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Positive effects of multiple *pch* genes on expression of the locus of enterocyte effacement genes and adherence of enterohaemorrhagic *Escherichia coli* O157 : H7 to HEp-2 cells

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Enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC and EHEC, respectively) genomes contain a pathogenicity island, termed the locus of enterocyte effacement (LEE), which encodes genes involved in the formation of attaching and effacing lesions on epithelial cells. To elucidate the regulatory mechanism of the LEE genes in EHEC, an EHEC O157 genomic library was screened for clones which modulated expression of the LEE genes. From more than 5000 clones, a DNA fragment was obtained containing a *perC* homologue as a positive regulator for the LEE genes. In EPEC, *perC* is known to be part of the *per* operon, along with *perA* and *perB*, located on the EPEC adherence factor plasmid, which is not found in EHEC. However, the complete genome sequence of EHEC O157 Sakai strain reveals that there are five *perC*-like sequences, but no *perA* and *perB*, on the chromosome. These five *perC* homologues were characterized, and it was found that three of the homologues (renamed *perC* homologue *pchA*, *pchB* and *pchC*) encoded 104 aa proteins, and when expressed on a multicopy plasmid enhanced the expression of LEE genes. In contrast, *perC* homologues encoding proteins of 89 and 90 aa, renamed *pchD* and *pchE*, respectively, had no significant effect. Deletion mutants of the *pch* genes were constructed, and the effect on the expression of LEE-encoded type III effector proteins, such as EspA, B and D, and adhesion phenotype to HEp-2 cells was examined. Deletion of *pchA* or *pchB*, but not *pchC*, decreased the expression of Esp proteins and adhesion to HEp-2 cells. Such effects were more apparent with mutants carrying double deletions of *pchA*/*pchB* or *pchA*/*pchC*, suggesting that *pchA*/*B*/*C* are all necessary for full expression of the LEE genes and adhesion to HEp-2 cells. Further study demonstrated that the positive effect of *pchA*/*B*/*C* was caused by enhanced transcription of the LEE-encoded regulatory gene, *ler*. Introduction of a multicopy plasmid carrying each *pchA*/*B*/*C* gene significantly induced microcolony formation by EHEC O157 on HEp-2 cells. These results suggest that the *pchABC* genes are necessary for full virulence of EHEC O157.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) is a major cause of haemorrhagic colitis and haemolytic uraemic syndrome (reviewed by Nataro & Kaper, 1998; Frankel *et al.*, 1998). Though the most important virulence determinant responsible for severe illness is Shiga toxin (Stx), other virulence factors also contribute to the EHEC infection process. Adherence of EHEC and enteropathogenic *E. coli* (EPEC) to intestinal epithelial cells is characterized by the formation of attaching and effacing (AE) lesions (Moon *et al.*, 1983; Tzipori *et al.*, 1986), which cause destruction of microvilli and the formation of pedestal-like structures, triggered by rearrangement of cytoskeletal proteins (Knutton *et al.*, 1987; Rosenshine *et al.*, 1996). The genes involved in the formation of AE lesions are encoded within a locus of enterocyte effacement (LEE), which exists in the chromosome of many strains of EHEC and EPEC (McDaniel *et al.*, 1995; Nataro & Kaper, 1998; Frankel *et al.*, 1998).

LEE contains more than 40 ORFs, which are thought to constitute at least five operons, LEE1–5 (Elliott *et al.*, 1998, 1999a; Sanchez-SanMartin *et al.*, 2001). Most of the genes within the LEE1, 2 and 3 operons encode structural and auxiliary proteins necessary for the formation of dedicated

Abbreviations: AE (lesion), attaching and effacing; EPEC, enteropathogenic *Escherichia coli*; EHEC, enterohaemorrhagic *Escherichia coli*; LEE, locus of enterocyte effacement.

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type III secretion machinery (Jarvis *et al.*, 1995). The LEE4 operon encodes several effector proteins, such as EspA, EspB, EspD and EspF, all of which are exported from the bacterial cell through the type III secretion machinery (Kenny & Finlay, 1995; Kenny *et al.*, 1996; Lai *et al.*, 1997; McNamara & Donnenberg, 1998). LEE5 encodes an adhesion factor called intimin (Jerse *et al.*, 1990; Jerse & Kaper, 1991), as well as Tir, which is also translocated through the type III secretion machinery and acts as a receptor for intimin at the host-cell membrane (Kenny *et al.*, 1997).

Previous reports demonstrated that the expression of LEE genes is under the positive and negative control of several regulatory genes (Shin *et al.*, 2001; Tatsuno *et al.*, 2003). Ler, which is encoded by the first gene of the LEE1 operon, has been shown to be essential as a transcriptional activator for the expression of all the LEE genes (Mellies *et al.*, 1999; Friedberg *et al.*, 1999). Among regulatory proteins encoded outside LEE, several nucleoid proteins such as Fis (Goldberg *et al.*, 2001), H-NS (Bustamante *et al.*, 2001; Umanski *et al.*, 2002) and IHF (Friedberg *et al.*, 1999) have been shown to be involved in the positive or negative control of LEE expression. Recent findings indicate that several quorum sensing-related genes, such as *luxS* (Sperandio *et al.*, 1999), *sdiA* (Kanamaru *et al.*, 2000) and *qseA* (Sperandio *et al.*, 2002), are important for the regulation of LEE expression, and most of these regulatory mechanisms are thought to be common to both EHEC and EPEC. However, other studies indicate that there are some differences between these two organisms in the regulatory mechanism of LEE. For example, the EPEC adherence factor (EAF) plasmid is widely distributed in EPEC strains (Sohel *et al.*, 1996; Stone *et al.*, 1996), but not found in EHEC. The EAF plasmid contains *perA*, *B* and *C* (Gómez-Duarte & Kaper, 1995), also called *bfpT*, *V* and *W* (Tobe *et al.*, 1996), in addition to other *bfp* genes, including *bfpA*, which encodes the major subunit of bundle-forming pili (Sohel *et al.*, 1996; Stone *et al.*, 1996). It has been demonstrated that *perA* and *perC* are important for full activation of the expression of *bfpA* (Tobe *et al.*, 1996) and LEE genes (Gómez-Duarte & Kaper, 1995; Mellies *et al.*, 1999). In EHEC, it had been presumed that there were no *per* homologues, based on a Southern hybridization study using the *perA* and *B* genes as DNA probes (Gómez-Duarte & Kaper, 1995). However, the recently published whole-genome sequence of EHEC O157 Sakai strain revealed that there are at least five *perC*-like sequences on the chromosome (accession no. NC_002695, available at http://genome.gen-info.osaka-u.ac.jp/bacteria/o157/). As a phenotypic difference, a cloned LEE region of EPEC on a cosmid vector conferred the AE phenotype on *E. coli* K-12 strain (McDaniel & Kaper, 1997), while the LEE region from EHEC did not (Elliott *et al.*, 1999b). In the present study, we isolated a *perC* homologue from an EHEC O157 genomic library as a positive regulatory gene for the expression of LEE genes, and showed that all *perC* homologues encoding 104 aa proteins enhance transcription of the LEE genes and adhesion to HEp-2 cells. These results suggest that these genes may play an important role in the full virulence not only of EPEC but also of EHEC O157.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used in this study are summarized in Table 1. For wild-type EHEC O157 : H7 strains, we used EDL933 (Perna *et al.*, 2001) and Sakai (Hayashi *et al.*, 2001a, b). *E. coli* K-12 strain JM109 (Yanisch-Perron *et al.*, 1985) was used for DNA manipulation. Unless otherwise specified, bacteria were grown in Luria broth (LB). LB agar plates were prepared by adding agar (Shoei, Japan) to LB at a final concentration of 1·2 % (w/v). As required, antibiotics or 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) were added at the following final concentrations: ampicillin (Ap), 100 μg ml^{-1}; chloramphenicol (Cm), 25 μg ml^{-1}; kanamycin (Km), 50 μg ml^{-1}; tetracycline (Tc), 20 μg ml^{-1}; X-Gal, 50 μg ml^{-1}.

DNA manipulation. Standard DNA manipulation was performed as described previously (Iyoda & Kutsukake, 1995; Iyoda *et al.*, 2001). PCR amplification was performed on a thermal cycler PE9600 (Perkin Elmer) with *TaKaRa EX Taq* DNA polymerase (Takara). DNA sequence analysis was performed with a PE 310 DNA autosequencer (Perkin Elmer).

