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Regulation of virulence by butyrate sensing in enterohaemorrhagic *Escherichia coli*

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Enterohaemorrhagic *Escherichia coli* (EHEC) colonizes and proliferates at the mucosal surface, inducing severe diarrhoea. Short-chain fatty acids (SCFAs) are abundant in the intestine owing to the metabolic activity of microflora, and are important for colonic health. We found that, although a high concentration of SCFAs inhibited the growth of EHEC, at low concentrations, the SCFAs markedly enhanced the expression of the virulence genes required for cell adherence and the induction of attaching and effacing (A/E) lesions. Of the SCFAs tested, butyrate markedly enhanced the expression of these virulence-associated genes, even at the low concentration of 1.25 mM, but acetate and propionate showed only a small effect at concentrations higher than 40 mM. Butyrate enhanced the promoter activity of the *LEE1* operon, which encodes a global regulator of the LEE genes, Ler. This enhancement was dependent on a regulator, PchA. Butyrate sensing was completely abrogated by the deletion of *lrp*, the gene for the leucine-responsive regulatory protein, Lrp. Expression of a constitutively active mutant of Lrp enhanced the expression of the LEE genes in the absence of butyrate, and a response-defective Lrp derivative reduced the response to butyrate. Thus, upon entering the distal ileum, EHEC may respond to the higher butyrate level via Lrp by increasing its virulence expression, leading to efficient colonization of the target niche.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) is a human pathogen that causes a wide spectrum of illnesses, ranging from mild diarrhoea to severe diseases such as haemorrhagic colitis and haemolytic uraemic syndrome. The hallmark of EHEC pathogenicity is the formation of attaching and effacing (A/E) lesions on intestinal epithelial cells (Nataro & Kaper, 1998). A/E lesions are characterized by the localized destruction of brush border microvilli and the intimate attachment of bacteria to the membrane of host cells. The formation of A/E lesions is mediated by the type III secretion system (T3SS), which translocates virulence factors called effectors into host cells, and the outer-membrane protein intimin, which is necessary for intimate attachment. These genes are encoded in the LEE pathogenicity island, a chromosomal locus in enteropathogenic strains of *E. coli*. The LEE genes consist of five operons and several cistrons, and their expression is coordinately regulated at the level of transcription (Mellies *et al.*, 2007). The expression of the LEE genes is regulated by a variety of environmental factors through a cascade controlled by two O157-specific virulence regulators. The LEE-encoded protein Ler activates the transcription of the LEE genes, except for the *LEE1* operon genes, including the *ler* gene, whose transcription is activated by the Pch regulators, which are encoded by extra-LEE chromosomal loci (Iyoda & Watanabe, 2004). The combined actions of the Ler and Pch regulators control the expression of many virulence-associated genes, along with the LEE genes (Abe *et al.*, 2008).

The environment of the human large intestine involves a complex bacterial ecosystem composed mainly of anaerobic bacteria. The activities of these organisms have a major influence on the nutrition and health of the host and

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; LEE, locus of enterocyte effacement; T3SS, type III secretion system; SCFA, short-chain fatty acid.

The GEO accession number for the microarray data associated with this paper is GSE12903.

Four supplementary figures are available with the online version of this paper.

the intestinal environment (Topping & Clifton, 2001). Dietary carbohydrates – specifically, resistant starches and dietary fibre – are substrates for a fermentation process that produces short-chain fatty acids (SCFAs) as end products (Louis *et al.*, 2007). Concentrations of SCFAs in the intestine vary depending on the activity of microflora and nutrient conditions, ranging from 20 to 140 mM (Cummings & Macfarlane, 1991). SCFAs, mainly acetate, propionate and butyrate, contribute to normal large bowel function and prevent pathology (Wong *et al.*, 2006). In particular, butyrate is thought to help maintain a normal colonocyte population, because it is the preferred energy source for colonocytes and has been implicated in protection against colitis and colorectal cancer (Hamer *et al.*, 2008). Therefore, probiotics and prebiotics that target the colon alter the environment by enhancing the production of SCFAs, especially butyrate (Wong *et al.*, 2006). Such manipulation of the SCFA levels through diet is a potentially useful approach to preventing intestinal colonization by pathogens (Van Immerseel *et al.*, 2005).

Here we studied the effect of SCFAs on the expression of EHEC virulence genes and found that LEE gene expression and the capacity to adhere to epithelial cells were enhanced in response to butyrate. We also showed that leucine-responsive regulatory protein (Lrp) plays a central role in regulating the butyrate response.

METHODS

Bacterial strains, plasmids and culture conditions. EHEC O157 Sakai (RIMD 0509952) (Hayashi *et al.*, 2001) and its derivative strains, and *E. coli* K-12 strains used in this study are listed in Table 1. Deletion mutants of EHEC O157 Sakai were constructed using the method and plasmids of Datsenko & Wanner (2000). pWKS-*pchA* was constructed by inserting DNA fragment containing *pchA* gene, which was isolated by PCR with primers GCGCGGATCCCATTTTTTTGACCGCGCG TTTCCGG and GCGCAAGCTTCACAGGAATATATCCGTACCC. pMW-*pchA* was constructed by inserting a DNA fragment containing the *pchA* gene of pWKS-*pchA* (*Eco*RI fragment). pWKS-P*tac-pchA* was constructed by replacing the P*tac* promoter DNA with upstream sequence of *pchA* on pWKS-*pchA*. pWKS-*lrp* was constructed by inserting a DNA fragment containing the *lrp* gene, which was isolated by PCR with primers CTCCCGTCATTATCACCTCT and AAACGG ACAACAGTACCAGG. Bacteria were grown overnight in LB, diluted 100-fold, and then incubated at 37 °C with shaking to OD_{600} 0.9–1.0 in DMEM (Sigma) containing 0.1 M MOPS (pH 6.7) and sodium chloride (Wako) or the sodium salts of SCFAs: sodium acetate (Wako), sodium propionate (Sigma) or sodium butyrate (Wako). To examine the effect of leucine on the expression of LEE genes, bacteria grown overnight in LB and diluted 100-fold were then grown at 37 °C with shaking to OD_{600} 0.9–1.0 in DMEM containing 0.1 M MOPS (pH 6.7) and 20 mM L-leucine (Wako).

