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Regulation of virulence and motility by acetate in enteropathogenic *Escherichia coli*

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ABSTRACT

Enteropathogenic *Escherichia coli* colonizes the human small intestine and causes severe diarrhea. Short-chain fatty acids are abundant in the intestine owing to the metabolic activity of the microflora and are important for intestinal health. Here, we found that acetate promotes the adherence of enteropathogenic *E. coli* O127:H6 to Caco-2 intestinal epithelial cells and its motility on semi-solid Luria–Bertani agar by activating the expression of locus of enterocyte effacement genes and flagellar genes, respectively. The effect of acetate on locus of enterocyte effacement gene expression is mediated by Ler, the master regulator of locus of enterocyte effacement genes, whereas the regulation of flagellar genes by acetate is dependent on the RNA polymerase sigma factor FliA. Conversely, formate, propionate, and butyrate had little or no effect on enteropathogenic *E. coli* O127:H6 adherence and motility. Finally, the acetate-mediated regulatory pathway was found to be a widespread mechanism used by a range of enteropathogenic *E. coli* to mediate bacterial virulence and motility. Therefore, upon entering the human small intestine, enteropathogenic *E. coli* may respond to the higher acetate level to enhance its virulence and motility, leading to efficient colonization of the target niche.

1. Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a common cause of infant diarrhea in developing countries, often associated with high mortality rates of between 10% and 40% (Chen and Frankel, 2005). Although humans are considered the primary reservoir of EPEC that cause human illness, animals such as dogs and cattle do carry EPEC strains that may pose a threat to human health (Spears et al., 2006). The most frequent EPEC serogroups implicated in human disease include O55, O86, O111ab, O119, O125ac, O126, O127, O128ab, and O142 (Nataro and Kaper, 1998; Yoon and Hovde, 2008). EPEC colonizes the human small intestine and produces a characteristic histopathological lesion referred to as an attaching and effacing (A/E) lesion (Clarke et al., 2003; Kaper et al., 2004; Spears et al., 2006). The A/E lesion is characterized by effacement of microvilli and intimate adherence of bacteria to the intestinal epithelial cells (Kaper et al., 2004; Spears et al., 2006). Attached bacteria stimulate host cytoskeletal actin polymerization accumulation, resulting in a raised attachment pedestal (Kaper et al., 2004; Spears et al., 2006). The genetic determinants involved in the development of A/E lesions are mainly located in a pathogenicity island called the locus of enterocyte effacement (LEE), which is conserved in all A/E-forming pathogens including enterohemorrhagic *Escherichia coli* (EHEC), EPEC, and *Citrobacter rodentium* (Garmendia et al., 2005; McDaniel et al., 1995; Vallance et al., 2002). The LEE genes encode a type III secretion system (T3SS), translocator and effector proteins, chaperones, and transcriptional regulators (Garmendia et al., 2005; McDaniel et al., 1995; Vallance et al., 2002). The regulation of LEE is a very complex process governed by many environmental factors and global and special regulators (review by (Clarke et al., 2003; Franzin and Sircili, 2015; Mellies et al., 2007)).

Flagellar motility also plays diverse roles in bacterial pathogenesis, including migration to an optimal site in the host, colonization or invasion, survival at the infection site, and post-infection dispersal

Abbreviations: A/E, attaching and effacing; CFU, colony-forming units; DMEM, Dulbecco's modified Eagle medium; EHEC, enterohemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; LB, Luria–Bertani; LEE, locus of enterocyte effacement; OD_{600}, absorbance at 600 nm; SCFAs, short-chain fatty acids

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(Chaban et al., 2015). A flagellum consists of a basal body, hook, and filament formed through polymerization of flagellin (FliC) (Erhardt et al., 2010). More than 50 genes in *E. coli* have been demonstrated to be involved in formation and operation of the flagellum (Chaban et al., 2015). The bacterial flagellum is strictly regulated to prevent expression until environmental conditions are optimal in order to avoid high metabolic cost and ensure survival in different environments (Chevance and Hughes, 2008). Coordinated expression of flagellar genes is controlled by regulatory proteins, with FlhDC and FliA as master transcriptional activators (Claret and Hughes, 2000). FliA boosts expression of flagellar genes directly, whereas FlhDC exerts its regulatory function either directly or via FliA (Chevance and Hughes, 2008).

Short-chain fatty acids (SCFAs) are organic fatty acids with one to six carbon atoms that arise from bacterial fermentation of polysaccharide, oligosaccharide, and glycoprotein precursors in the human intestine (Wong et al., 2006). Among these SCFAs, formate, acetate, propionate, and butyrate are the most abundant (Cummings and Macfarlane, 1991). Additionally, the levels of individual SCFAs vary in different regions of the intestinal tract, with formate and acetate predominating in the small intestine, while levels of propionate and butyrate are higher in the large intestine (Cummings and Macfarlane, 1991; Keeney and Finlay, 2011). SCFAs are known to have a profound influence upon the pathogenicity of intestinal pathogens. In *Salmonella enterica* serovar Typhimurium (a small intestinal pathogen), formate and acetate act as intestinal signals to induce the expression of invasion genes, while butyrate represses invasion genes (Gantois et al., 2006; Huang et al., 2008; Lawhon et al., 2002). Conversely, exposure of EHEC (a large intestinal pathogen) to butyrate promotes LEE gene expression and enhances adherence to Caco-2 intestinal epithelial cells (Nakanishi et al., 2009). Nevertheless, the effect of SCFAs on EPEC pathogenicity is not fully understood.