Construction of plasmids. The reporter plasmid pLEE19 carries a 10·8 kbp *Sac*I fragment containing a part of the LEE region (including the 3′ region of the *tir* gene and several intact genes, such as *cesT*, *eae*, *escD*, *sepL*, and *espA*, *D*, *B* and *F*) of EDL933 on cloning vector pSSVI215 (Favre & Viret, 1996). The *espB–lacZYA* transcriptional fusion gene on pLEE19 was constructed by inserting Tn*3-lacZYA bla*, as described previously (Stachel *et al.*, 1985). Sequence analysis revealed that the Tn*3-lacZYA bla* is inserted 200 bp downstream from the *espB* translational start site (data not shown). A cloning vector, pBR322C, was constructed by cloning the *Pst*I fragment, which carries the chloramphenicol acetyltransferase gene (*cat*) of pKRP10 (Reece & Phillips, 1995), into the *Pst*I site of pBR322 (Bolivar *et al.*, 1977) in the same orientation as the *bla* gene. Plasmids pGEMLER, pACLER, $pBRCC_{EDL}$, pBRCC89, pBRCC90, pBRCC104a, pBRCC104b, pBRCC104c and pFZLERp were constructed by cloning the amplified PCR fragments into pGEM-T-Easy (Promega), pACYC184 (Chang & Cohen, 1978), pBR322C or pFZY1 (Koop *et al.*, 1987). To obtain pGEMLER, a 930 bp DNA fragment of EDL933 containing the *ler* gene (including 378 bp upstream from the initiation codon of *ler*) amplified with LERC1 and LERC2 primers was cloned into pGEM-T-Easy. The transcriptional direction of the *ler* gene was the same as that of the *lac* promoter on pGEM-T-Easy. To obtain pACLER, the *ler*-containing *Eag*I fragment of pGEMLER was recloned into the same site of pACYC184. The transcriptional direction of the *ler* gene on pACLER was opposite to that of the *tetA* gene. To obtain $pBRCC_{EDL}$, a 620 bp DNA fragment containing the *pchB* sequence (including 280 bp upstream from the initiation codon of *pchB*) of the pSPH2 plasmid (see Results) amplified with PERC1BHI and PERC2STU primers, was digested with *Bam*HI and *Stu*I, and cloned into the *Eco*RV–*Bam*HI sites of pBR322C. To obtain pBRCC89 and pBRCC90, a 400 bp DNA fragment of Sakai carrying *pchD* or *pchE* (including 84 bp or 80 bp upstream from the initiation codon of *pchD* or *pchE*, respectively) amplified with the primer sets PERC89STU/PERC89BHI (for *pchD*) or PERC90STU/PERC90BHI (for *pchE*) was digested with *Bam*HI and *Stu*I and recloned into the *Bam*HI–*Nru*I sites of pBR322C. To obtain pBRCC104a, pBRCC104b or pBRCC104c, a 420 bp DNA fragment (including 37 bp upstream from the initiation codon of *pchABC*) of Sakai strain amplified with PERC3BHI and PERC4STU primers was initially ligated into pGEM-T-Easy. After the confirmation of the DNA sequence of each clone, a *Stu*I–*Bam*HI fragment

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant phenotype	Source or reference
Strain		
EDL933	Wild-type EHEC O157 : H7	CDC
ATCC 700927	EDL933 (genome sequenced strain)	ATCC
EDL933-1	EDL933 Δ(*ler*) : : *kan*	This study
EDL933-5141	EDL933 Δ(*lac*)	Unpublished data
Sakai	Wild-type EHEC O157 : H7 (Same as RIMD0509952)	T. Hayashi*
SKI-1	Sakai Δ(*ler*) : : cat	This study
SKI3-89	Sakai Δ(*pchD*) : : *kan*	This study
SKI3-90	Sakai Δ(*pchE*) : : *kan*	This study
SKI3-8990	Sakai Δ(*pchD*) : : *kan* Δ(*pchE*) : : *cat*	This study
SKI3-104a	Sakai Δ(*pchA*) : : *kan*	This study
SKI3-104b	Sakai Δ(*pchB*) : : *kan*	This study
SKI3-104c	Sakai Δ(*pchC*) : : *kan*	This study
SKI3-104ab	Sakai Δ(*pchB*) : : *kan* Δ(*pchA*) : : *cat*	This study
SKI3-104bc	Sakai Δ(*pchB*) : : *kan* Δ(*pchC*) : : *cat*	This study
SKI3-104ca	Sakai Δ(*pchC*) : : *kan* Δ(*pchA*) : : *cat*	This study
SKI-5141	Sakai Δ(*lacZYA*) : : *kan*	This study
SKI-5168a	SKI-5141 Δ(*pchA*) : : *kan*	This study
SKI-5168b	SKI-5141 Δ(*pchB*) : : *kan*	This study
SKI-5168c	SKI-5141 Δ(*pchC*) : : *kan*	This study
MC4100	F^- *araD*139 Δ(*argF-lac*)*U169 deoC1 thiA flbB5301 ptsF25 relA1 rpsL150 tonA21*	H. Takahashi†
Plasmid		
pUC4K	Kan gene cassette, Ap^r, Km^r	Pharmacia
pKD3, pKD4, pKD13	Template plasmid for lambda Red recombination system, Ap^r	Datsenko & Wanner, 2000
pKD46	Red recombinase expression plasmid, Ap^r	Datsenko & Wanner, 2000
pSSVI215	Cloning vector, Km^r	Favre & Viret, 1996
pLEE19	pSSVI215-LEE, *espB* : : Tn*3-lacZYA bla*, Km^r, Ap^r	This study
pGEM-T-Easy	TA cloning vector, Ap^r	Promega
pGEMLER	pGEM-T-Easy-*ler*	This study
pACYC184	Cloning vector, Cm^r, Tc^r	Chang & Cohen, 1978
pACLER	pACYC184-*ler*	This study
pSPH2	10·1 kbp *Sph*I fragment from EDL933, cloned into pACYC184	This study
pBR322	Cloning vector, Ap^r, Cm^r, Tc^r	Bolivar *et al.*, 1977
pKRP10	*cat* gene cassette, Cm^r, Ap^r	Reece & Phillips, 1995
pBR322C	Cloning vector, Ap^s, Cm^r, Tc^r	This study
pBRCC89	pBR322C-*pchD*	This study
pBRCC90	pBR322C-*pchE*	This study
pBRCC104a	pBR322C-*pchA*	This study
pBRCC104b(=$pBRCC_{EDL}$)	pBR322C-*pchB*	This study
pBRCC104c	pBR322C-*pchC*	This study
pFZY1	Promoter probe vector, Ap^r	Koop *et al.*, 1987
pFZLERp	pFZY1-*ler* promoter region	This study

containing *pchA*, *pchB* or *pchC* was recloned into the *Eco*RV–*Bam*HI sites of pBR322C. To construct the *ler*–*lacZYA* fusion plasmid pFZLERp, a 1·46 kbp DNA fragment containing the predicted promoter region of the *ler* (Sperandio *et al.*, 1999) of EDL933 amplified with LERKPN and LERC2 primers was digested with *Kpn*I and *Sph*I, and the resulting 890 bp fragment, corresponding to 15 to 904 bp upstream of the translational start site of *ler*, was ligated into the corresponding site of pFZY1.

Shotgun cloning. In order to identify new regulator(s) other than *ler*, chromosomal DNA from EDL933-1, a *ler*-deleted mutant of EDL933, was used. This DNA was digested with *Sph*I and ligated with pACYC184. A *lac*-negative derivative of EDL933, designated EDL933-5141 (unpublished data) was transformed with pLEE19, and the resultant tester strain was electroporated with the above ligation mixture. The β-galactosidase activities of shotgun clones were examined on LB agar plates containing X-Gal, Ap, Km and Cm. Introduction of pACLER into the tester strain enhanced activity of the *espB*–*lacZYA* more than tenfold compared to that carrying pACYC184 (Table 4), indicating that the *espB*–*lacZYA* gene on pLEE19 can be used as an indicator for enhanced expression of the LEE genes.