Analysis of proteins in culture supernatants and whole-cell lysates. Bacteria were harvested by centrifugation, and the cell pellet was dissolved in SDS sample buffer. To prepare the proteins from the culture supernatant, 10 ml of the culture was spun, and the supernatant was passed through a filter (0.22 μm pore size; Millipore). Proteins in the filtered supernatant were precipitated by 6 % (final) trichloroacetic acid, and dissolved in SDS sample buffer. The concentration of each sample was normalized to the OD_{600} of the culture, and samples prepared from similar numbers of cells were analysed by immunoblotting by SDS-polyacrylamide (12 % or 10 %) gel electrophoresis (SDS-PAGE) and transfer onto an Immobilon membrane (Millipore). The proteins were detected with antibodies specific for EspB, Tir (Tatsuno *et al.*, 2000) or DnaK (Calbiochem) and a horseradish-peroxidase-conjugated secondary antibody, followed by visualization with the ECL detection kit (Amersham Biosciences).

Transcript analysis by DNA microarray. Total RNA from EHEC was isolated with TRI reagent (Sigma) using the method recommended by the

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
E. coli		
Sakai (RIMD 0509952)	Wild-type EHEC O157 : H7	Hayashi *et al.* (2001)
SKI 0352	Sakai Δ*ler*	Nakanishi *et al.* (2006)
SKI 0863	Sakai Δ*pchA*	Tobe *et al.* (2005)
SKI 0742	Sakai Δ*pchB*	Tobe *et al.* (2005)
SKI 1012	Sakai Δ*pchC*	Nakanishi *et al.* (2006)
SKI 0215	Sakai Δ*lrp*	This study
BW25113	K-12 strain	Datsenko & Wanner (2000)
BW25113 Δ*lrp*	BW25113 Δ*lrp*	This study
Plasmids		
pOSK1-P*LEE1*	P*LEE1-lacZ* on pACYC184	Tobe *et al.* (2005)
pMW-*pchA*	pMW119 with *pchA*	This study
pWKS-*pchA*	pWKS130 with *pchA*	This study
pWKS-P*tac-pchA*	pWKS130 with P*tac-pchA*	This study
pWKS-*lrp*	pWKS130 with *lrp*	This study
pWKS-P*ara*	pWKS130 with P*ara*	This study
pWKS-P*ara-lrp*(WT)	pWKS130 with P*ara-lrp*(WT)	This study
pWKS-P*ara-lrp*(V76A)	pWKS130 with P*ara-lrp*(V76A)	This study
pWKS-P*ara-lrp*(M124R)	pWKS130 with P*ara-lrp*(M124R)	This study

manufacturer. The RNA was purified again after treatment with RNase-free DNase I (Takara). Cy-3- or Cy-5-labelled cDNA was synthesized from the total RNA and hybridized to an O157 DNA microarray. cDNA synthesis, microarray hybridization, data capture and data analysis were performed as described previously (Tobe *et al.*, 2005). We used a DNA microarray with oligo DNAs specific for each of the ORFs of EHEC O157 Sakai (Tobe *et al.*, 2005). The expression ratios were the mean values from two replicate experiments with signal intensities greater than the mean + 1 SD of the negative control. The raw and processed data are available in the GEO (Gene Expression Omnibus) database of NCBI (GSE12903). The clustering of genes based on changes in transcript levels among acetate, propionate and butyrate was performed by the software Gene Tree with Pearson correlation in Gene Spring GX 7.3 (Agilent Technologies), and a group of genes clustered with LEE genes was selected for further screening of regulators involved in the butyrate response. Among them genes with transcript levels increased more than 1.5-fold by butyrate and with annotation of transcription regulation were selected for further screening by experiments with mutants.

Promoter activity assay. The P*LEE1-lacZ* operon fusion plasmid, pOSK1-P*LEE1*, was used as described previously to measure the promoter activity of the *LEE1* operon (Tobe *et al.*, 2005). Briefly, bacteria were grown overnight in LB and diluted 100-fold with DMEM containing 0.1 M MOPS (pH 6.7) and 20 mM SCFAs. At the sampling time points, an aliquot of culture was removed, the OD_{600} was measured, and the bacterial culture was mixed with Z-buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol) containing chloroform and SDS. The β-galactosidase activity of the culture was measured as described by Miller (1972).

Adherence assay. The adherence assay was performed as described previously (Nakanishi *et al.*, 2006), with a slight modification. Bacteria were grown overnight in LB, diluted 100-fold in DMEM with 0.1 M MOPS (pH 6.7) and 20 mM sodium chloride, or the sodium salt of SCFAs, and incubated at 37 °C for 3 h with shaking. Caco-2 cells in a confluent monolayer were infected at an m.o.i. of 100 ± 15, for 2 h at 37 °C. The cells were washed with PBS and then incubated in fresh medium for an additional 3 h at 37 °C. After another PBS wash, the cells were fixed and stained with Giemsa. Clusters containing at least eight bacteria were counted as microcolonies. The microcolonies and Caco-2 cells in five microscopic fields, by use of × 40 objective, were counted. The number of microcolonies that developed on a Caco-2 cell monolayer was adjusted for the number of Caco-2 cells in a given field.

Isolation of Lrp activation mutant and leucine response mutant. Based on information about the Lrp mutation reported by Platko & Calvo (1993), we replaced T227 and T371 of *lrp* with C and G, respectively, to obtain genes encoding V76A (activation mutant) and M124R (leucine response mutant) derivatives of Lrp. Targeted mutagenesis was performed on the pGEM-*lrp* plasmid by PCR with primers GCATCACTTCTGGCATTCGTTGAGATT and AATCTC AACGAATGCCAGAAGTGATGC (for V76A) or GCGTGCCGGATA GGTCAGCCTACCG and CGGTAGGCTGACCTATCCGGCACGC (for M124R), followed by treatment with *Dpn*I. The plasmids were then introduced into *E. coli* DH5α cells. A DNA fragment including the *lrp* (V76A) or *lrp* (M124R) gene was placed under control of the *araBAD* promoter on the pWKS-P*ara* plasmid, which was constructed by inserting P*araBAD* promoter DNA at the *Eco*RI site of pWKS130.