In this study, we demonstrated that acetate positively regulates the expression of LEE genes and flagellar genes to promote EPEC O127:H6 adherence and motility. Acetate activated LEE genes and flagellar genes via Ler and FliA, respectively. On the other hand, propionate and butyrate had no obvious effect on EPEC O127:H6 adherence and motility. Finally, this acetate-mediated regulatory pathway was found to be present in an additional nine representative EPEC strains from seven serotypes. We propose that this regulatory pathway is used for the site-specific colonization of the small intestine by EPEC.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains, plasmids, and primers used in this study are summarized in Tables 1 and 2. Mutant strains were generated using the λ Red recombinase system, and all strains were verified via PCR amplification and sequencing. All strains were maintained at −80 °C in Luria–Bertani (LB) broth with 20% glycerol and grown in LB broth or Dulbecco's modified Eagle medium (DMEM) when required. As necessary, antibiotics were added at the following final concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 15 μg ml^{-1}; and kanamycin, 50 μg ml^{-1}.

2.2. Bacterial adherence assays

Adherence assays were performed using a previously described method (Dibb-Fuller et al., 2001) with some modifications. Briefly, Caco-2 cells, obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), were grown in DMEM (Hyclone; #SH30243.01B) with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 until confluent. Before infection, Caco-2 cells were washed three times with pre-warmed PBS, and the medium was replaced with fresh DMEM without antibiotics and fetal bovine serum. Monolayers of cells were infected with bacterial cultures in exponential phase (10^8 colony-forming units (CFU)/well), and cultures were incubated for 3 h at 37 °C in a 5% CO_2 atmosphere. After incubation, unattached bacteria were removed by washing the wells six times with PBS. Caco-2 cells were then lysed with 0.1% SDS at 37 °C for 5 min. The resulting lysates were serially diluted and plated on LB agar. Attachment efficiency was calculated by counting the CFU per milliliter.

Table 1
Strains and plasmids used in this study.

	Genotype or description
Strains	
EPEC O127 WT	Wild-type EPEC O127:H6 E2348/69, *eae*+, *stx*1-, *stx*2-
EPEC O127 Δler	*ler* deletion mutant in EPEC O127 WT
EPEC O127 Δ*fliA*	FliA deletion mutant in EPEC O127 WT
O55-1	EPEC O55:H7 (*eae*+, *stx*1-, *stx*2-)
O55-2	EPEC O55:H7 (*eae*+, *stx*1-, *stx*2-)
O127	EPEC O127:H- (*eae*+, *stx*1-, *stx*2-)
O86-1	EPEC O86:H34 (*eae*+, *stx*1-, *stx*2-)
O86-2	EPEC O86:H34 (*eae*+, *stx*1-, *stx*2-)
O114	EPEC O114:H2 (*eae*+, *stx*1-, *stx*2-)
O128	EPEC O128:H2 (*eae*+, *stx*1-, *stx*2-)
O142	EPEC O142:H6 (*eae*+, *stx*1-, *stx*2-)
O145	EPEC O145:H34 (*eae*+, *stx*1-, *stx*2-)
O157	EHEC O157:H7 (*eae*+, *stx*1+, *stx*2+)
O26	EHEC O26:H11 (*eae*+, *stx*1+, *stx*2-)
O103	EHEC O103:H2 (*eae*+, *stx*1+, *stx*2-)
Plasmids	
pKD46	Red recombination plasmid, ApR
pKD3	Containing a chloramphenicol resistance cassette and the flippase recognition sites, CmR
pKD4	Containing a kanamycin resistance cassette and the flippase recognition sites, KmR
pCP20	FLP expression plasmid, ApR, CmR

2.3. Growth curves

Overnight cultures of EPEC O127:H6 wild-type strains were subcultured 1:100 into fresh DMEM supplemented with different concentrations of sodium acetate for an additional 24 h at 37 °C and 180 rpm. The absorbance at 600 nm (OD_{600}) of 1-ml aliquots of the culture was measured regularly over this period. The results represent the average values of three independent experiments.

2.4. Quantitative RT-PCR (qRT-PCR)

Bacteria were collected at an OD_{600} of 0.8, and the total RNA was extracted with TRIzol® LS Reagent (Invitrogen) following the manufacturer's instructions. Total RNA was then treated with RNase-Free DNase I (Qiagen; #79254) to eliminate genomic DNA contamination. First-strand cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara; #D6110 A) and analyzed by qRT-PCR on an ABI 7500 system (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems; #4367659). The 16S rRNA gene (*rrsH*) was used as a reference gene to standardize the samples (Yang et al., 2015). The relative difference in gene expression was calculated by the cycle threshold method ($2^{\Delta\Delta ct}$) (Livak and Schmittgen, 2001).

2.5. Motility assays

Swimming motility was assessed using 0.25% LB agar plates (Yang et al., 2018). Overnight cultures were standardized to an OD_{600} of 1.0. The semi-solid LB agar plates were stab inoculated with 1 μl of standardized cultures using a sterile pipette tip. The plates were then incubated for 8 h at 30 °C, and the diameter of the swimming zone around the inoculation site was measured.

Table 2
Primers used in this study (5′-3′).