Construction of mutant strains. To construct EDL933-1, SKI-1, SKI-5141, SKI3-89, SKI3-90, SKI3-8990, SKI3-104a, SKI3-104b,

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SKI3-104c, SKI3-104ab, SKI3-104bc and SKI3-104ca strains, we employed a one-step inactivation method (Datsenko & Wanner, 2000). PCR products containing the Km or Cm resistance cassette flanked by 50 bp of homology to each gene at the 5′ and 3′ ends were electroporated into the EDL933 or Sakai strain harbouring pKD46, a helper plasmid encoding λ Red, Gam and Bet (Datsenko & Wanner, 2000). DNA sequences of the primers are indicated in Table 2. To construct EDL933-1 and SKI-1, PCR products containing Km (for EDL933-1) or Cm (for SKI-1) resistance cassettes flanked by 50 bp of homology to *ler* at the 5′ and 3′ ends, amplified from pKD13 using the primers LERP1 and LERP4 (for EDL933-1) or amplified from pKD3 using the primers LERP5 and LERP6 (for SKI-1), were used. The resultant disrupted *ler* locus was verified by PCR with LERC1 and LERC2 primers (data not shown). To construct SKI-5141, PCR products of the Km resistance cassette flanked by 50 bp of homology to *lacI* and *lacA*, amplified from pKD4 using the primer set LACIP7 and LACAP8, was used. The resultant Km-resistant colonies were examined for their Lac-negative phenotype on LB agar plates containing X-Gal. To construct SKI3-89 and SKI3-90, PCR products of the Km resistance cassette flanked by 50 bp of homology to *pchD* or *pchE* at the 5′ and 3′ ends, amplified from pKD4 using the primer sets PERC89P1/PERC89P2 or PERC90P1/PERC90P2, respectively, were used. The resultant disrupted *pchD* or *pchE* locus was verified by PCR with the PERC89BHI/PERC89STU or PERC90BHI/PERC90STU primer sets, respectively (data not shown). To construct double-deletion mutant SKI3-8990, PCR products containing the Cm resistance cassette flanked by 50 bp of homology to *pchE* at the 5′ and 3′ end, amplified from pKD3 using the primers PERC90P5 and PERC90P6, were electroporated into SKI3-89. To construct SKI3-104a, SKI3-104b or SKI3-104c, PCR products containing the Km resistance cassette flanked by 50 bp of homology to *pchC* at the 5′ and 3′ ends, amplified from pKD4 using the primers PERCP1 and PERCP2, were used. To construct double-deletion mutants SKI3-104ab, SKI3-104bc and SKI3-104ca, PCR products containing the Cm resistance cassette flanked by 50 bp of homology to *pchC* at the 5′ and 3′ end, amplified from pKD3 using the primers PERCP5 and PERCP6, were electroporated into SKI3-104b (to obtain SKI3-104ab and SKI3-104bc) or SKI3-104c (to obtain SKI3-104ca). To check the resultant disrupted locus of each *pch* allele, DNA sequences of PCR products amplified with the PERCC2/P1R or PERCC2/P6R primer sets were determined (data not shown). We further confirmed the DNA sequences of the intact *pchABC* loci in each mutant, which were amplified by PCR using the primers PERC3BHI and PERC2STU (data not shown).

β-Galactosidase assay. The activity of β-galactosidase was assayed as described previously (Iyoda *et al.*, 2001). Bacteria were grown until they reached an OD_{600} of 0·7 in Dulbecco's modified Eagle medium (DMEM; Gibco/Invitrogen) supplemented with 0·3 %

Table 2. Oligonucleotide primers used in this study

Priming sites on pKD3, pKD4 or pKD13 are underlined. Restriction sites are shown in bold type.

Primer	DNA sequence (5′–3′)
LERP1	TTTCATCTTCCAGCTCAGTTATCGTTATCATTTAATTATTTCATGGTGTAGGCTGGAGCTGCTTC
LERP4	GTTGGTCCTTCCTGATAAGGTCGCTAATAGCTTAAAATATTAAAGATTCCGGGGATCCGTCGACC
LERP5	TTTCATCTTCCAGCTCAGTTATCGTTATCATTTAATTATTTCATGCTTACGCCCCGCCCTGCCAC
LERP6	GTTGGTCCTTCCTGATAAGGTCGCTAATAGCTTAAAATATTAAAGCTACCTGTGACGGAAGATCA
LACIP7	GCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGATCCCCTCACGCTGCCGCA
LACAP8	CATATCGGTAAATAGCTTGCCTGCTTTTATTCTTTCTGTCATCGACATGTTTCGAACCCCAGAGTCCCGC
PERC89P1	GAGTTAATGTTTTGAGCAGGAAAAAGAGTACCCCGGAAGTTGTGGAAGACGTGTAGGCTGGAGCTGCTTC
PERC89P2	TATACTGTTTGCCATTCCTTGCTTCATCATTAATACCTGTTACCTGAATACATATGAATATCCTCCTTAGT
PERC90P1	GGGAGGCGATGACCCTTACGGTGACGGTATTCCGTTCTGAGGGGGTGGCGGTGTAGGCTGGAGCTGCTTC
PERC90P2	CCATACCGTTTCAACTGGTGCAAAAAAAGCCGGATTTCTCCGGCTGTTGACATATGAATATCCTCCTTAGT
PERC90P6	GGGAGGCGATGACCCTTACGGTGACGGTATTCCGTTCTGAGGGGGTGGCGCTACCTGTGACGGAAGATCA
PERC90P5	CCATACCGTTTCAACTGGTGCAAAAAAAGCCGGATTTCTCCGGCTGTTGACTTACGCCCCGCCCTGCCAC
PERCP1	AAAGGAAGGTAAATTCAGGATAGCAGTCTGTAGATAATCGGAGGTCACTTGTGTAGGCTGGAGCTGCTTC
PERCP2	TAAAGTCGCTTTTTCTTATGGTAACAGGCAATAACGCTCTCAGATATTTTCATATGAATATCCTCCTTAGT
P1R	GAAGCAGCTCCAGCCTACAC
P6R	ACAGGGACACCAGGATTTAT
PERCC2	TTGTATGGGCAGAGAAGGCA
LERKPN	GG**GGTACC**CCGTGATGCGGCAATTAGGACT
LERC1	GAAACGGTTCAGCTTGGTTT
LERC2	ATCACACGCAATGAGCAGTT
PERC89BHI	CG**GGATCC**CGCGCTGAGACCGTCAGATATG
PERC89STU	GA**AGGCCT**TCCTGTTTGCCATTCCTTGCTT
PERC90BHI	CG**GGATCC**CGCACAGGCACAGGGTAATC
PERC90STU	GA**AGGCCT**TCACCGTTTCAACTGGTGCAAA
PERC1BHI	CG**GGATCC**CGCAGGCAATAACGCTCTCAGA
PERC2STU	GA**AGGCCT**TCTGTCCGTATTGTATGGGCAG
PERC3BHI	CG**GGATCC**CGAAGCGACCACTAAAGTCGCT
PERC4STU	GA**AGGCCT**TCTTCAGGATAGCAGTCTGTAG

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(w/v) glycerol and appropriate antibiotics without shaking at 37 °C in the presence of 5 % CO_2. All assays were done in triplicate and were repeated at least three times.

Analysis of proteins in culture supernatant and whole-cell lysates. Bacteria were grown under the same culture conditions as those for the β-galactosidase assay until they reached an OD_{600} of 0·7. The bacteria were removed by centrifugation (10 000 ***g*** for 20 min), and the proteins secreted in the culture supernatant were precipitated with 10 % (w/v) TCA and separated on a 12 % (w/v) SDS-polyacrylamide gel, as described previously (Iyoda & Kutsukake, 1995; Iyoda *et al.*, 2001). Western blotting was performed with anti-EspB and anti-Stx2A polyclonal antibodies to probe for each protein in whole-cell lysates derived from equal amounts of bacteria normalized by measurement of OD_{600}. Binding of secondary anti-mouse IgG antibody conjugated to horseradish peroxidase was detected using ECL Western blotting detection reagents (Amersham). All assays were performed in triplicate and were repeated at least three times. The protein bands were scanned by densitometer (Cool saver analyser; ATTO, Japan), and the scanned data were shown as percentage intensity with standard errors of each band (wild-type = 100 %).

Assay of enterohaemolysin activity. Production of haemolysin from EHEC was examined on enterohaemolysin test plates (EHT plate, Kyokuto, Japan) which contained 5 % washed sheep erythrocytes. The size of the haemolytic lysis zone was recorded after incubation for 3 h (for α-haemolytic activity) or 18 h (for enterohaemolysin activity) at 37 °C with or without 5 % CO_2. The same experiments were repeated twice, using at least four independent colonies of each tester strain: wild-type Sakai, SKI3-104a, SKI3-104b and SKI3-104c.

HEp-2 adhesion assay. HEp-2 cells maintained in DMEM supplemented with 10 % (v/v) fetal bovine serum (FBS) were plated onto cover slips in six-well plastic plates at 2×10^5 cells ml^{-1} and incubated for 24 h in the presence of 5 % CO_2. After inoculation of 10^7 bacteria into each well, the plastic plate was centrifuged at 1000 ***g*** for 5 min and incubated for 1 h at 37 °C in the presence of 5 % CO_2. The cells were then washed three times with PBS and incubated for another 6 h. The monolayers were washed six times with PBS, fixed with 100 % methanol, and then stained with Giemsa's solution for observation by phase-contrast microscopy. We defined a microcolony as a cluster consisting of more than ten bacteria associated with HEp-2 cells. The assay was performed in triplicate and was repeated at least three times. The number of microcolonies per adhesion site ($n > 200$) was shown as a percentage. Statistical analysis of the results was performed by Student's two-tailed *t* test on data recorded from at least three independent assays.