RESULTS

Virulence gene expression in response to SCFAs

To elucidate the effect of SCFAs on virulence gene expression, we compared the levels of LEE-encoded virulence factors, which are necessary for adherence to the intestinal mucosa, in EHEC grown in DMEM with various concentrations of an SCFA mixture containing sodium acetate, sodium propionate and sodium butyrate, in a molar ratio of 3 : 1 : 1. The sodium salts of the SCFAs were used to avoid any effect from pH changes, and the DMEM was supplemented with 0.1 M MOPS (pH 6.7) for the same reason. Moreover, sodium chloride was added to the control medium to avoid the effect of difference in osmolarity or levels of sodium ion (see Supplementary Fig. S2, available with the online version of this paper) (Lawhon *et al.*, 2002). The bacterial growth rate was reduced by the addition of 50 mM or more of the SCFA mixture (Fig. 1a): the doubling time was 22 or 24 min for bacteria grown in medium containing 50 mM or 100 mM SCFA mixture, respectively; it was 20 min in the control medium. The effect of SCFAs on the production of virulence factors was determined by comparing the amounts of the LEE-encoded EspB or Tir proteins. The addition of the SCFAs at final concentrations ranging from 6.25 to 25 mM increased the amounts of both EspB and Tir, as compared with EHEC in control medium, although higher concentrations of the SCFA mixture reduced the levels of these proteins (Fig. 1b). The amount of DnaK proteins, as internal control, in EHEC was not affected by these conditions, indicating that the effect was specific for a set of genes including *tir* and *espB*. Since 50 mM or more of sodium chloride, the control, reduced the expression of EspB and Tir, their reduced expression in response to 50 mM or more of the SCFA mixture was a non-specific effect.

To examine the effect of SCFAs on the production of LEE-encoded virulence factors, we determined which SCFAs contributed to the enhanced expression of Tir and EspB. EHEC was grown in DMEM containing various concentrations of sodium acetate, sodium propionate or sodium butyrate, and the bacterial growth and amounts of EspB and Tir in the EHEC were monitored. The bacterial growth rate was reduced by 40 mM or more acetate, and by 80 mM or more propionate or butyrate (Supplementary Fig. S1). The individual SCFAs had different effects on production of LEE-encoded proteins. The level of LEE-encoded protein production was reduced slightly in the presence of acetate at concentrations higher than 20 mM, and increased slightly in the presence of propionate at concentrations higher than 20 mM. On the other hand, even at the low concentration of 1.25 mM, butyrate enhanced the production of the LEE-encoded proteins (Fig. 2a). To clarify their effects on production of LEE-encoded virulence factors, all the SCFAs were tested at 20 mM, a concentration that did not affect EHEC growth. The levels of EspB and Tir in the bacteria and culture supernatant were higher for the EHEC grown with sodium butyrate than for EHEC grown with sodium chloride or sodium propionate, and acetate repressed their levels slightly (Fig. 2b; see also Supplementary Fig. S2).

The effect of the SCFAs on transcription of EHEC genes was determined as the changes of transcript levels relative

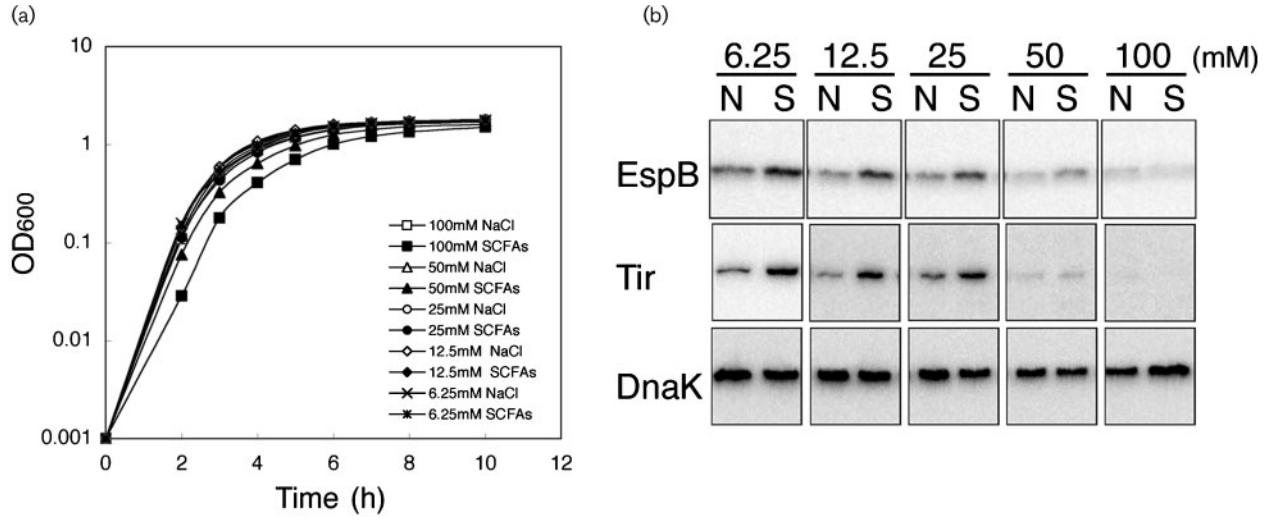

Fig. 1. Effect of a mixture of SCFAs on the expression of LEE genes. (a) Growth of EHEC O157 : H7 Sakai in DMEM containing mixed SCFAs (acetate : propionate : butyrate, 3 : 1 : 1) at 6.25–100 mM, as indicated. The control was NaCl at 6.25–100 mM. The graph is representative of three independent experiments. (b) Effect of SCFA mixture on EspB and Tir expression. The levels of EspB or Tir in bacteria grown in DMEM containing SCFAs (S) or NaCl (N) were determined by immunoblotting with EspB- or Tir-specific antiserum. DnaK was the quantitative control.

