4) on the circular chromosome. The term 'mismatched gap' is being applied to regions on the optical map where the restriction sites between the two maps show no similarities over a stretch of the genome. Map homology reappears on either side of the mismatched gap region. All three regions of mismatched gaps in H10407 are larger than the corresponding regions in *E. coli* K-12 and the extra DNA at these sites contributes to the greater length of the ETEC genome. The extra DNA could include single and/or multiple copies of novel gene sequences, pseudogenes, IS elements, phage and prophage genes. Annotation of the mismatched gap regions indicates that these sites overlap phage and prophage sequences present in the K-12 strain genome, including cryptic prophage CP4-6 in the G2 region, CP4-44 in G3 and KpLE2 phage-like element in G1+G4. A recent microarray study has shown that several ORFs corresponding to these prophages are missing in strain H10407 (Fukuya *et al.*, 2004), signifying that the mismatched gap regions are made up of both gene loss and gene gain. It is also interesting to note that G1+G4 overlap *thrW*, *pheU* and *leuX* tRNA sequences and G3 overlaps *asnT* tRNA sequence on the *E. coli* genome. This is significant because several PAIs have inserted adjacent to these sequences in EPEC, EHEC, *Shigella* and UPEC strains. We predict that most of the genes corresponding to the unique sequences in the H10407 SSH

Fig. 1. Genome-wide optical map comparison. (a). The *Bam*HI *in silico* map of *E. coli* K-12 MG1655 was compared to the optical map of ETEC H10407. (b). The *Bam*HI *in silico* map of *E. coli* K-12 was compared to the optical map of ETEC H10407, and the *in silico* maps of *S. flexneri* strain 301, *S. flexneri* 2a strain 2457T, UPEC strain CFT073, and EHEC strains O157:H7 (from Sakai outbreak) and O157:H7 EDL933. Single matched areas are shown in green, multiple match areas in red, and non-match areas in white. G-mismatch gap: I, insertion; D deletion. The gaps and insertions of strain H10407 are underlined. The deletions of strain H10407 are indicated by solid triangles at the corresponding positions of *E. coli* K-12.

Table 3. Identification of mismatched gaps, deletions and insertions of ETEC H10407 relative to *E. coli* K-12 MG1655 by optical mapping

Strains		Start–ending positions (size of gap, del. or ins. in kb)		Predicted regions	
		<i>Bam</i> HI	<i>Bsi</i> WI		
Gaps					
G1	K-12	1–24 (24)	1–51 (51)	Part of the gap contains deletion of CP4-6 prophage (Fukiya <i>et al.</i> , 2004) and presence of a complete <i>eaeH</i> gene*	
	H10407	1–21 (21)	1–27 (27)		
Gap2	K-12	243–323 (80)	246–305 (59)		
	H10407	240–390 (150)	219–335 (116)		
G3	K-12	2000–2213 (213)	1948–2222 (274)	Part of the gap contains deletion of CP4-44 prophage (Fukiya <i>et al.</i> , 2004)	
	H10407	2088–2422 (334)	2016–2415 (399)		
G4	K-12	4342–4645 (303)	4349–4664 (315)	Part of the gap contains deletion of KpLE2 phage-like element (Fukiya <i>et al.</i> , 2004)	
	H10407	4758–5110 (352)	4767–5126 (359)		
Deletions					
D1	K-12	1393–1418 (25)	1407–1450 (43)	Deletion of Rac prophage	
	H10407	1526	1515–1534 (19)		
D2	K-12	1629–1650 (21)	1626–1660 (34)	Deletion of Qin prophage	
	H10407	1743	1714–1727 (13)		
Insertions					
I1	K-12	807	800–816 (16)	Part of the insertion contains <i>gsp</i> †	
	H10407	860–907 (47)	825–890 (65)		
I2	K-12	1182	1175–1210 (35)		<i>tia</i> and <i>leoA</i> PAI (Fleckenstein <i>et al.</i> , 2000)
	H10407	1283–1315 (32)	1254–1318 (64)		
I3	K-12	2609	2629		
	H10407	2796–2841 (45)	2802–2845 (43)		
I4	K-12	2699	2688	Part of the insertion contains <i>gsp</i> †	
	H10407	2927–2968 (41)	2902–2941 (39)		
I5	K-12	3127	3088		<i>tia</i> and <i>leoA</i> PAI (Fleckenstein <i>et al.</i> , 2000)
	H10407	3398–3482 (84)	3348–3439 (91)		
I6	K-12	3820–3860 (40)	3833		
	H10407	4161–4243 (82)	4164–4207 (43)		
I7	K-12	4255	4256		
	H10407	4640–4670 (30)	4630–4672 (42)		

*The placement of the *eaeH* gene in H10407 is based on the location of the truncated *eaeH* gene in *E. coli* K-12.