Primers for gene mutation		
ler	F	TTCCAGCTCAGTTATCGTTATCATTTAATTATTTTATGGTGTAGGCTGGAGCTGCTTCG
ler	R	ATAAGGATAAGGTCGCTAATAGCTTAAAATATTAAAGCATGGGAATTAGCCATGGTCC
fliA	F	TCCTGGTAGTCAAAGTTAAAATGCGGCATTTACTGACGGTGTAGGCTGGAGCTGCTTCG
fliA	R	AATCATGCCGATAACTCATATAACGCAGGGCTGTTTATCATGGGAATTAGCCATGGTCC
Primers for mutant verification		
ler	F	GGATTTCGTTCTTTTTGTCG
ler	R	ATTTTAAGGTGGTTGTTTGATG
fliA	F	CAATGTAAATCACCGCAAA
fliA	R	ACCCCCAAATAACCCCT
Primers for qRT-PCR		
rrsH	F	GAAAGCGTGGGGAGCAAAC
rrsH	R	ACATGCTCCACCGCTTGTG
ler	F	CAGGAAGCAAAGCGACTG
ler	R	ACCAGGTCTGCCCTTCTT
eae	F	GACGGTAGTTCACTGGACTTCTT
eae	R	TCGCCACCAATACCTAAACG
tir	F	AGCTTTCATCGGGTATTGGTT
tir	R	GCGTCTTCTGCTCTTGTGGC
escT	F	GCAATAGATGCGGCTGGGC
escT	R	TCGGCTTGTAATGGTAATATCTCG
espB	F	GCTTCTCAGGTAGCTGAAGAGGC
espB	R	CTGGTAACAACAATAAATGGCGT
fliC	F	CGGCACCAGGGGGAAC
fliC	R	CATCAGAAGCGGCGGAATA
motA	F	TTGGAGCACTCTATCAACCCG
motA	R	AGCGAAAACATCCCCATCTG
flhD	F	ACCTCCGAGTTGCTGAAACAC
flhD	R	TTGCTGGAGATCGTCAACGC
flhC	F	CCCGCAAGCAGAAGAAGGA
flhC	R	GCTGGTGAGCGTGGGTAATAA
fliA	F	GGATAAACACTCGCTGTGGCAG
fliA	R	GAAGTTCATCCAGCATAGCGCC
bfpA	F	GCTGCCACCGTTACCGC
bfpA	R	CCTACATTTAATTCCCCCCCA
Primers for EHEC/EPEC identification		
mdh	F	AGGCGCTTGCACTACTGTTA
mdh	R	AGCGCGTTCTGTTCAAATG
eae	F	ATTATGGAACGGCAGAGGTTAAT
eae	R	ATCCCCATCGTCACCAGAGG
stx1 A	F	CATTCGCTCTGCAATAGGTA
stx1 A	R	AACTCGCGATGCATGATGA
stx2 A	F	TATCTGGCGTTAATGGAGTT
stx2 A	R	CCTGTCGCCAGTTATCTGAC

2.6. Statistical analysis

Statistical analysis was conducted using the software MedCalc (v12.3.0.0). The mean ± SD from three independent experiments is shown in figures. Differences between two mean values were evaluated by two-tailed Student's *t*-test. A P value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. The effect of SCFAs on the adherence of EPEC O127:H6 to host cells

To elucidate the effect of SCFAs on bacterial adherence, we compared the ability of EPEC O127:H6 to adhere to Caco-2 intestinal epithelial cells in DMEM supplemented with various concentrations of sodium formate, sodium acetate, sodium propionate, and sodium butyrate. The sodium salts of the SCFAs were used to avoid any adverse effect from pH changes. As shown in Fig. 1A, EPEC O127:H6 adherence to Caco-2 intestinal epithelial cells slightly increased in the presence of acetate at the low concentration of 5 mM but significantly increased at concentrations higher than 10 mM. The growth of EPEC O127:H6 in LB medium and DMEM was not affected by the presence of various concentrations of sodium acetate (Fig. 1B and C), indicating that the positive effect of acetate on EPEC O127:H6 adherence was not due to different growth rates. Conversely, formate, propionate, and butyrate had little or no effect on EPEC O127:H6 adherence to Caco-2 cells (Fig. 1D–F).

3.2. Acetate promotes EPEC O127:H6 adherence by controlling LEE genes

Considering that the ability of EPEC to adhere to host cells and induce A/E lesions is conferred by the LEE pathogenicity island, we therefore investigated the effect of SCFAs on EPEC O127:H6 LEE gene expression. The transcriptional levels of five representative LEE genes, including *ler* (the master regulator of LEE genes), *eae* (intimin), *tir* (intimin receptor), *escT*, and *espB*, were determined in the presence of different concentrations of sodium SCFAs (0 or 20 mM). The expression of LEE genes was significantly increased in the presence of 20 mM sodium acetate in EPEC O127:H6, in comparison with that observed at 0 mM (Fig. 2A). This result indicates that the positive effect of acetate on EPEC O127:H6 adherence was due to the positive regulation of LEE genes by acetate. In accordance with the adherence assays, the ability of EPEC O127:H6 to express LEE genes was unaffected by the presence of formate, propionate, and butyrate (Fig. 2B–D).