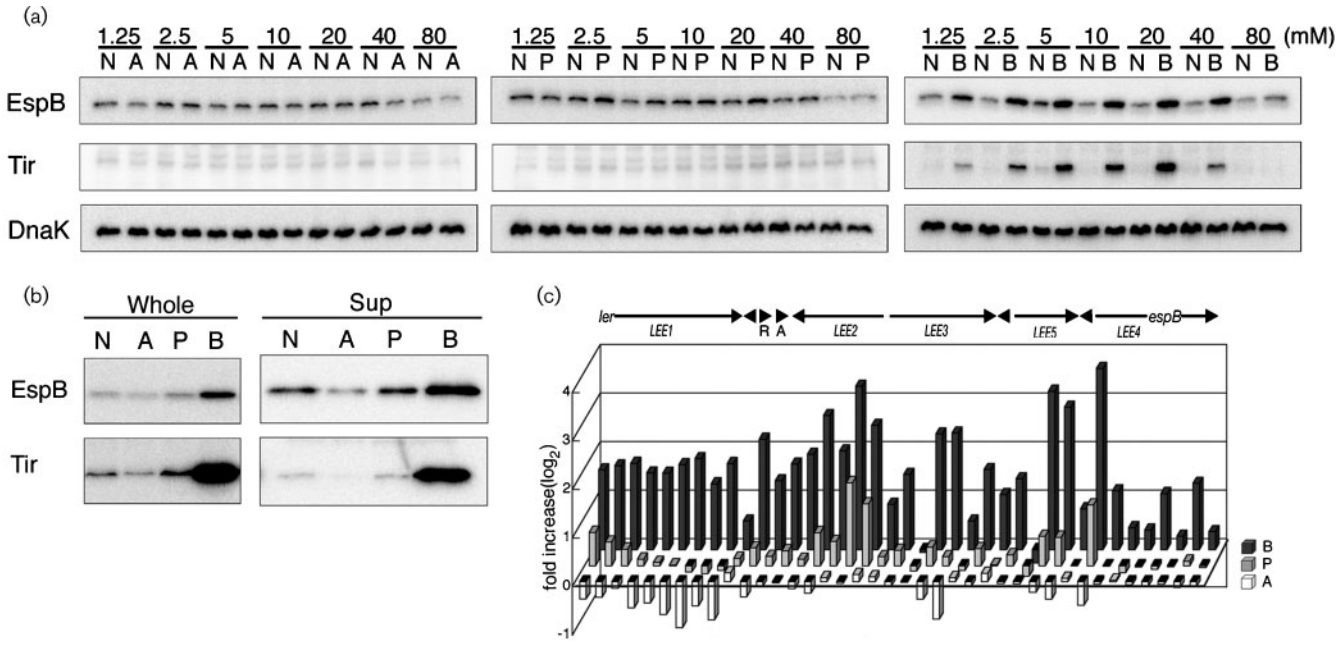

Fig. 2. Effect of SCFAs on the expression of LEE genes. (a) EspB and Tir expression levels in bacteria grown with SCFAs (1.25, 2.5, 5, 10, 20, 40, or 80 mM) or NaCl, determined by immunoblotting with EspB- or Tir-specific antiserum. N, NaCl; A, acetate; P, propionate; B, butyrate. DnaK was the quantitative control. (b) Effect of each SCFA (20 mM) on the secretion of EspB and Tir. Immunoblots of bacterial whole-cell extract (whole) or culture supernatant (Sup) are shown. (c) Effect of SCFAs on LEE gene transcription. The relative amounts of transcript in EHEC grown in DMEM containing 20 mM sodium chloride, sodium acetate (A), sodium propionate (P), or sodium butyrate (B) are presented as the fold increase (in $\log_2$ scale) over the level in EHEC grown with sodium chloride. The positions of *ler*, *espB*, *grlR* (R) and *grlA* (A) are indicated along with the LEE operons (arrows) at the top of the panel.

to those in EHEC grown with sodium chloride, by using a DNA microarray. Consistent with the changes seen in the LEE-encoded proteins, the levels of transcription of LEE genes were increased in EHEC grown in butyrate-supplemented medium, compared with EHEC grown in control medium (Fig. 2c). The increase in the secreted proteins in the butyrate-containing medium suggested that a functional T3SS was produced (Fig. 2b). These results indicated that LEE gene expression was coordinately enhanced by butyrate at the transcription step. Conversely, acetate and propionate had little or no effect on the expression of LEE genes.

One of the important steps in EHEC infection is the bacteria's adherence to and colonization of intestinal epithelial cells, for which LEE-encoded proteins are required. To demonstrate the effect of SCFAs on virulence expression in EHEC, the bacteria's ability to adhere to and form microcolonies on epithelial cells was examined. Since formation of microcolonies on cells is dependent on LEE-encoded virulence factors mediating intimate attachment (Tatsuno *et al.*, 2000), we compared the microcolony formation ability in the presence and absence of SCFAs. The adherence of EHEC grown with butyrate was 10-fold higher than that of EHEC grown without SCFAs (Fig. 3). Only a slight effect was observed for EHEC grown with propionate or acetate (Fig. 3). These results indicated that butyrate enhanced EHEC's colonization ability as well as the expression of LEE genes, whereas acetate and propionate affected the expression of the genes and proteins to a much lesser extent.