RESULTS

Identification of the *perC* homologue as a putative transcriptional activator necessary for the expression of LEE genes in EHEC

To clarify the regulatory mechanism of the LEE genes in EHEC, we screened a genomic library of the EDL933-1 strain, which has a deletion in the *ler* gene, as described in Methods. We examined more than 5000 transformants and identified several clones that increased or decreased the expression of *espB–lacZYA* on pLEE19 (data not shown). Among them, we focused on the characterization of one plasmid clone that contained a 10·1 kbp *Sph*I fragment (designated pSPH2). pSPH2 had the ability to activate the expression of *espB–lacZYA* more than fourfold compared to pACYC184 in EDL933-5141 and SKI-5141 background (data not shown; Table 4). Sequence analysis showed that the inserted fragment in pSPH2 contained a *perC*-like sequence (data not shown). As *perC* has been shown to be responsible for the full activation of LEE expression in EPEC (Gómez-Duarte & Kaper, 1995; Mellies *et al.*, 1999), we next examined whether this *perC* homologue was able to upregulate the expression of *espB–lacZYA* in EHEC O157. For this purpose, we amplified only the *perC*-like sequence from pSPH2 (extracted from *E. coli* K-12 strain JM109) by PCR, using the primers PERC1BHI and PERC2STU, and recloned it into pBR322C. The resultant plasmid, $pBRCC_{EDL}$, enhanced the expression of *espB–lacZYA* more than fourfold compared to pBR322C in EDL933-5141 or SKI-5141 (data not shown; Table 4), suggesting that this plasmid contains a functional *perC* homologue of EHEC O157.

PerC homologues found in the EHEC O157 Sakai and EDL933 genomes

The complete genome sequence of Sakai strain reveals that there are five *perC* homologues on the chromosome (accession no. NC_002695): three of them code for 104 aa proteins, and the other two encode proteins of 89 and 90 aa, respectively. In this study, we renamed the first three genes *pchA* (ORF number of Sakai genome, ECs1091), *pchB* (ECs2182) and *pchC* (ECs2737), respectively, and the last two genes *pchD* (ECs1388) and *pchE* (ECs1588) (Table 3). However, these *pch* genes have not been annotated on the published genome sequence of strain EDL933 (accession no. NC_002655). We therefore searched the EDL933 genome for *pch* sequences and found that it contains two *pchD* sequences (located between ORFs Z1204 and Z1205, and between ORFs Z1644 and Z1645), one *pchE* (between ORFs Z1845 and Z1846) and one *pchC* (between ORFs Z2366 and Z2367) sequence (summarized in Table 3). This analysis indicated that EDL933 had only one *pch* gene, which encoded 104 aa (corresponding to *pchC*), on the genome. However, we also found that the *pch* gene cloned on pSPH2 was not *pchC*, but *pchB* (data not shown). This discrepancy prompted us to examine whether there are three different

Table 3. Location of *pch* genes on the EDL933 genome

EDL933 genome position refers to the nucleotide sequence of accession no. NC_002655. ND, Not determined.

Sakai genome		EDL933 genome	
Gene	ORF	Located between ORFs:	Position (start–end)
pchA	ECs1091	ND	ND
pchB	ECs2182	ND	ND
pchC	ECs2737	Z2366 and Z2367	2139137–2138823
pchD	ECs1388	Z1204 and Z1205	1127902–1128171
		Z1644 and Z1645	1523508–1523777
pchE	ECs1588	Z1845 and Z1846	1685607–1685879

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Table 4. Effect of multicopy *pch* genes on the expression of a *espB–lac* fusion gene

Enzyme activities (Miller units) are the mean of three independent experiments. SD values shown in parentheses. ND, Not determined.

Plasmid	*β*-Galactosidase activity of *espB–lac* in:	
	SKI-5141	MC4100
pACYC184	27·4 (3·4)	7·99 (0·27)
pSPH2	88·2 (4·6)	ND
pACLER	215 (1·4)	264 (37)
pBR322C	38·1 (1·1)	4·61 (0·20)
pBRCC89	22·5 (2·3)	4·75 (0·58)
pBRCC90	52·2 (6·6)	5·12 (1·2)
pBRCC104a	202 (8·3)	6·4 (0·48)
$pBRCC_{EDL}$(=pBRCC104b)	173 (2·0)	5·97 (0·29)
pBRCC104c	189 (3·2)	6·3 (0·52)

pch (*pchABC*) genes encoding 104 aa proteins on the EDL933 genome. Sequence analysis of the PCR products amplified with the PERC3BHI and PERC2SPH primers clearly showed that the EDL933 strain used in this study possessed *pchA*, *B* and *C* genes on the genome (data not shown). In order to further confirm this result, we obtained the genome-sequenced strain of EDL933 (ATCC 700927) from ATCC. We confirmed that ATCC 700927 certainly contains *pchA*, *B* and *C* (data not shown), though the location of *pchA* and *pchB* on the genome is currently unknown. These results indicated that the published genome sequence of EDL933 lacked the sequence information corresponding to the *pchA* and *pchB* genes. Because sequence information of *pchA*, *pchB* and *pchC* was currently available for the Sakai strain, we further characterized the *pch* genes of Sakai.

Multicopy plasmids carrying *pchA*, *B* or *C* enhanced the transcription of *espB–lacZYA*

We cloned all the *pch* genes from the Sakai genome onto pBR322C to test whether they all act as positive regulatory genes. All the multicopy plasmids pBRCC104a, pBRCC104b (exactly the same as $pBRCC_{EDL}$) and pBRCC104c enhanced transcription of *espB–lacZ* more than fourfold compared to pBR322C (Table 4) in a SKI-5141 background. On the other hand, these plasmids did not activate *espB–lacZ* in a MC4100 background (Table 4), although the introduction of pACLER carrying the *ler* gene significantly enhanced the expression of *espB–lacZ* compared to pACYC184 (Table 4). The multicopy plasmids pBRCC89 and pBRCC90 did not significantly affect the expression of *espB–lacZYA* (Table 4). Thus, *pchABC* genes, at least, seem to be functional in the upregulation of LEE genes in EHEC O157. Consistent with these results, the predicted sequence for the PerC of the EPEC E2348/69 strain showed a slightly higher similarity to those of PchA, PchB and PchC (47 % identical and 67 % similar over the homologous region) than that of PchD (25 % identical and 54 % similar over the homologous region) or PchE (39 % identical and 65 % similar over the homologous region), although the PerC of E2348/69 encodes 89 aa (Fig. 1A, B). Comparison of the amino-acid sequence of PchA in the BLAST database with BLASTP revealed sequence similarity with several other hypothetical proteins. These include YfdN of uropathogenic *E. coli* strain CFT073 (42 % identical and 54 % similar over the homologous region), YfdN of *E. coli* K-12 (38 % identical and 50 % similar over the homologous region) and Sb43 of *Salmonella enterica* serovar Typhimurium phage ST64B (38 % identical and 54 % similar over the homologous region), although the functions of these proteins are currently unknown.

Effect of deletion(s) in *pch* genes on the expression of Esp proteins

We next examined the effect of mutation(s) in *pch* genes on the expression of LEE genes. For this purpose, we constructed the SKI3-89, SKI3-90, SKI3-8990, SKI3-104a, SKI3-104b and SKI3-104c strains. Proteins in the culture supernatants from equal amounts of these strains were analysed by SDS-PAGE (Fig. 2). As suggested in the above experiment using the plasmid pBRCC89 or pBRCC90, a deletion in *pchD* or *pchE* alone, or deletions in both genes did not affect the expression of Esp proteins (EspP, D, B and A, based on their mobility on the gel) under our assay conditions (Fig. 2A). In contrast, a deletion in *pchA* or *pchB* reduced the expression of Esp proteins compared to the wild-type strain (Fig. 2B, lanes 1, 3 and 4), and this reduction was complemented by the introduction of pBRCC104a, pBRCC104b or pBRCC104c (Fig. 2B, lanes 3, 4, 7 and 8; data not shown). All four Esp proteins were detected in larger amounts in the culture supernatant of strains carrying pBRCC104a than those carrying pBR322C (Fig. 2B). The amounts of EspB in whole-cell lysates derived from equal amounts of those strains were further examined by Western blotting using a polyclonal antibody against EspB (Fig. 3A, B). In strains SKI3-104a or SKI3-104b carrying pBR322C, EspB was detected in a lower amount than that in the parent strain harbouring

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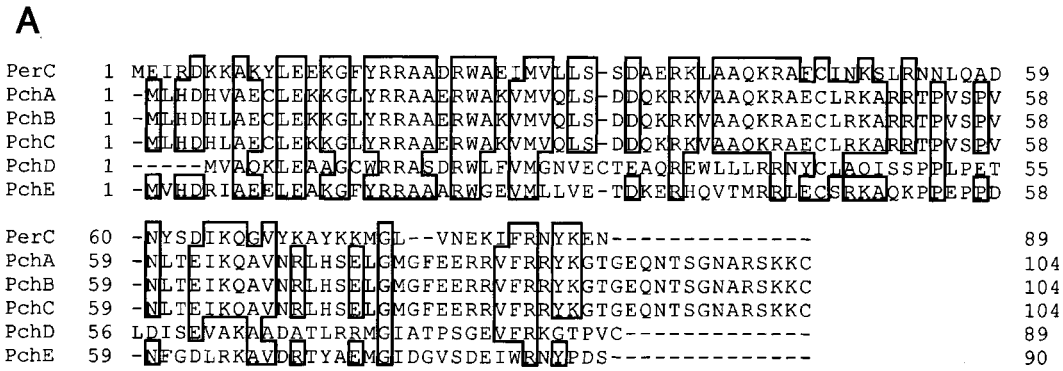