†The placement of *gsp* island for type II secretion pathway, is based on the sequence done by Tauschek *et al.* (2002, GenBank accession no. AY056599).

library will be located at the insertion sites (I1–I7) and mismatched gap regions (G1–G4) identified in this study.

H10407 chromosome is highly homologous to *E. coli* K-12

In order to validate the efficacy of using optical maps to evaluate genomic variation, the *in silico* map of strain MG1655 was also compared with the *in silico* maps of the chromosomes of *S. flexneri* 2a strains 2457T and 301, UPEC strain CFT073, and EHEC strains O157:H7 and O157:H7 EDL933 (Fig. 1b). In contrast to the high degree of map homology observed between strains H10407 and MG1655,

these *E. coli* pathogens show a much lower degree of map homology, 12–34 %, to strain MG1655 (Fig. 1b, Table 4). Previously, genome sequence comparison has shown that 74–85 % of the genome of the different *E. coli* pathogens contain *E. coli* K-12 backbone sequences (Hayashi *et al.*, 2001; Jin *et al.*, 2002; Perna *et al.*, 2001; Wei *et al.*, 2003; Welch *et al.*, 2002). Reduced restriction-enzyme-site-based map homology suggests that significant restriction enzyme site polymorphism has occurred due to vertically acquired mutations, deletions, acquisition of DNA and gross rearrangements on the genome of the different *E. coli* pathogens. Using a less stringent programme for comparing such maps, Zhou *et al.* (2004a) predicted that the *S. flexneri* 2a

Table 4. Correlation of optical map-based chromosomal homology to sequence identity

Strain	Map identity (%) [*]		Sequence identity (%)
	<i>Bam</i> HI	<i>Bsi</i> WI	
<i>E. coli</i> H10407	77	76	96†
<i>S. flexneri</i> 301	26	20	85‡
<i>S. flexneri</i> 2a 2457T	28	23	82#
<i>E. coli</i> CFT073	18	12	75§
<i>E. coli</i> O157:H7	32	34	75¶
<i>E. coli</i> O157:H7 EDL933	32	32	74#

^{*}The *in silico* map of MG1655 was compared with the optical map of strain H10407 and the *in silico* restriction maps of the sequenced genomes of the other pathogenic strains. Homology to strain MG1655 was identified under the default scores. The percentage map identity for each strain is $100 \times (\text{total length of homologous regions})/(\text{length of the chromosome})$.

†Predicted based on a theoretical model curve correlating percentage map identity to percentage sequence identity and derived from known genome sequences of *E. coli* pathogens and *in silico*-derived restriction maps. The curve is based on the assumption that each base pair match is independent of any of the other matches, and that the variation between two genomes is randomly distributed across the genome. Whether these assumptions hold will depend on more work to be done to quantify differences between genomes at the map level and at the sequence level. At this point, these data are not readily available; hence the model curve is at best a working model.

‡Jin *et al.* (2002).

#Wei *et al.* (2003).

§Welch *et al.* (2002).

¶Hayashi *et al.* (2001).

#Perna *et al.* (2001).

genome sequence is 47% similar to that of *E. coli* K-12. However, using a more stringent programme to compare sequences, as has been done in our study, the *Shigella* genome is 28% similar to that of *E. coli* K-12, as mentioned above. In striking contrast, the *Bam*HI- and *Bsi*WI-generated optical maps of H10407 show 77% and 76% similarity to the *E. coli* K-12 genome, respectively, suggesting that fewer restriction enzyme site polymorphisms have occurred on the ETEC chromosome (Fig. 1b, Table 4). Based on the high level of optical-map-based similarity between the genome of ETEC and *E. coli* K-12, comparisons with other sequenced genomes and working with a model curve that correlates sequence identity with map identity (Table 4), one can make a tentative prediction that the two genomes share >96% sequence similarity, a prediction that awaits confirmation from the availability of the completed ETEC genome sequence.