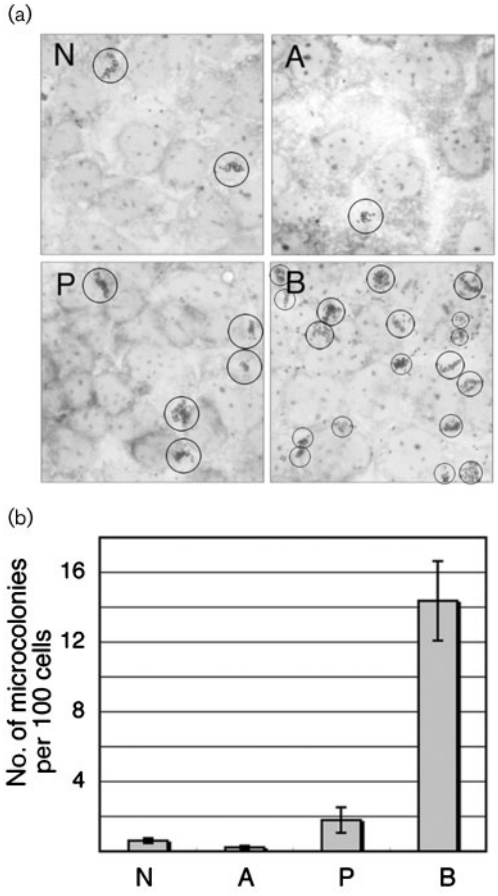

Fig. 3. Adherence of EHEC grown with SCFAs. (a) EHEC microcolonies on Caco-2 cells. Bacteria were grown with 20 mM sodium chloride (N), sodium acetate (A), sodium propionate (P) or sodium butyrate (B) and incubated with Caco-2 cells. Microcolonies (circled) were stained with Giemsa and counted. (b) Efficiency of adherence. The number of microcolonies formed was determined from the experiments shown in (a). The data are expressed as the mean number of microcolonies per 100 cells. A total of 15 microscope fields were examined. In this and subsequent figures, error bars represent SD.

Regulators of LEE genes mediate the butyrate response

The transcription of LEE genes is regulated by dual positive steps: Pch regulators activate transcription of the *LEE1* operon, which encodes the transcriptional regulator Ler, which in turn regulates the other LEE operons. We examined Ler's role in the butyrate-regulated expression of LEE genes. The deletion mutation of a *ler* gene completely abolished the response of LEE-encoded EspB and Tir production to butyrate (Fig. 4a). Next, we examined the involvement of Pch regulators in the butyrate-elicited response. The EHEC Sakai strain has three *pch* genes that are involved in the positive regulation of *ler* transcription: *pchA*, *pchB* and *pchC* (Iyoda & Watanabe, 2004). We thus examined the effect of butyrate on EspB and Tir production in *pch* mutants. In the Δ*pchA* mutant, butyrate did not enhance EspB or Tir production, but the Δ*pchB* and Δ*pchC* mutants continued to respond to butyrate (Fig. 4b). The activity of the *LEE1* promoter, which is positively regulated by Pch, was measured using a P*LEE1-lacZ* fusion gene. With the wild-type *pch* gene, butyrate enhanced the promoter activity twofold over its control level (Fig. 4c). In the Δ*pchA* mutant, the promoter activity was lower than in wild-type, and remained at the same low level even when butyrate was added. The response was rescued by introducing the *pchA* gene on a plasmid (Fig. 4c).

Next, we sought to reconstruct the regulation of the *LEE1* promoter in an *E. coli* K-12 background. In the absence of the *pchA* gene, the promoter activity in *E. coli* K-12 was comparable to that in the Δ*pchA* mutant of EHEC and did not respond to butyrate. Introduction of the *pchA* gene enhanced the promoter activity, and butyrate enhanced the activity threefold more (Fig. 4d). We further examined whether the enhancement of the *LEE1* promoter activity by butyrate occurred through the enhancement of *pchA* transcription or through the activation of some other factor(s). To do this, we compared the butyrate response of the *LEE1* promoter in a strain harbouring a P*tac-pchA* fusion gene, which did not carry the *pchA* promoter