B

Identical aa / homologous region (%)
Similar aa / homologous region (%)

	PchA	PchB	PchC	PchD	PchE
PerC	41 / 87 (47) 59 / 87 (67)	41 / 87 (47) 59 / 87 (67)	41 / 87 (47) 59 / 87 (67)	21 / 81 (25) 44 / 81 (54)	35 / 89 (39) 58 / 89 (65)
PchA		103 / 104 (99) 104 / 104 (100)	102 / 104 (98) 104 / 104 (100)	23 / 81 (27) 44 / 81 (53)	36 / 87 (41) 57 / 87 (65)
PchB			103 / 104 (99) 104 / 104 (100)	22 / 83 (26) 44 / 83 (53)	36 / 87 (41) 57 / 87 (65)
PchC				22 / 83 (26) 44 / 83 (53)	36 / 87 (41) 57 / 87 (65)
PchD					32 / 82 (39) 47 / 82 (57)

Fig. 1. Sequence similarity of PerC homologues. GENETYX software (GENETYX, Japan) was used for analysis. (A) Alignment of predicted amino-acid sequences of PerC of EPEC strain E2348/69 (accession no. CAA88447), PchD (BAB34811), PchE (BAB35011), PchA (BAB34514), PchB (BAB35605) and PchC (BAB36160). Amino acid residues conserved in more than four proteins are indicated by boxes. Bars indicate gaps. (B) Pairwise similarities of PerC of E2348/69 and PchABCDE, as described in (A). The numbers of residues identical (upper) and similar (lower) between two sequences over the homologous region are indicated. Numbers in parentheses indicate percentage of identity (upper) or similarity (lower).

pBR322C (Fig. 3B, lanes 1, 3 and 4), and this reduction was complemented by the introduction of pBRCC104a (Fig. 3B, lanes 7 and 8), as shown in Fig. 2B. We also examined the expression of Esp proteins in strains containing different combinations of double deletions in *pchABC* (Fig. 4). For this purpose, we constructed the SKI3-104ab, SKI3-104bc and SKI3-104ca strains. The amounts of Esp proteins in culture supernatant from equal amounts of SKI3-104ab and SKI3-104ca culture (Fig. 4, lanes 5 and 6) were lower than those from each single mutant (Fig. 4, lanes 2–4), and comparable to those from SKI-1, which carries a deletion in the *ler* gene (Fig. 4, lane 8).

Effect of a deletion in *pchABC* on the expression of other virulence factors

Because Stx is responsible for severe clinical symptoms associated with EHEC infection, we examined whether the dosage of *pchA*/*B*/*C* affects the expression level of Stx. As shown in Fig. 3C, the expression level of Stx2, probing with anti-Stx2A polyclonal antibody, was not significantly changed by a deletion in any *pchABC* gene or by overexpression of *pchA*. We also examined the effect of a deletion in *pchA*, *B* or *C* on the expression of enterohaemolysin activity on EHT plates, as described in Methods, and observed that all the deletions in *pchA*/*B*/*C* did not

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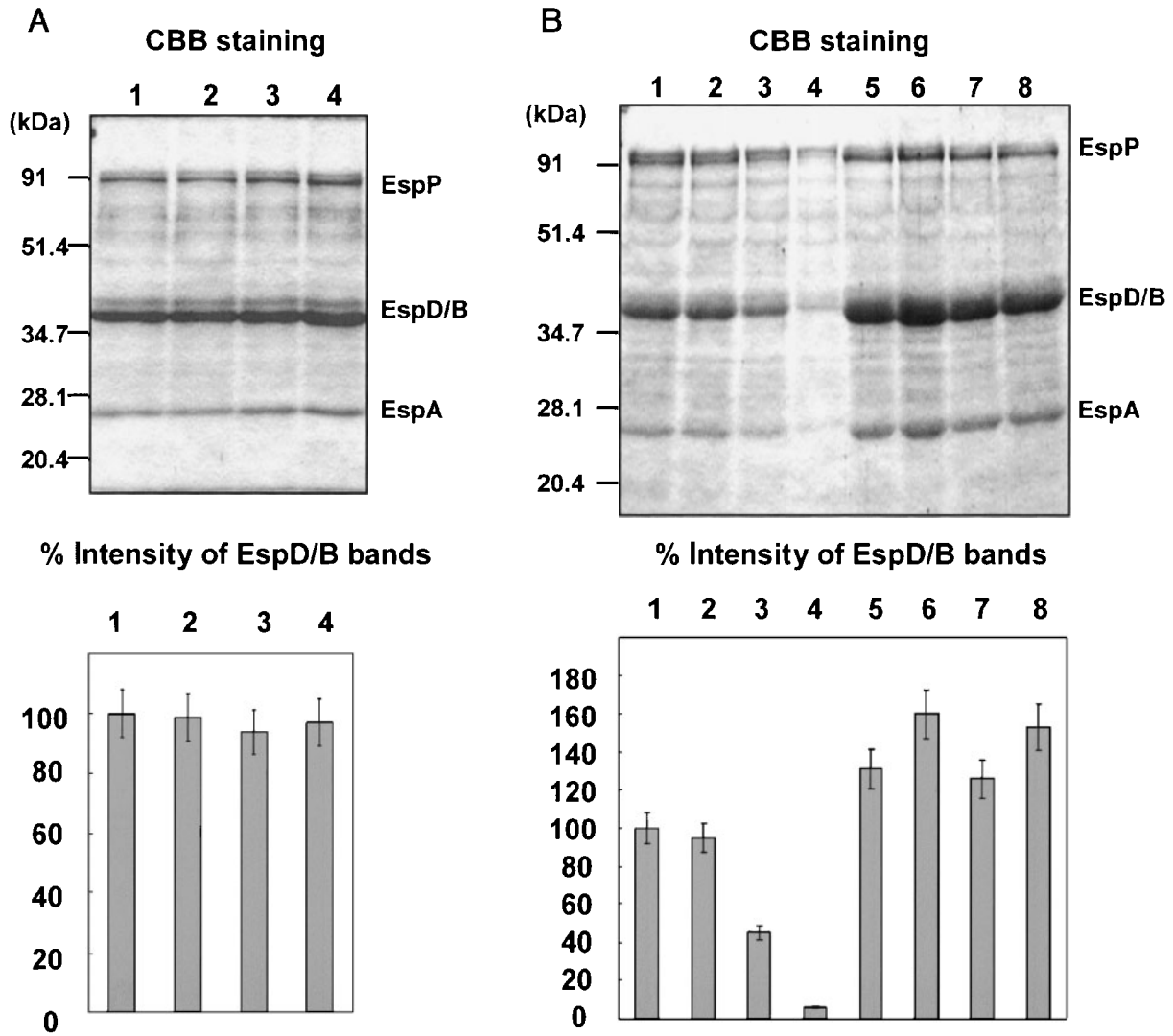

Fig. 2. Effect of *pch* mutation(s), or a multicopy plasmid carrying *pchA*, on the secretion of Esp proteins. Upper two panels show Coomassie Brilliant Blue staining SDS-PAGE profiles of the Esp proteins secreted in the culture supernatant, derived from equal amounts of wild-type Sakai or *pch* mutants, as described in Methods. Lower two panels show relative band intensity of EspD/B bands, with standard errors, scanned by densitometer (Sakai or Sakai/pBR322C = 100 %). Strains used in (A): lane 1, Sakai; lane 2, SKI3-89; lane 3, SKI3-90; lane 4, SKI3-8990. (B): lane 1, Sakai/pBR322C; lane 2, SKI3-104c/pBR322C; lane 3, SKI3-104b/pBR322C; lane 4, SKI3-104a/pBR322C; lane 5, Sakai/pBRCC104a; lane 6, SKI3-104c/pBRCC104a; lane7, SKI3-104b/pBRCC104a; lane 8, SKI3-104a/pBRCC104a.

seem to affect the enterohaemolysin activity of Sakai (data not shown).