DISCUSSION

The whole genome sequence of ETEC strain H10407 (currently being sequenced) will aid in the complete

characterization and localization of the genes that comprise the group I–V category of sequences isolated by SSH. Based on the strikingly high genomic map homology between strain H10407 and *E. coli* K-12, we predict that most of these sequences, as well as additional gene sequences that may be identified, will reside at the few discrete regions identified on the H10407 optical map where the genome differs from *E. coli* K-12. Besides unique chromosomal sequences, strain H10407 has four plasmids, a non-conjugative 92 kb plasmid (NTP113 or pCS1) that encodes the CFA/I, ST1b, *astA*, which encodes an enterotoxin EAST1, the autotransporter gene *eatA*, and *longus*, a type IV pilus-encoding gene, a conjugative 64 kb plasmid (NTP104 or pYJ11) that encodes LT and reportedly a second ST gene, a second 64 kb plasmid (pTRA1) and a 6 kb plasmid of unknown composition. The complete sequence of the ETEC plasmids, when available, might demonstrate the presence of additional virulence-associated genes.

The chromosomal SSH library contains several sequences that are representative of genes seen in the genomes and PAIs of other *E. coli* pathogenic strains. These are carbohydrate-utilizing and fatty acid biosynthesis genes, adhesins, auto-transporters, various restriction-modification systems, and hypothetical and unknown genes. However, the optical map suggests that large-scale acquisition of H10407-specific islands of genes, or the extensive gene duplications, inversions and translocations that contribute to the genomic variation previously reported in the genomes of other *E. coli* pathogens, has not occurred in strain H10407. Furthermore, the backbone sequence of the ETEC chromosome is almost identical to that of *E. coli* K-12, indicating that few vertically acquired base pair substitutions have occurred in conserved genes. Therefore, from an evolutionary standpoint, the main event in the derivation of ETEC from *E. coli* occurred with the acquisition of virulence plasmids encoding CF adhesins and enterotoxins. Similar observations have been made for *Shigella*, EHEC and other pathogens, although more extensive alterations have occurred in the genomes of these pathogens. Pathogenic *E. coli* such as EHEC, UPEC, EPEC and *Shigella* strains, have evolved through a complex process. While the *E. coli* backbone has undergone slow accumulation of vertically acquired changes, numerous independent horizontal gene transfer events at discrete sites have conferred unique pathogenic properties to each *E. coli* pathogen. The result is a mosaic genome structure where a core *E. coli* signature chromosome that maintains its characteristic physiology is punctuated by combinations of islands of genes that confer to each pathogenic strain a characteristic disease-causing lifestyle. For example, a comparison of the EHEC O157:H7 EDL933 genome sequence with *E. coli* K-12 revealed a surprising level of diversity between two members of the same species. Although they shared a common collinear backbone sequence, interrupted by a single 422 kb inversion, hundreds of islands of EHEC-specific DNA (O-islands, total 1.34 Mb encoding 1387 new genes) and loss of K-12-specific sequences (K-islands, total 0.53 Mb) are distributed throughout both genomes (Perna

et al., 2001). The O-islands contained genes encoding putative virulence factors such as toxins, urease genes, a type III secretion system with associated substrates and effector proteins, the LEE island, a fatty acid biosynthesis system and adhesins. About 33 % of the O-islands contain genes of unknown function. A similar pattern of variation was seen in the genome sequence of UPEC strain CFT073, which is 590 kb longer than strain K-12: it had 247 CFT073-specific DNA segments > 50 bp and 60 segments > 4 kb encoding potential virulence genes, inserted or substituted into a conserved backbone sequence (Welch *et al.*, 2002). The atypical base composition of most of the islands in EHEC, UPEC and *Shigella* indicate that they were acquired by horizontal transfers. Single nucleotide polymorphisms are distributed throughout the homologous backbone, contributing to the restriction enzyme site polymorphism, and as expected most differences are synonymous changes at third codon positions (Perna *et al.*, 2001). The number of *Shigella*-specific islands is smaller than those in *E. coli* strains CFT073 and EDL933, and a larger proportion of the genome (82 %) is backbone compared to 75 % for strains CFT073 and EDL933 (Wei *et al.*, 2003). Based on the optical map homology described in this study, the H10407 chromosome is much more similar to that of strain MG1655 than to strain CFT073, *S. flexneri* 2a or EHEC. Fewer backbone substitutions and changes have occurred and the ETEC chromosome is predicted to be > 96 % identical in sequence to the *E. coli* K-12 chromosome.