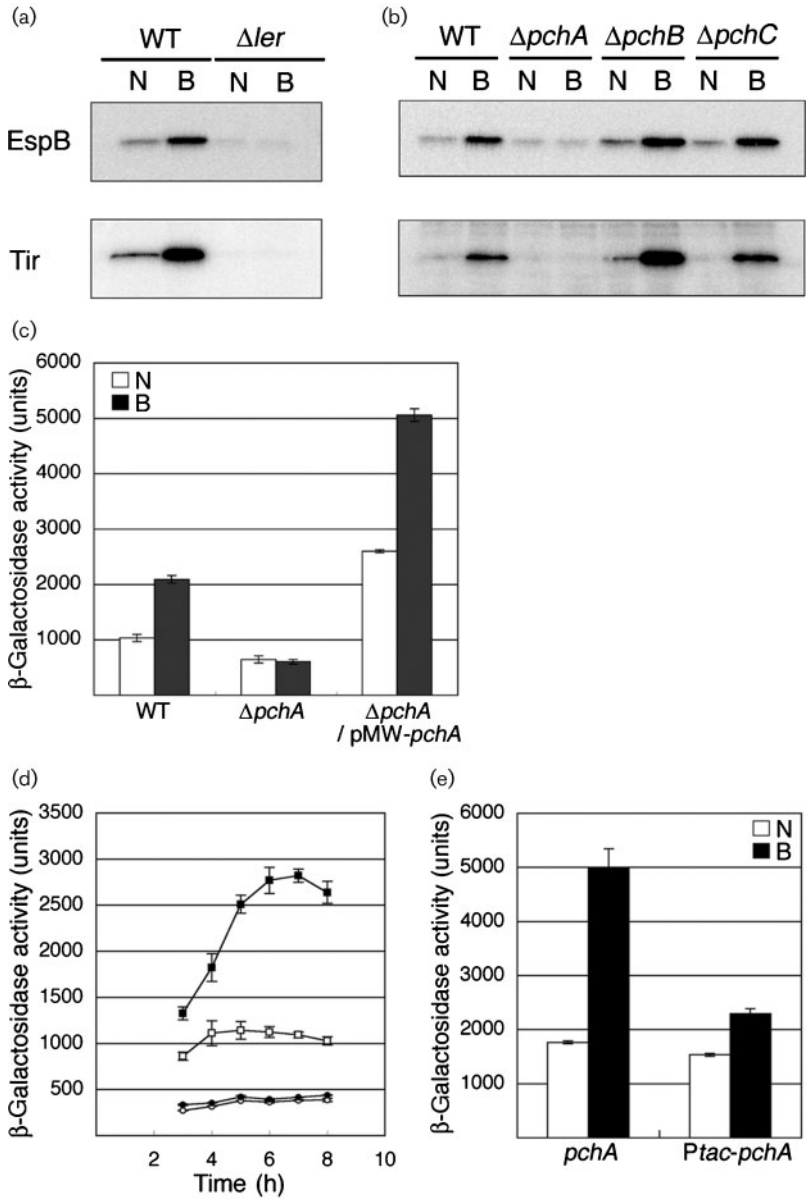

Fig. 4. Role of regulatory genes in the butyrate response. (a) Response of *ler* mutant to butyrate. Immunoblots of whole-cell extracts of wild-type or Δ*ler* mutant EHEC grown in DMEM with 20 mM sodium chloride (N) or sodium butyrate (B). (b) Response of Δ*pch* mutants to butyrate; details as for (a). (c) Promoter activity of the *LEE1* operon in wild-type EHEC or the Δ*pchA* mutant or the Δ*pchA* mutant harbouring pMW-*pchA* grown in DMEM containing sodium chloride (N) or sodium butyrate (B). Promoter activity is presented as units of β-galactosidase activity. (d) Reconstruction of the regulatory system in *E. coli* K-12. Promoter activity of the *LEE1* operon in bacteria grown with 20 mM NaCl (open symbols) or sodium butyrate (filled symbols) at 3, 4, 5, 6, 7, and 8 h from the start of the culture. The following strains were used: BW25113 with pWKS130 (circles) and BW25113 with pWKS-*pchA* (squares). (e) Abolition of the *LEE1* promoter response to butyrate in the absence of *pchA* promoter sequence. BW25113 expressing the *pchA* gene from a native promoter, or expressing the *pchA* gene from a different promoter (P*tac-pchA*), was grown with 20 mM sodium chloride (N) or sodium butyrate (B). To express *pchA* at a level comparable to that in wild-type, 0.1 μM IPTG was added to the medium for the strain harbouring the P*tac-pchA* gene.

sequences, with that in a strain carrying the native *pchA* gene, which carries the native *pchA* promoter. The *LEE1* promoter activity increased only slightly in response to butyrate in the P*tac-pchA* strain (Fig. 4e). Thus, the native *pchA* promoter sequence was required for the butyrate response of the *LEE1* promoter. Furthermore, these results showed that a regulatory system common to both *E. coli* K-12 and EHEC Sakai was involved in the butyrate-enhanced expression of genes.

Lrp mediates the butyrate response

To identify the gene(s) responsible for the butyrate-enhanced expression, we screened for genes that are common to both *E. coli* K-12 and EHEC Sakai, and whose transcript levels changed coordinately with those of the LEE genes, using the transcriptome data of EHEC Sakai grown with or without SCFAs. Examination of deletion mutants of the 14 candidate genes for the response to butyrate revealed that only the Δ*lrp* mutation affected the response to butyrate, although the change of the *lrp* transcript level in response to butyrate was not remarkable (1.8-fold increase over control). The level of the LEE-encoded proteins and activity of the *LEE1* promoter in the Δ*lrp* mutant remained the same whether or not the EHEC was grown with butyrate (Fig. 5a, b). The *lrp* gene encodes the leucine-responsive regulatory protein, Lrp, which is a DNA-binding protein involved in regulation of many genes (Brinkman *et al.*, 2003). To explore the involvement of Lrp and other EHEC-specific factors, we examined the response of the *LEE1* promoter in *E. coli* K-12 to butyrate. The addition of butyrate did not enhance the promoter activity

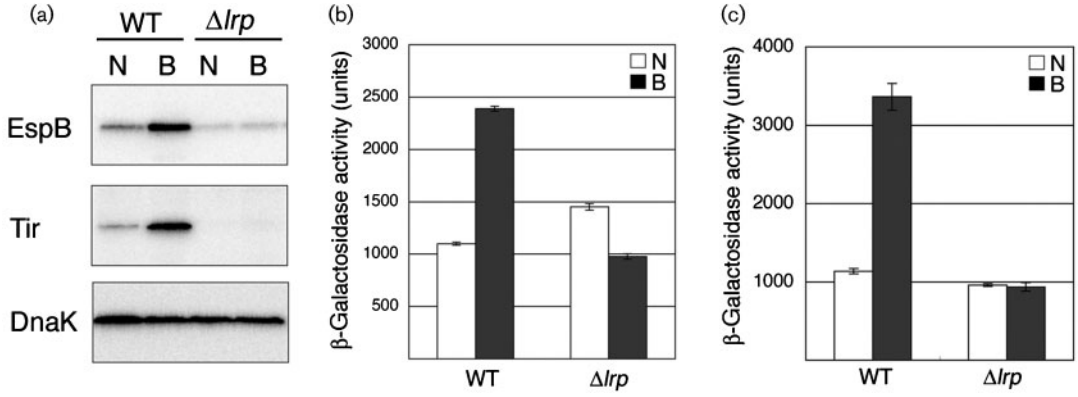

Fig. 5. Necessity for Lrp in the butyrate response. (a) Butyrate response assessed by EspB and Tir expression in wild-type EHEC and the Δ*lrp* mutant. (b) Butyrate response of the *LEE1* promoter in EHEC and the Δ*lrp* mutant, grown in DMEM containing sodium chloride (N) or sodium butyrate (B). Promoter activity is expressed as units of β-galactosidase activity. (c) Butyrate response of the *LEE1* promoter in *E. coli* K-12 strain BW25113 and its Δ*lrp* mutant, harbouring pWKS-*pchA*, grown in DMEM containing sodium chloride (N) or sodium butyrate (B).

in the *E. coli* K-12 Δ*lrp* mutant, but the activity increased with the addition of butyrate in the wild-type strain (Fig. 5c). These results strongly suggested that Lrp plays a central role in the butyrate-response regulation of the LEE genes.