The positive regulatory effect of *pchABC* on LEE gene expression is mediated through the upregulation of *ler*

In the experiments mentioned above, a deletion or deletions in *pchA* and/or *pchB*, or overexpression of *pchA*, decreased or increased, respectively, the expression of all Esp proteins (EspA, B, D and P), suggesting that these effects are mediated through upregulation of a central regulator of LEE genes. As Ler is a transcriptional activator for the LEE genes in both EPEC and EHEC (Elliott *et al.*, 2000), we examined whether *ler* is under the positive regulation of *pchABC*. For this purpose, we constructed a single-copy plasmid carrying the promoter region of *ler* fused to promoter-less *lacZYA* genes (designated pFZLERp), as described in Methods. We examined the effect of pBRCC104a on transcription from the *ler* promoter in both SKI-5141 and MC4100 backgrounds. Activity of the *ler* promoter on pFZLERp in SKI-5141 was more than eight times higher than that in MC4100 (Table 5), suggesting that the transcription of *ler* in EHEC is under the

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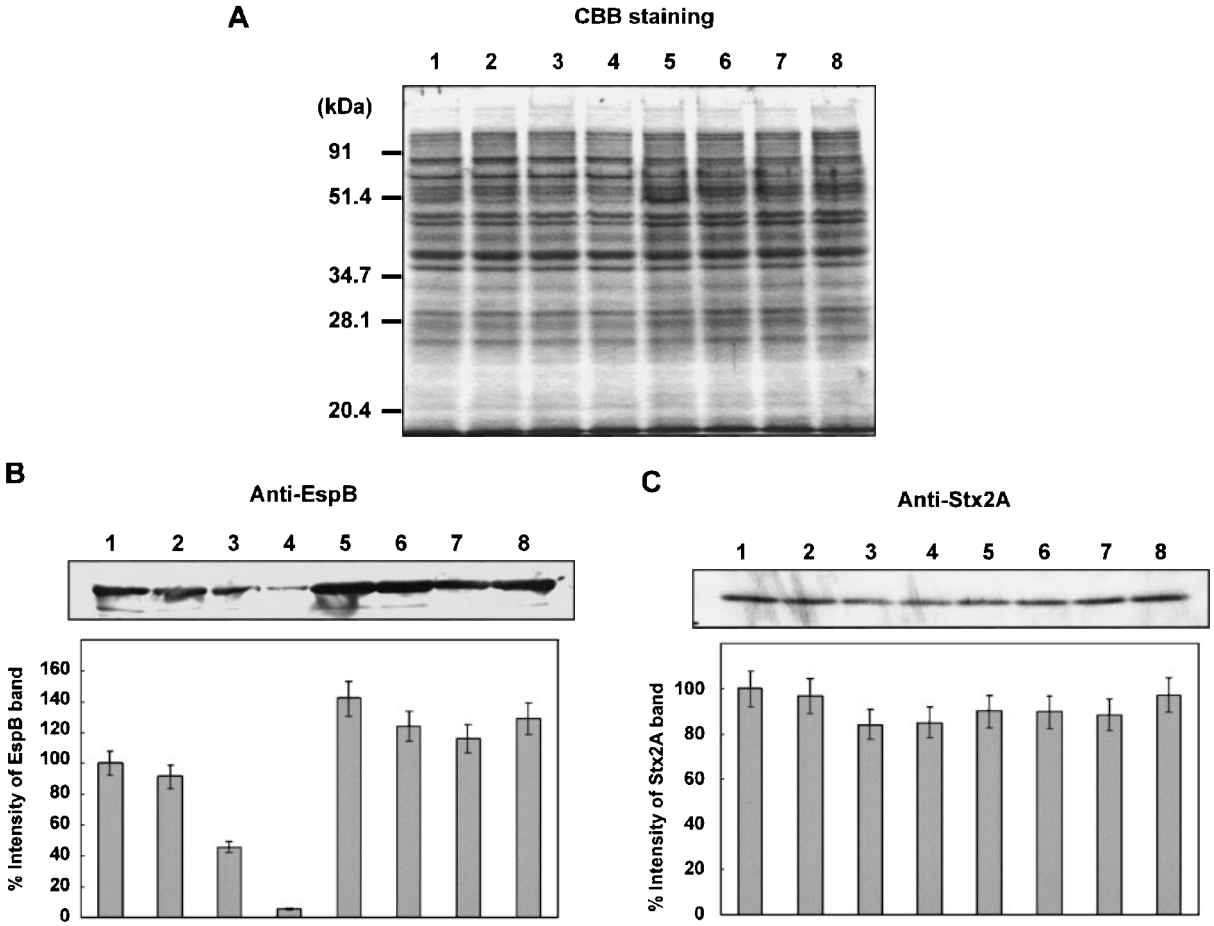

Fig. 3. Effect of deletion in *pchABC*, or a multicopy plasmid carrying *pchA*, on the expression of EspB and Stx2A. Coomassie Brilliant Blue staining SDS-PAGE profiles (A) and Western blotting analysis of EspB (B) and Stx2A (C) of the whole-cell lysates derived from equal amounts of the same strains, as described in Fig. 2(B). Lower two panels of (B) and (C) are relative intensity of each band, with standard errors, scanned by densitometer (Sakai/pBR322C = 100 %).

positive control of regulator(s) that are not found in the *E. coli* K-12 strain. Introduction of the plasmid pBRCC104a enhanced transcription from the *ler* promoter more than fivefold compared to pBR322C in both SKI-5141 and MC4100 backgrounds (Table 5). We also examined the effect of a deletion in *pchA*/*B*/*C* on the transcription of *ler*. For this purpose, we constructed SKI-5168a, SKI-5168b and SKI-5168c strains. The activity of the *ler* promoter in SKI-5168a, which carried the *pchA* deletion most effective towards the expression of Esp proteins, as described above, was three times lower than that in wild-type background (Table 6). These results indicated that the positive effect of *pchABC* on the expression of *espB* was mediated through the activation of *ler* transcription in EHEC O157.

Effect of deletion(s) in *pchABC* on adhesion of Sakai to HEp-2 cells

Previous studies in EPEC have shown that the *perA* and *perC* genes enhance localized adhesion of EPEC to HEp-2 cells (Gómez-Duarte *et al.*, 1995). We examined whether a deletion in *pchA*/*B*/*C* affects adhesion of Sakai to HEp-2 cells. As shown in Fig. 5A, the wild-type Sakai adhered to HEp-2 cells and formed microcolonies. The SKI3-104a and SKI3-104b mutant strains showed reduced adhesion to HEp-2 cells compared to wild-type or SKI3-104c (Fig. 5A–D): the number of microcolonies formed by SKI3-104a or SKI3-104b per adhesion site was less than that of wild-type or SKI3-104c ($P<0{\cdot}05$ in each case, Fig. 5K). Double deletions in *pchA*/*pchB* or *pchA*/*pchC* further decreased adhesion to HEp-2 cells compared to each single mutation, and, similar to the situation observed for the *ler*-deleted strain SKI-1 (Fig. 5E, F, H), few microcolonies were observed ($P<0{\cdot}05$ in each case, Fig. 5K). Furthermore, introduction of a *ler*-carrying plasmid, pGEMLER, restored microcolony formation in all single or double mutants of *pchABC* ($P<0{\cdot}05$ in each case, Fig. 5I; data not shown), suggesting that the effect of *pchABC* on adhesion to HEp-2 cells was mediated through activation of *ler* expression. Additionally, mutants carrying

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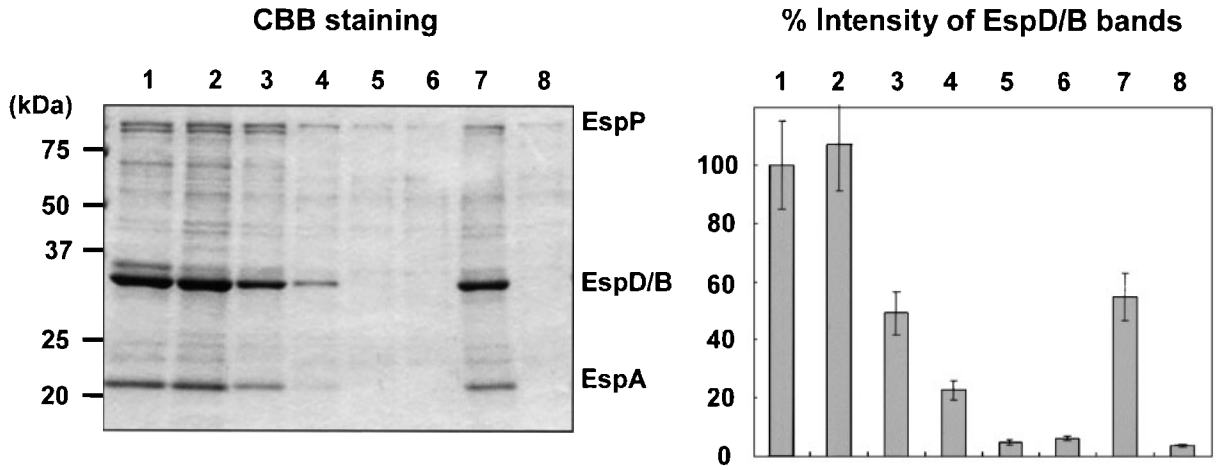

Fig. 4. Effect of double deletions in *pchABC* genes on the secretion of Esp proteins. Left panel shows Coomassie Brilliant Blue staining SDS-PAGE profile of the Esp proteins secreted in the culture supernatant derived from equal amounts of tester strains. Right panel shows relative band intensity of EspD/B bands, with standard errors, scanned by densitometer (Sakai = 100 %). Strains used: lane 1, Sakai; lane 2, SKI3-104c; lane 3, SKI3-104b; lane 4, SKI3-104a; lane 5, SKI3-104ab; lane 6, SKI3-104ca; lane 7, SKI3-104bc; lane 8, SKI-1.

pBRCC104a, b, or c formed dense microcolonies on the surface of HEp-2 cells (Fig. 5J; data not shown) with larger numbers observed ($P<0{\cdot}0003$ in each case; Fig. 5J, K), indicating that *pchABC* positively regulates adhesion of EHEC O157 to HEp-2 cells.