The virulence plasmid-encoded bundle-forming pilus (BFP) is also an important virulence factor that plays a crucial role in the initial adherence of EPEC to intestinal epithelial cells (Knutton et al., 1999). However, the transcriptional levels of *bfpA*, which encodes the major

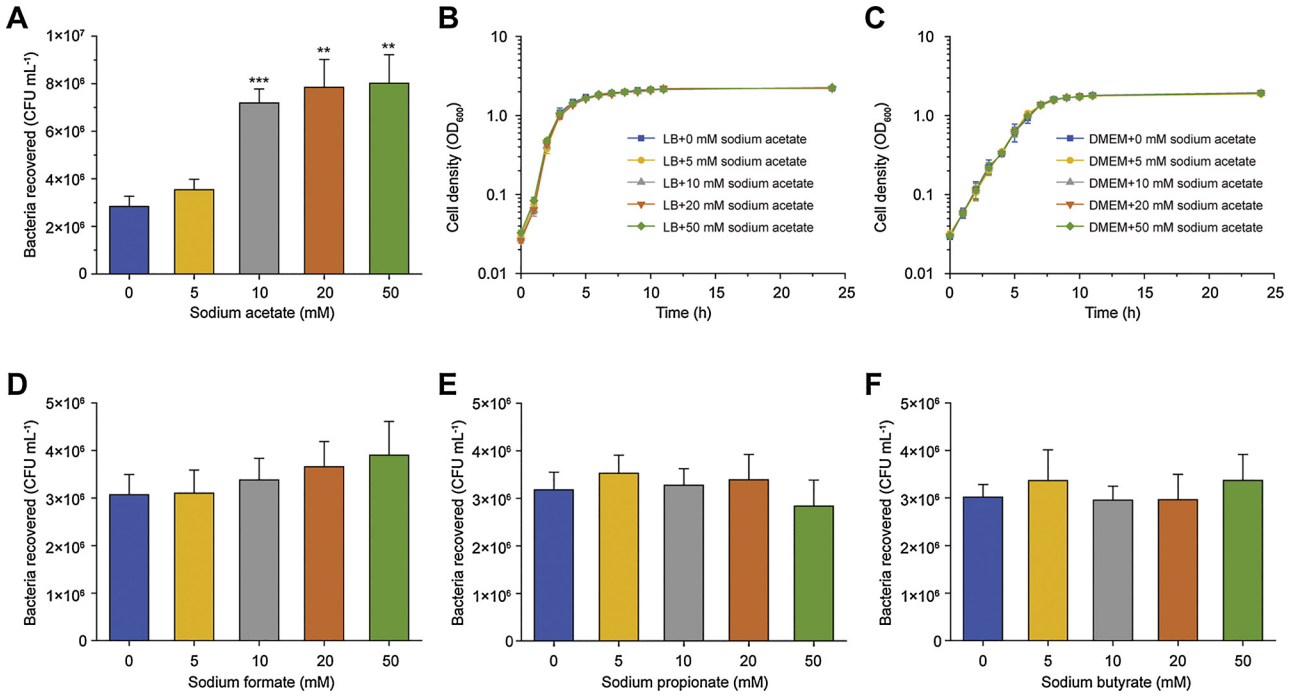

Fig. 1. Effect of SCFAs on the adherence of EPEC O127:H6 to host cells. (A) Adhesion of EPEC O127:H6 to Caco-2 intestinal epithelial cells in DMEM supplemented with different concentrations of sodium acetate. (B–C) The growth of EPEC O127:H6 in LB (B) or DMEM (C) supplemented with different concentrations of sodium acetate. (D–F) Adhesion of EPEC O127:H6 to Caco-2 intestinal epithelial cells in DMEM supplemented with different concentrations of sodium formate (D), sodium propionate (E), or sodium butyrate (F). Data are presented as means ± s.d.; n = 3. ** $P \leq 0.01$; *** $P \leq 0.001$ by Student's *t*-test.

pilin subunit of BFP, were unchanged in the presence of 0 or 20 mM sodium SCFAs (Supplementary Fig. S1).

3.3. Ler mediates acetate-induced EPEC O127:H6 LEE gene activation

The first gene of the LEE pathogenicity island encodes the master regulator Ler, which activates the expression of genes from LEE1 to LEE5 (Franzin and Sircili, 2015). To further investigate whether acetate regulated EPEC O127:H6 adherence and LEE gene expression through *ler*, a Δler mutant was constructed. Deletion of *ler* resulted in significant reduction in the ability of EPEC O127:H6 to adhere to Caco-2 cells and express LEE genes (Fig. 3A and B), which is in line with the fact that Ler is a positive LEE regulator. Furthermore, neither the adherence capacity nor the LEE gene expression of the Δler mutant was affected by the presence of acetate (Fig. 3C and D), indicating that the positive effect of acetate on EPEC O127:H6 adherence and LEE gene expression is mediated by Ler.