Since the *lrp* transcript level increased 1.8-fold over the control level in EHEC grown with butyrate (see transcriptome data) and the amount of Lrp in EHEC slightly increased when the bacteria were grown with butyrate (Supplementary Fig. S3), we examined the effects of changing the expression level of the *lrp* gene. Introduction of an *lrp* clone on a multi-copy plasmid into the Δ*lrp* EHEC mutant increased the production of the LEE-encoded proteins, without butyrate, at a level comparable to that achieved with butyrate (Fig. 6a). Next, in order to achieve low level of Lrp production, we introduced a P*ara*-*lrp* fusion gene, in which transcription of the *lrp* gene was under the control of an arabinose-responsive promoter, into the Δ*lrp* mutant and controlled the expression levels of the *lrp* gene by adding arabinose to the medium. The addition of 3.2 mM arabinose alone slightly enhanced the production of Tir in EHEC grown without butyrate, whereas much more EspB and Tir were produced when butyrate was added along with the 3.2 mM arabinose (Fig. 6a). These results suggest that, at a level comparable to that in the wild-type strain, Lrp mediated the response to butyrate and enhanced the expression of LEE genes through the activation of Lrp at a post-transcriptional step.

The Lrp protein consists of a DNA-binding domain and a ligand-binding domain. The binding of leucine by Lrp either stimulates or reduces the promoter activity of its target genes or operons (Platko & Calvo, 1993). In addition, the non-specific binding of Lrp to DNA affects the expression of many genes, and this binding varies with the nutrient conditions and growth phase (Chen *et al.*, 2001). To confirm the involvement of Lrp in the regulation of the LEE genes, we examined the response of the LEE genes to leucine. With 20 mM leucine in the medium, the amounts of EspB and Tir proteins in EHEC were higher than in EHEC grown in control medium (Fig. 6b). This response was completely abrogated by the Δ*lrp* mutation (Fig. 6b).

To examine the role of Lrp in the butyrate-sensing process, we constructed a plasmid encoding one of two Lrp derivatives: a constitutively active mutant, Lrp (V76A), and a 'response' mutant, Lrp (M124R) (Platko & Calvo, 1993), which does not respond to leucine, and expressed them from the P*ara* promoter in the Δ*lrp* mutant in medium containing 3.2 mM arabinose. The amounts of EspB and Tir protein in the strain expressing Lrp (V76A) grown with sodium chloride were comparable to those in the strain expressing wild-type Lrp grown with butyrate (Fig. 6c). These results indicate that changing Lrp to its active form is sufficient for the enhancement of LEE gene expression. On the other hand, even with butyrate, the amounts of EspB and Tir proteins in the strain expressing Lrp (M124R) were lower than in EHEC expressing wild-type Lrp (Fig. 6c), and the response to the addition of leucine was also weak, comparable to the response to butyrate. These results suggest that the ligand-binding domain of Lrp is necessary for the bacteria's response to butyrate. They also strongly suggest that the Lrp protein works as a sensor for butyrate, just as for leucine, to activate the expression of LEE genes in EHEC.

To examine the possibility that Lrp directly regulates transcription of the *pchA* gene, we searched for an Lrp-recognition site in the DNA sequence around the *pchA* gene. However, we could not identify such a site in the *pchA* gene, including in its promoter region. Next, we searched for an Lrp-recognition site in the *LEE1* promoter

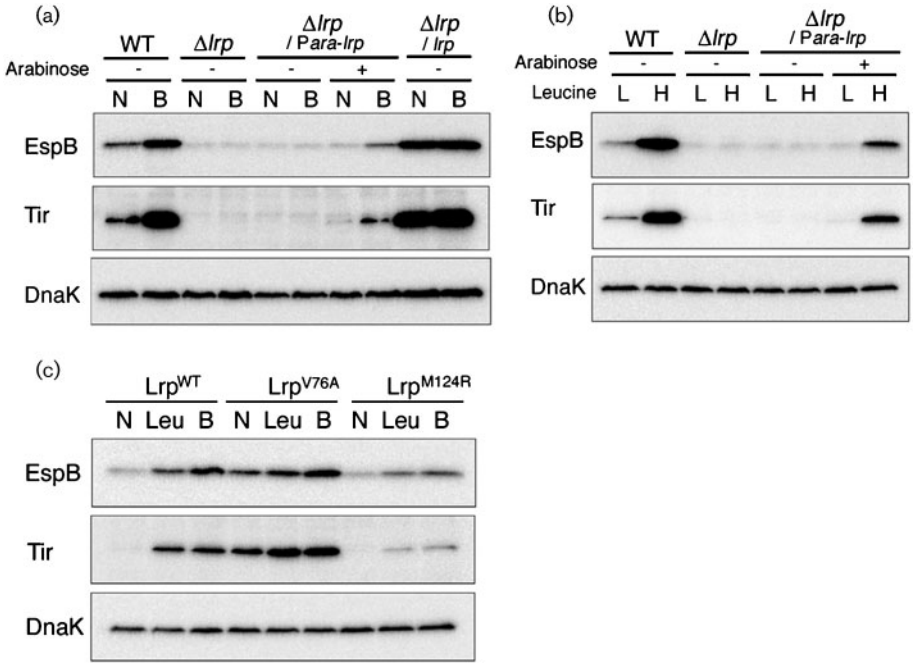

Fig. 6. Involvement of Lrp protein in butyrate sensing. Bacterial whole-cell extract was prepared and the EspB and Tir proteins were detected by immunoblotting. DnaK was the quantitative control. (a) Butyrate response with controlled *lrp* transcription. Wild-type EHEC (WT), the *lrp* mutant (Δ*lrp*), the *lrp* mutant harbouring pWKS-P*ara-lrp* (Δ*lrp*/P*ara-lrp*) or the *lrp* mutant harbouring pWKS-*lrp* (Δ*lrp*/*lrp*) were grown in DMEM containing 20 mM sodium chloride (N) or sodium butyrate (B) with or without 3.2 mM arabinose. (b) Response of the LEE genes to leucine. Wild-type EHEC (WT), the *lrp* mutant (Δ*lrp*), or the *lrp* mutant harbouring pWKS-P*ara-lrp* (Δ*lrp*/P*ara-lrp*) was grown in DMEM containing 0.8 mM (L) or 20 mM (H) leucine, with or without 3.2 mM arabinose. (c) Response to butyrate and leucine by EHEC expressing Lrp derivatives. The *lrp* mutant harbouring pWKS-P*ara-lrp* (WT) (Lrp^{WT}), pWKS-P*ara-lrp* (V76A) (Lrp^{V76A}) or pWKS-P*ara-lrp* (M124R) (Lrp^{M124R}) was grown in DMEM containing sodium chloride (N), leucine (Leu), or sodium butyrate (B). Proteins in whole-cell extracts were detected by immunoblotting with the EspB- or Tir-specific antiserum. DnaK was the quantitative control.

region; again we could not identify one. Furthermore, we could not detect any binding of Lrp to DNA fragments of these genes by chromatin immunoprecipitation from EHEC expressing an Lrp-FLAG fusion protein (Supplementary Fig. S4). These results suggested that the response of the LEE genes to butyrate was mediated by Lrp through the indirect regulation of the *pchA* gene.