Prevalence of *pch* genes in EHEC

Finally, we examined the prevalence of the *pchABC*, *pchD* and *pchE* genes among a total of 90 clinical isolates of LEE-positive EHEC (30 each of the O157, O26 and O111 serogroups), originating from epidemiologically unrelated cases (data not shown), by PCR with PERC3BHI/PERC4STU, PERC89BHI/PERC89STU or PERC90BHI/PERC90STU primer sets, respectively. All the strains were found to contain the *pchABC* genes (not determined whether *pchA*, *B* or *C*; data not shown), with most O157 and O26 (>80 %) strains possessing the *pchD* and *pchE* genes (data not shown).

DISCUSSION

Identification of the functional *pch* genes in EHEC

Though there have been many studies of the regulatory mechanism of LEE expression in EPEC, it remains unclear whether these regulatory systems are all applicable to EHEC. To clarify the regulatory mechanisms of the LEE genes in EHEC, we screened an EHEC O157 genomic library, and identified a *perC* homologue (*pch*) as a positive regulatory gene for LEE expression in EHEC O157. It was shown that five *pch* genes are found in the Sakai genome sequence, while these are not annotated on the EDL933 genome sequence (Table 3). We demonstrated that the EDL933 genome has *pchA*, *B* and *C* genes on the genome, although the location of *pchA* and *pchB* has not been assigned. Perna *et al.* (2001) stated that the genome sequence of EDL933 has two physical

Table 5. Effect of a multicopy plasmid carrying *pchA* on the expression of a *ler–lac* fusion gene

Enzyme activities (Miller units) are the mean of three independent experiments. SD values shown in parentheses.

Plasmid	*β*-Galactosidase activity of *ler–lac* in:	
	SKI-5141	MC4100
pBR322C	633 (37)	75·2 (1·7)
pBRCC104a	3410 (320)	436 (12)

Table 6. Effect of *pchA/B/C* deletion on the expression of a *ler–lac* fusion gene

Enzyme activities (Miller units) are the mean of three independent experiments. SD values shown in parentheses.

Strain	*β*-Galactosidase activity of *ler–lac*
SKI-5141	710 (7·5)
SKI-5168a	226 (33)
SKI-5168b	695 (5·6)
SKI-5168c	731 (28)

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gaps that correspond to phage-related sequences. The published genome sequence of Sakai indicates that all the *pch* genes, except *pchD*, are carried by prophage-like sequences (*pchE* is located on prophage Sp7, *pchA* on Sp4, *pchB* on Sp11, and *pchC* on Sp14 [Hayashi *et al.*, 2001b; accession no. NC_002695]). Thus, the missing *pchA* and *pchB* genes on the EDL933 genome may also be carried by phage-associated sequences like other *pch* sequences of EDL933 (*pchE* is located on prophage CP-933C, and *pchC* on CP933-R [accession no. NC_002655]).

A deletion in *pchA* or *pchB* decreased expression of the Esp proteins and the number of microcolonies on HEp-2 cells under our assay conditions (Figs 2, 3 and 5), and these effects were more prominent in combination with an additional deletion in *pchB* or *pchA*, respectively (Figs 4 and 5). These results suggest that the functions of *pchA* and *pchB* are important for LEE expression and adhesion to HEp-2 cells, and the effect of each gene is additive. While the effect of a single deletion in *pchC* on the expression of LEE and adhesion phenotype was not significant under our assay conditions, an effect was observed in a *pchA*-deletion background (Figs 4 and 5). Additionally, *pchA*, *B* or *C* genes on a multicopy vector enhanced the expression of LEE genes (Table 4). These results suggest that *pchC* is also functional, but its expression on the chromosome may be low or induced under different conditions which we have not examined. In the LEE-regulation system in EPEC, the *per* locus is essential for the expression of bundle-forming pili, which is thought to be an initial attachment factor of EPEC to the surface of epithelial cells (Gómez-Duarte & Kaper, 1995; Tobe *et al.*, 1996). It is possible that an unspecified pilus factor involved in the adhesion of EHEC O157 to HEp-2 cells is under the control of the *pchABC* genes. Further study to clarify this hypothesis is now in progress.

The effect of the *pch* gene on LEE expression is mediated through *ler*

Our results also showed that positive regulation by *pchABC* on the expression of LEE genes in EHEC O157 is mediated through the enhanced transcription of *ler* (Tables 4, 5 and 6), as described for EPEC (Mellies *et al.*, 1999). Since the positive regulation by *pchABC* on transcription from the *ler* promoter was also observed in an MC4100 background (Table 5), PchABC may directly regulate *ler* transcription, or induce another regulator common to both the EDL933 and MC4100 genomes.

Prevalence of *pch* in EHEC

We found that a total of 90 EHEC strains (30 each of the O157, O26 and O111 serogroups) possessed *pchA*, *B* and/or *C* gene(s) (data not shown). These results suggest that the *pch*-mediated transcriptional activation of LEE genes may not be specific to EPEC, but rather a general mechanism in LEE-positive EHEC strains. We could not demonstrate a significant effect of *pchD* and *pchE* on the expression of LEE genes, even when each gene was introduced by a multicopy plasmid (Table 4) or when both genes were disrupted (Fig. 2). Alignments of predicted amino-acid sequences of PerC homologues indicated that identities between PchD/E and others (PchABC or PerC of E2348/69) are in the range of 25 to 41 %, while those between PerC of E2348/69 and each PchABC are all 47 % (summarized in Fig. 1B). As there are several amino-acid residues conserved between PerC of E2348/69 and PchABC, but not found in PchD or PchE, these residues may be necessary for upregulation of the LEE genes in EHEC. Additionally, we detected the *pchD* and *pchE* genes with high frequency among EHEC O157 and O26 strains (data not shown), all of which were isolated from patients. Thus, we cannot currently rule out the possibility that they are functional under conditions which we have not examined.

Gene organization of the *pchABC* genes

As described above, all the *pch* genes, except *pchD*, appear to be carried by prophage-like elements on the Sakai genome. Ohnishi *et al.* (2001) hypothesize that multiple prophages integrating on the EHEC O157 genome may contribute to variation of the EHEC genome due to possible recombination events among similar prophage genomes or their horizontal transfer. If this occurs frequently, the copy number of *pch* per genome may not be fixed, and more or fewer *pch* genes may be found in other EHEC genomes. More careful genetic and biochemical study, including prevalence of the *pch* in EHEC, will elucidate the role of multiple sets of *pch* on the regulation of LEE genes and their contribution to the virulence phenotype of EHEC. *perC* is a member of the *per* operon on the EAF plasmid in EPEC, together with the *perA* and *B* genes (Gómez-Duarte & Kaper, 1995). *perA* encodes an AraC-like transcriptional regulator that activates transcription from its own promoter, and this autoactivation is presumed to be necessary for full activation of the LEE genes by *perC* (Martinez-Laguna *et al.*, 1999). In several EHEC strains, including Sakai and EDL933, however, there are no *perA*- and *B*-like sequences (Gómez-Duarte & Kaper, 1995; Perna *et al.*, 2001; Hayashi *et al.*, 2001a, b). As potential transcriptional terminator sequences are found in the upstream regions of all the *pchABC* genes (data not shown), they do not seem to constitute an operon with other regulatory genes as seen with the EAF plasmid. Thus, the regulatory mechanism for the expression of *pchABC* itself in EHEC should be different from that of EPEC. An unidentified AraC-like transcriptional regulator may be involved in the regulation of *pch* expression and subsequent activation of LEE expression in EHEC. Alternatively, the multiplicity of *pchABC* may simply compensate for the absence of a *perA*-like gene for the expression of LEE in EHEC.