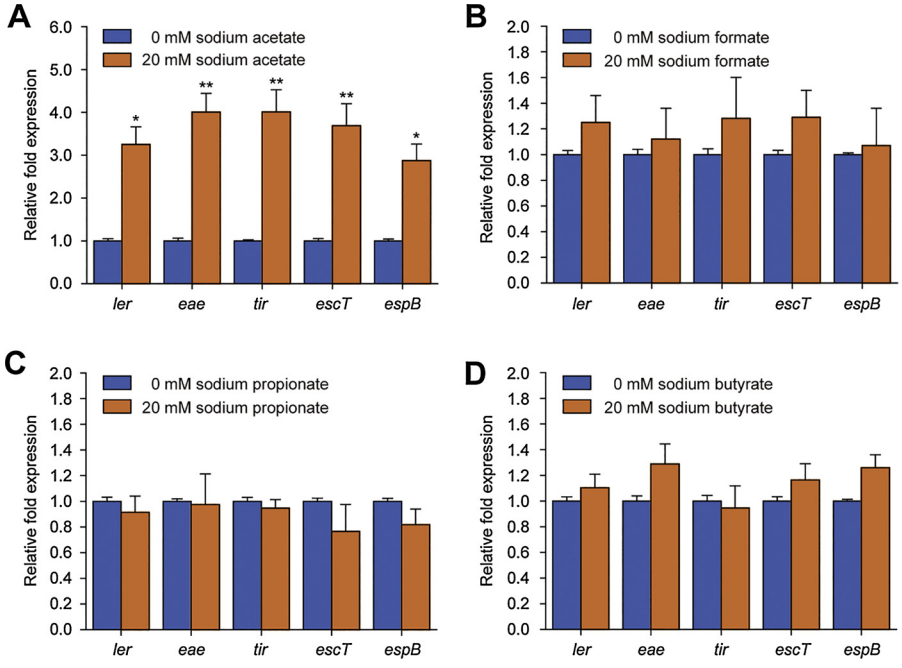

Fig. 2. Effect of SCFAs on LEE gene expression in EPEC O127:H6. (A–D) qRT-PCR was performed to measure the expression of LEE genes in EPEC O127:H6 grown in DMEM supplemented with 0 or 20 mM sodium acetate (A), sodium formate (B), sodium propionate (C), or sodium butyrate (D). Data are presented as means ± s.d.; n = 3. * $P \leq 0.05$; ** $P \leq 0.01$ by Student's *t*-test.

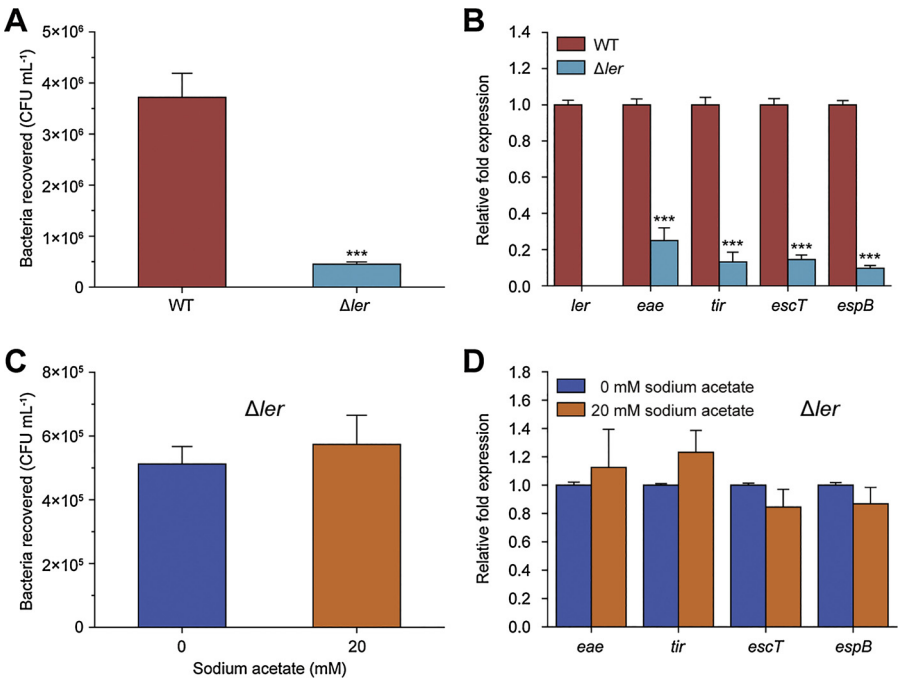

Fig. 3. Activation of EPEC O127:H6 adherence and LEE gene expression by acetate is mediated by Ler. (A) Adhesion of EPEC O127:H6 wild-type strain and Δ*ler* mutant to Caco-2 intestinal epithelial cells. (B) qRT-PCR quantification of changes in the expression of LEE genes in EPEC O127:H6 wild-type strain and Δ*ler* mutant. (C) Adhesion of Δ*ler* mutant to Caco-2 intestinal epithelial cells in DMEM supplemented with 0 or 20 mM sodium acetate. (D) qRT-PCR quantification of the change in LEE gene expression in Δ*ler* mutant grown in DMEM supplemented with 0 or 20 mM sodium acetate. Data are presented as means ± s.d.; n = 3. *** P ≤ 0.001 by Student's *t*-test.

3.4. The effect of SCFAs on EPEC O127:H6 motility and flagellar biosynthesis

To further investigate whether SCFAs regulate EPEC O127:H6 motility, swimming motility assays were carried out in semi-solid LB agar plates supplemented with different concentrations of SCFAs. It was found that EPEC O127:H6 motility on semi-solid LB agar was not affected by the presence of low levels of acetate (less than 10 mM) but significantly increased in the presence of high levels of acetate (above 20 mM; Fig. 4A). Accordingly, the expression of two representative flagellar genes, fliC (encoding the major flagellin) and motA (encoding the flagella motor), was significantly increased in the presence of 20 mM acetate, in comparison with the expression levels observed at 0 mM (Fig. 4B). This result indicated that acetate promotes EPEC O127:H6 motility and flagellar biosynthesis. In contrast, formate, propionate, and butyrate had no obvious effect on EPEC O127:H6 motility