DISCUSSION

The fermentation of carbohydrates by microflora in the intestine produces SCFAs, primarily acetate, propionate and butyrate, as end products. The amounts of SCFAs vary through the gastrointestinal tract. The levels in the distal ileum are low, between 20 and 40 mM total SCFAs (Argenzio *et al.*, 1974), but they are higher in the proximal colon, about 70 to 140 mM, and 20 to 70 mM in the distal colon (Wong *et al.*, 2006). In this study, we showed that the presence of SCFAs at a level ranging from 6.25 to 25 mM enhanced the expression of the LEE genes in EHEC without affecting bacterial growth, although growth was negatively affected at SCFA concentrations of 50 mM or higher. In the process of infection, EHEC travels through the gastrointestinal tract, encountering a variety of environmental factors, including SCFAs at the optimal concentrations for enhancing LEE gene expression in the distal ileum. It is therefore likely that the expression of adherence-related EHEC genes is enhanced, and EHEC adherence capacity increased, by the SCFAs in the distal ileum. In support of this idea, in an *in vitro* organ culture EHEC can colonize the follicle-associated epithelium of the distal ileum at the initial stage of infection (Chong *et al.*, 2007). Taken together with our findings, the evidence is consistent with the distal ileum being a primary target of EHEC infection from which, once the EHEC colonization has become established, it can spread to the proximal colon.

We propose that butyrate, one of the major SCFAs, acts as a signalling molecule to trigger the full expression of adherence-related genes and the initiation of colonization in the distal ileum. Furthermore, EHEC O157 cannot grow in minimal medium in which butyrate is the sole carbon source (N. Nakanishi, unpublished). This strongly suggests that butyrate-induced gene expression in EHEC is not due

to the stimulation of the butyrate-associated metabolic pathway, but rather is attributable to butyrate acting as an environmental signal that does not alter metabolic activity, at least at relatively low concentrations. We also found that Lrp, a leucine-responsive regulatory protein, plays a key role in the EHEC response to butyrate. Lrp is known to be a global regulator, controlling genes involved in amino acid metabolism, transport, and the synthesis of various cell appendages (Brinkman *et al.*, 2003). Leucine is required for the binding of Lrp to some of its DNA target sites. However, at other sites leucine inhibits DNA binding, and at still others it has no effect at all. The activity of the *LEE1* promoter was not enhanced in a null *lrp* mutant of EHEC or *E. coli* K-12. The response to butyrate of the *LEE1* promoter was restored by introducing a *pchA* gene into *E. coli* K-12, indicating that one or more genes common to EHEC O157 : H7 Sakai and *E. coli* BW25113 are required, but the Sakai-specific genes, except for the *pchA* genes, are unnecessary for the response. The expression of an activation or response mutant of the Lrp protein in the Δ*lrp* mutant clearly showed that the response of the LEE genes to butyrate occurred via a modulation of Lrp activity. These results strongly suggested that the activity of Lrp was modulated by the interaction of butyrate with Lrp's ligand-binding domain. Consistent with these results, the expression of the LEE genes was activated at high concentrations of leucine, through Lrp. Thus, the LEE genes are coordinately regulated with other genes belonging to the Lrp-regulon, suggesting that the regulation of physiological activity is closely linked to the regulation of virulence genes in EHEC. Our findings also provide important information for further understanding of ligand recognition by Lrp. Its ligand-binding domain should recognize a molecular structure common to butyrate and leucine but not shared by acetate and propionate, such as CH_3–CH–CH. In conclusion, Lrp acts as a sensor to transduce stimulation by butyrate into the PchA-Ler regulatory network that governs the LEE genes.

The production of SCFAs depends on the metabolic activity of intestinal microflora and is also affected by dietary changes. Although the amount of butyrate is lower than that of the other SCFAs, it plays more important roles in the maintenance of colonic health as an energy source, as a regulator of gene expression and cell differentiation, and as an anti-inflammatory agent (Wong *et al.*, 2006). Butyrate has been shown to prevent the development of colon cancer, and to reduce bowel inflammation (Hamer *et al.*, 2008). Therefore, an increase in butyrate concentration is believed to be beneficial to colonic health. Furthermore, increasing the amount of butyrate *in vivo* reduces *Salmonella* infection in poultry (Van Immerseel *et al.*, 2005) and protects epithelial cells from infection with *Campylobacter jejuni in vitro* (Van Deun *et al.*, 2008). Hence, prebiotics and probiotics are sometimes used to influence the production and composition of SCFAs towards increased butyrate. In contrast, we found that 1.25–40 mM butyrate enhances the expression of virulence genes involved in colonization of the gut by EHEC, and therefore the amount of butyrate in the intestine may affect the infectivity of EHEC. At concentrations of up to 25 mM total SCFAs, the higher the ratio of butyrate produced by the microflora, the greater the adherence capacity of the EHEC. Therefore, both the composition of dietary carbohydrates and the species and activity of microflora might affect EHEC infection at the initial stage of colonization. An implication of these data is that the susceptibility to EHEC might be affected by nutrient conditions. Thus, our findings indicate that probiotics and prebiotics should be used with caution to increase butyrate in the intestinal environment, because these agents might cause an unexpected enhancement of EHEC virulence.

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