Regulation of *pch* expression

Previous reports have indicated that several transcriptional regulators are involved in the expression of LEE genes, some

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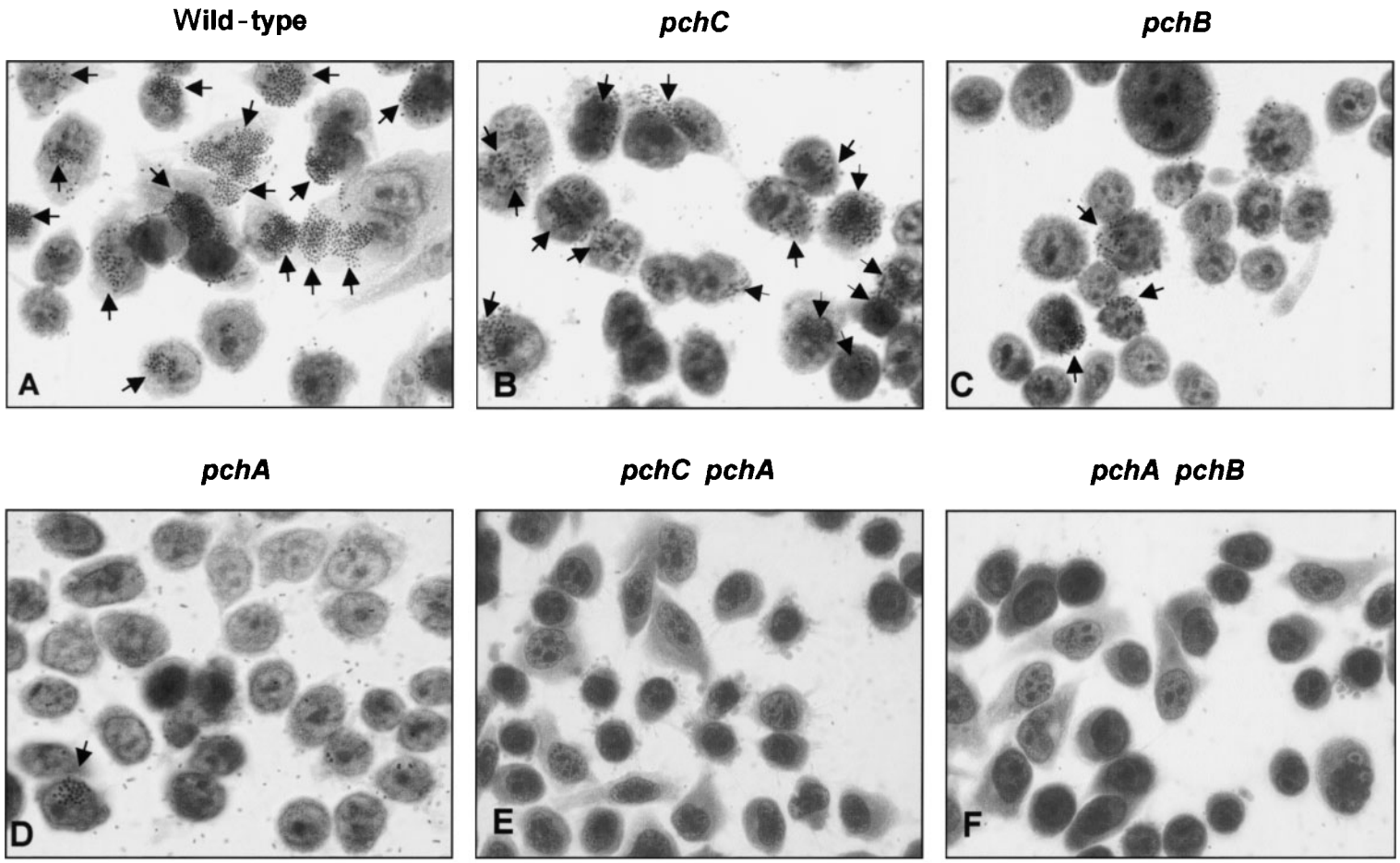

Fig. 5. Effect of deletion(s) of *pchABC* genes on adherence to HEp-2 cells. Strains used: (A), Sakai/pBR322C; (B), SKI3-104c/pBR322C; (C), SKI3-104b/pBR322C; (D), SKI3-104a/pBR322C; (E), SKI3-104ab/pBR322C; (F), SKI3-104ca/pBR322C; (G), SKI3-104bc/pBR322C; (H), SKI-1/pBR322C; (I), SKI3-104a/pGEMLER; (J), SKI3-104a/pBRCC104a. Each photograph presented is representative of one of three to five independent assays with comparable results. Black arrows indicate microcolonies developed on HEp-2 cells. The number of microcolonies per adhesion site ($n > 200$) is shown in (K): the data shown are means of three to five independent assays with standard errors.

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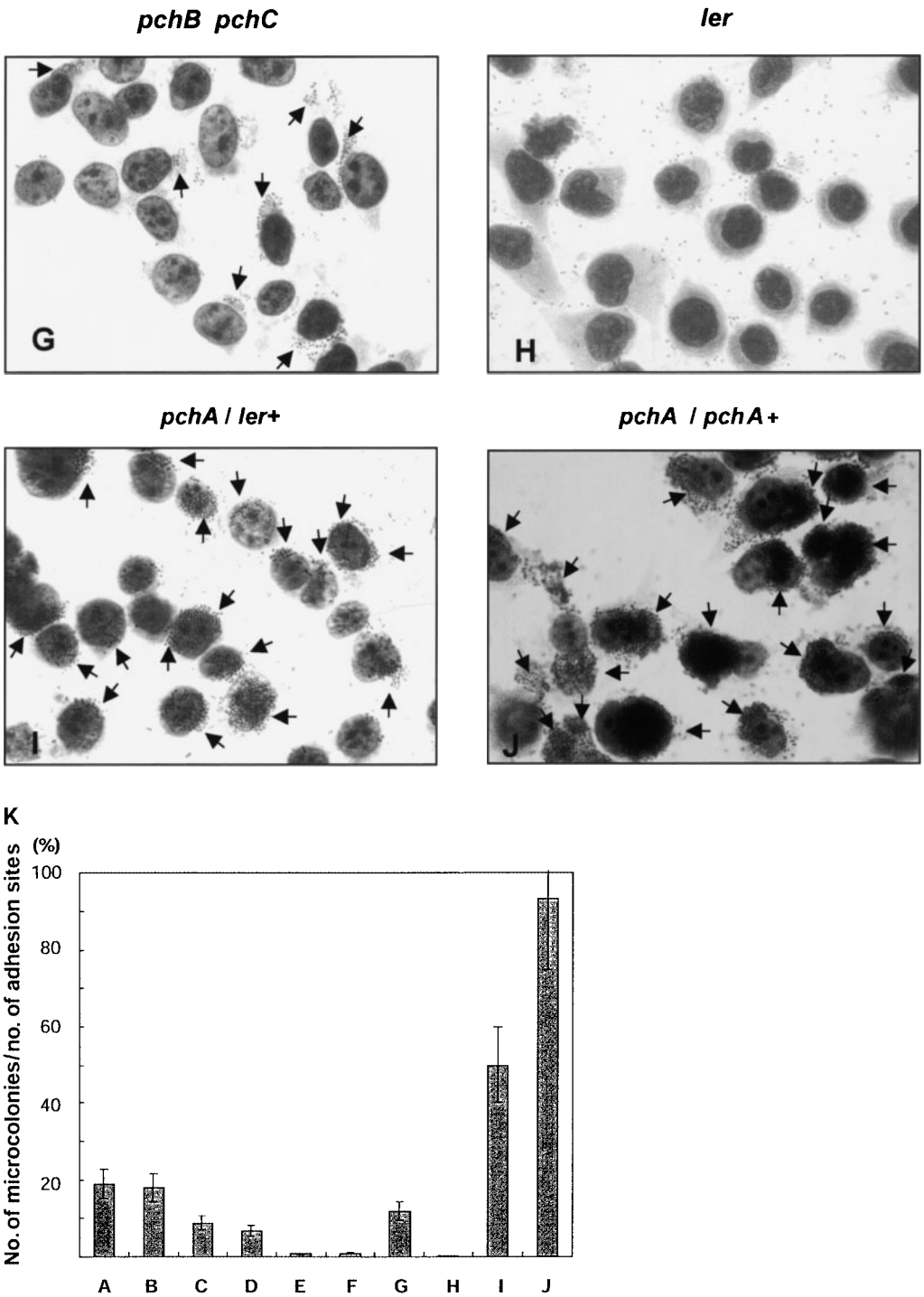

of which have been shown to bind directly to the regulatory region of the LEE operon (Friedberg *et al.*, 1999; Sperandio *et al.*, 2000; Umanski *et al.*, 2002; Haak *et al.*, 2003), while others remain to be proved. It is possible to hypothesize that some of these transcriptional regulators affect LEE expression via direct regulation of one or more *pch* in EHEC. Further analysis of the regulatory mechanism of *pch* expression is needed for better understanding of the specific regulation of LEE expression in EHEC.

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