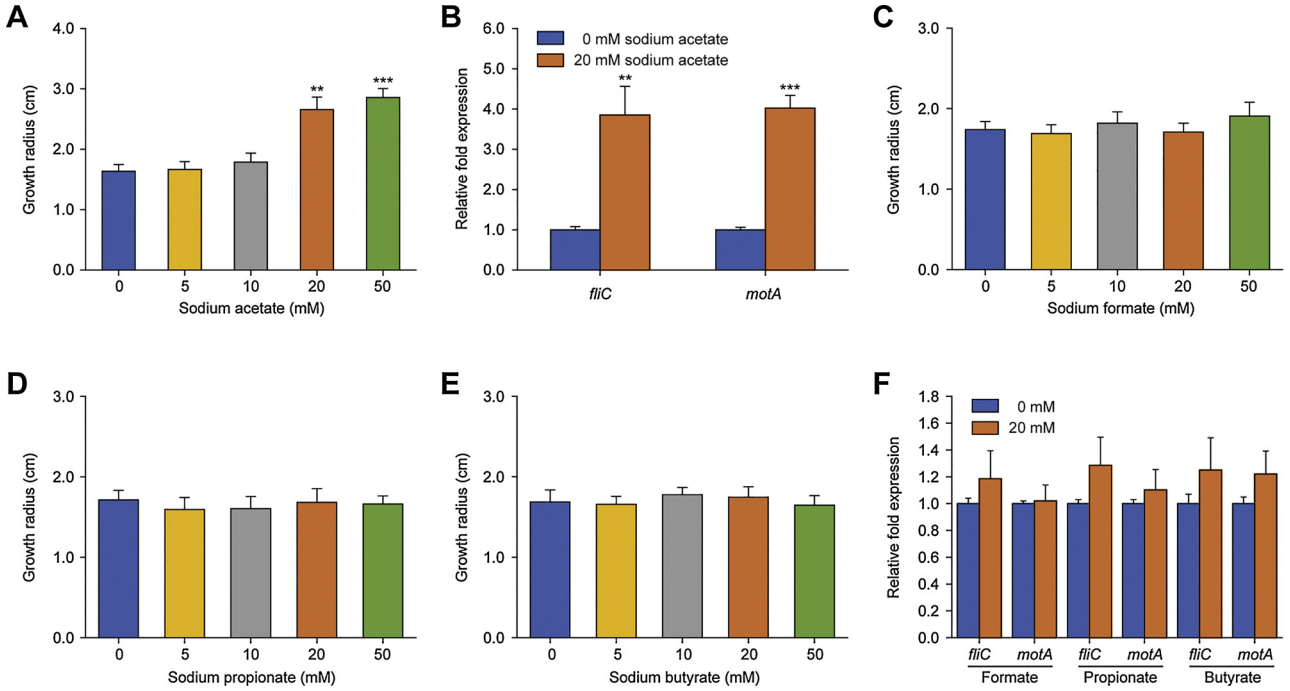

Fig. 4. Effect of SCFAs on EPEC O127:H6 motility and flagellar biosynthesis. (A) Growth radius of EPEC O127:H6 on motility agar supplemented with different concentrations of sodium acetate after 8 h at 30 °C. (B) qRT-PCR was performed to measure the expression of *fliC* and *motA* in EPEC O127:H6 grown in the presence of 0 or 20 mM sodium acetate. (C–E) Growth radius of EPEC O127:H6 on motility agar supplemented with different concentrations of sodium formate (C), sodium propionate (D), or sodium butyrate (E) after 8 h at 30 °C. (F) qRT-PCR was performed to measure the expression of *fliC* and *motA* in EPEC O127:H6 grown in the presence of 0 or 20 mM sodium formate, sodium propionate, or sodium butyrate. Data are presented as means ± s.d.; n = 3. ** P ≤ 0.01; *** P ≤ 0.001 by Student's *t*-test.