Many pathogen-specific island locations are at the same relative backbone positions although the island contents are unrelated. For example, at the *selC* tRNA site, strain H10407 has a 45 kb island of genes that include the *tia* and *leoA* genes, EPEC and EHEC strains contain the LEE PAI, UPEC strain 536 has PAI-I, *Salmonella* has SPI-3 and *Shigella* has the SHI-2 island of genes (Schmidt & Hensel, 2004). This pattern of insertion at tRNA gene sites, which are also frequently associated with phage attachment sites, is typical of many horizontally acquired PAIs and genes. The molecular basis for this insertion is not completely understood. One hypothesis suggests that the tRNA gene structure provides a conserved motif for the integration of PAIs and phages (Reiter *et al.*, 1989).

The difference in genome size between strains H10407 and *E. coli* K-12 can be accounted for by summing up the differences between the mismatched gap regions and insertions as well as the deletions. The SSH library of sequences does not comprise complete genes. Nor is the SSH library in this study a complete representation of all unique chromosomal sequences in strain H10407. The sequences in the SSH library constitute ~ 60 kb of unique strain H10407 sequences. If a mean *E. coli* ORF is assumed to be about 800 bp and the mean size of the insert fragments in the SSH library is ~ 390 bp, then the SSH library accounts for a minimum of ~ 120 kb of unique gene sequences. However, redundancies in bacterial genes, phage, prophage, transposases and IS elements are common in enterobacterial genomes and may add to the

increased length of the genome. We do not know which of the SSH sequences identified in this study are linked as island genes on the H10407 chromosome. That information will come from genome sequencing analysis.

It is interesting to note that ETEC strain B7A (O148:H28, CS6) lacks several H10407-specific chromosomal sequences in addition to the absence of three previously described genes *eatA*, *tia* and *tibA*. Many studies around the world have shown that ETEC-induced diarrhoea may range from the very mild to the very severe (Qadri *et al.*, 2005). Reliable and reproducible animal models are lacking for studying human ETEC infections and comparing the virulence of different strains. In human challenge studies, strain B7A has been consistently less virulent than strain H10407. Strain B7A required 20-fold more organisms (1×10^{10} c.f.u.) than strain H10407 (5×10^8 c.f.u.) to induce diarrhoea in orally challenged human volunteers. Likewise, while strain H10407 caused diarrhoea in 100 % of volunteers, strain B7A produced diarrhoea in only 67 %, despite the higher dose (Levine *et al.*, 1979; Byrd *et al.*, 2003). While differences in CF type (CFA/I vs CS6), O and H antigens may account for some of the reduced virulence in strain B7A, lack of H10407-specific sequences may also explain differences in virulence properties. The variable occurrence of H10407-specific sequences, not only in strain B7A but also in other ETEC strains, indicates that there is probably a greater heterogeneity among ETEC strains than previously described. The optical map of strain H10407 can be compared with similar maps from other ETEC strains to determine the level of microbial variation as well as identify regions on the chromosome that may be hypervariable.

Our results with SSH and optical mapping generally corroborate data obtained using other approaches. Using comparative genomic hybridization, labelled genomic DNA from 22 pathogenic *E. coli* strains including strain H10407 were hybridized to an *E. coli* K-12 microarray (Fukuya *et al.*, 2004). Thirty per cent of the ORFs in the following five categories were frequently absent: cell envelope, central intermediary metabolism, energy metabolism, transport and binding proteins, and hypothetical and some regulatory genes. There were differences in the number of absent ORFs in the different strains and it is interesting to note that on average, the two ETEC strains tested had the least number of absent ORFs (230 in strain H10407 and 286 in ETEC 31-10) compared to the other *E. coli* pathogens, confirming that ETEC has undergone fewer chromosomal changes compared to *Shigella*, UPEC or EPEC strains. Microarray analysis indicated that less than 10 % of the *E. coli* K-12 prophages were present in the pathogenic *E. coli* including strain H10407, and this loss will also contribute to the observed genetic variation. Most of the ORFs of the prophages CP4-6 and CP4-57 were absent in the *E. coli* pathogens. The *rfa* and *rfb* gene clusters of *E. coli* K-12 involved in core and O-antigen biosynthesis were frequently absent in pathogenic strains. Such losses and gains of pathogen-specific sequences in the H10407 chromosome

would account for the differences seen by optical mapping analysis.

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