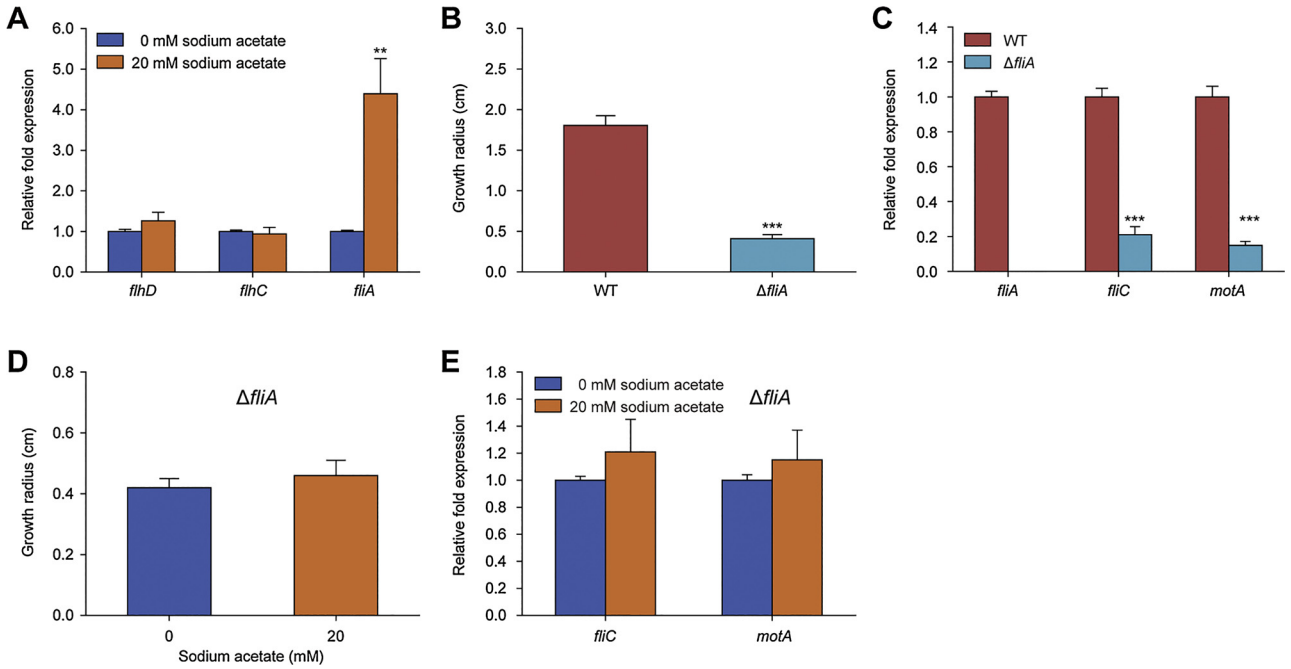

Fig. 5. Acetate activates EPEC O127:H6 flagellar gene expression via *fliA*. (A) qRT-PCR was performed to measure the expression of *flhC*, *flhD*, and *fliA* in EPEC O127:H6 grown in the presence of 0 or 20 mM sodium acetate. (B) Growth radius of EPEC O127:H6 wild-type strain and Δ*fliA* mutant on motility agar after 8 h at 30 °C. (C) qRT-PCR was performed to quantify the changes in the expression of *fliA*, *fliC* and *motA* in EPEC O127:H6 wild-type strain and Δ*fliA* mutant. (D) Growth radius of Δ*fliA* mutant on motility agar supplemented with 0 or 20 mM sodium acetate after 8 h at 30 °C. (E) qRT-PCR was performed to quantify the changes in the expression of *fliC* and *motA* in Δ*fliA* mutant grown in the presence of 0 or 20 mM sodium acetate. Data are presented as means ± s.d.; n = 3. ** $P \leq 0.01$; *** $P \leq 0.001$ by Student's *t*-test.

and flagellar gene expression (Fig. 4C–F).

3.5. Acetate activates flagellar gene expression via fliA

As expression of flagellar genes is directly controlled by FlhDC and FliA, we tested whether acetate activates flagellar gene expression to promote motility through either or both factors. It was found that the transcript levels of *fliA* were significantly more abundant in EPEC O127:H6 grown with 20 mM acetate, in comparison with those observed at 0 mM (Fig. 5A). In contrast, expression of flhD and flhC was comparable between EPEC O127:H6 grown in the absence and presence of 20 mM acetate (Fig. 5A). Additionally, inactivation of *fliA* in EPEC O127:H6 resulted in decreased bacterial motility capacity (Fig. 5B) and flagellar gene transcript levels (Fig. 5C), and neither feature was affected by the presence of acetate (Fig. 5D and E). These results suggested that the positive regulation of EPEC O127:H6 motility and flagellar gene expression by acetate is dependent on FliA.

3.6. Acetate-mediated regulation of bacterial virulence and motility is conserved in EPEC

The presence of the acetate-mediated regulatory pathway for the control of bacterial adherence and motility was investigated using an additional nine representative EPEC strains from seven serotypes. All these strains were first verified through serotyping using polyclonal O-antigen and H-antigen antisera. A multiplex PCR assay was then performed to confirm the presence of intimin and Shiga toxin genes following the method of the STEC center (http://www.shigatox.net/stec/cgi-bin/index). All of the strains used in this work contained the *eae* gene but no Shiga toxin genes (Table 1), confirming that these strains belonged to EPEC and not EHEC, which contain the *eae* gene and at least one Shiga toxin gene. The adherence and motility assays revealed that the adherence to Caco-2 intestinal epithelial cells and motility on semi-solid LB agar plates were significantly increased in the presence of 20 mM acetate for all nine EPEC strains (Fig. 6). In contrast, the

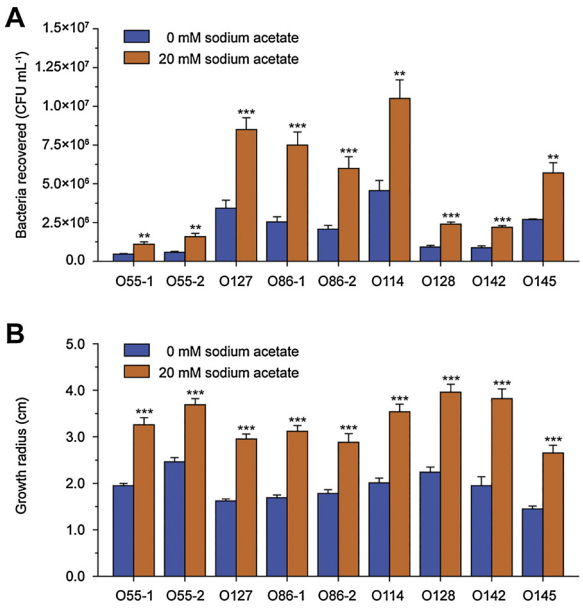

Fig. 6. The effect of acetate on the adherence and motility of other EPEC strains. (A) Adhesion of various EPEC strains to Caco-2 intestinal epithelial cells in DMEM supplemented with 0 or 20 mM sodium acetate. (B) Growth radius of various EPEC strains on motility agar supplemented with 0 or 20 mM sodium acetate after 8 h at 30 °C. Data are presented as means ± s.d.; n = 3. ** $P \leq 0.01$; *** $P \leq 0.001$ by Student's *t*-test.

adherence and motility of these EPEC strains were unaffected by the presence of 20 mM formate, propionate, or butyrate (Supplementary Fig. S2). These results suggested that the acetate-mediated regulatory pathway is a widespread mechanism used by EPEC to mediate bacterial virulence and motility.

4. Discussion

The fermentation of a wide variety of carbohydrates and possibly proteins by microflora in the human intestine results in the production of SCFAs—primarily formate, acetate, propionate, and butyrate—as end products along with various gases and energy, which the bacteria require for growth and maintenance of cellular function (Cummings et al., 1987; Wong et al., 2006). The amounts of SCFAs vary throughout the gastrointestinal tract, with formate and acetate abundant in the small intestine, while propionate and butyrate are higher in the colon (Cummings and Macfarlane, 1991; Keeney and Finlay, 2011). Here, we demonstrated that acetate positively regulates the virulence and motility of human small intestinal pathogen EPEC. It is proposed that when EPEC enter the small intestine, where the acetate level is high (about 10–30 mM in the distal ileum), the expression of *ler* and *fliA* is upregulated, ultimately activating the expression of LEE genes and flagellar genes. The resulting increase in adherence and motility then enables the pathogen to reach and adhere to colonization sites in the host. Nevertheless, further studies are required to reveal the precise regulatory mechanism of acetate in EPEC motility and pathogenesis, including investigation of whether other regulatory proteins are involved in this regulatory process.

In order to ensure successful colonization, the LEE and flagellar genes are subjected to strict regulation that assures expression only under optimal environmental conditions while avoiding intense metabolic cost and/or alert of the host immune system (Chevance and Hughes, 2008; Franzin and Sircili, 2015; Mellies et al., 2007). Expression of these virulence-related genes is affected by diverse environmental factors that include the bacterial growth phase, temperature, cation presence, pH, osmolarity, stress conditions, and nutrient availability (Chevance and Hughes, 2008; Franzin and Sircili, 2015). In this study, acetate was found to act as an intestinal signal sensed by EPEC to mediate bacterial expression of LEE genes and flagellar genes.

It is well known that FliA activates the transcription of the flagellar genes that encode flagellin and the motor and chemotaxis proteins once the hook-basal body has been assembled (Chevance and Hughes, 2008). A recent study identified 52 FliA-binding sites in the *E. coli* MG1655 genome by ChIP-seq; 35 of those were novel and unrelated to bacterial flagella synthesis (Fitzgerald et al., 2014). The presence of a large number of binding sites indicates that FliA may play multiple regulatory roles. Therefore, by interfering with FliA, acetate is expected to affect other FliA-regulated biological processes as well.

Previous studies revealed that formate and acetate act as intestinal signals to induce the expression of invasion genes in S. *typhimurium*, while butyrate, an SCFA present at higher concentrations in the large intestine, is known to repress invasion genes (Gantois et al., 2006; Huang et al., 2008). These results are consistent with a mechanism in which SCFAs serve as a signal for bacterial invasion of the ileum but reduce invasion in the colon and suggest an explanation for the long-held observation that Salmonella preferentially invade the distal ileum. Notably, the regulation of invasion genes by acetate is dependent on pH (Lawhon et al., 2002). The invasion genes can only be activated by acetate at pH 6.7 (pH of the distal ileum) and not pH 8.0 (Lawhon et al., 2002). In contrast with this finding, acetate activated the expression of LEE genes and flagellar genes in EPEC O127:H6 grown at different pH (Supplementary Fig. S3). These results indicated that the acetate-mediated virulence regulatory mechanisms in Salmonella and EPEC are inherently different. The concentration of butyrate in the human large intestine is much higher than that in the small intestine (Cummings and Macfarlane, 1991; Keeney and Finlay, 2011). A previous study revealed that butyrate induces LEE gene expression in large intestinal pathogen EHEC and enhances its adherence to Caco-2 cell monolayers (Nakanishi et al., 2009). In accordance with these results, we also found that the adherence capacity of all three tested EHEC strains (O157:H7, O26:H11, and O103:H2) was significantly increased in the presence of 20 mM sodium butyrate but not formate, acetate, and propionate (Supplementary Fig. S4). However, the positive effect of butyrate on bacterial adherence was not observed with small intestinal pathogen EPEC (Supplementary Fig. S2). It is proposed that EPEC and EHEC use different regulatory mechanisms to control the expression of LEE genes, leading to pathotype-specific colonization of the small and large intestines, respectively.

In summary, our results in this study demonstrated that acetate promotes EPEC adherence and motility by activating the expression of LEE genes and flagellar genes, respectively. The positive effect of acetate on the expression of LEE genes and flagellar genes is mediated by the master regulator of LEE genes, Ler, and the RNA polymerase sigma factor FliA. Our work largely enriches our understanding of the influence of intestinal signals on bacterial adherence and motility. The presence of this acetate-mediated regulatory pathway in all seven EPEC serotypes tested indicates that it is an important pathway for the evolution of this pathogen as an invader of the small intestine.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2018.07.010.

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