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Modulation of chemokine gene expression by Shiga-toxin producing *Escherichia coli* belonging to various origins and serotypes

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Abstract

Infection with Shiga-toxin producing *Escherichia coli* (STEC) may result in the development of the haemolytic-uremic syndrome (HUS), the main cause of acute renal failure in children. While O157:H7 STEC are associated with large outbreaks of HUS, it is difficult to predict whether a non-O157:H7 isolate can be pathogenic for humans. The mucosal innate immune response plays a central role in the pathogenesis of HUS; therefore, we compared the induction of IL-8 and CCL20 in human colon epithelial cells infected with strains belonging to different serotypes, isolated from cattle or from HUS patients. No correlation was observed between strain virulence and chemokine gene expression. Rather, the genetic background of the strains seems to determine the chemokine gene expression profile. Investigating the contribution of different bacterial factors in this process, we show that the type III secretion system of O157:H7 bacteria, but not the intimate adhesion, is required to stimulate the cells. In addition, H7, H10, and H21 flagellins are potent inducers of chemokine gene expression when synthesized in large amount.

Keywords: Epithelial cells; Shiga-toxin producing *Escherichia coli*; Chemokine; Locus of enterocyte effacement; Flagellin.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are emerging pathogens responsible for foodborne infections. Healthy rearing animals are the main STEC reservoir and human infection occurs through the ingestion of contaminated food. The presence of these bacteria in the gut can be asymptomatic or lead to diseases ranging from watery diarrhea to hemorrhagic colitis and life-threatening complications, such as hemolytic-uremic syndrome (HUS; [1,2]). Human isolates from patients are also referred to enterohemorrhagic *E. coli* (EHEC). STEC strains belonging to the O157:H7 serotype are frequently responsible for the majority of the cases of disease worldwide and for large outbreaks, whereas non-O157:H7 serotypes are often responsible for sporadic cases. The bacterial factors associated with pathogenicity are Shiga-toxin (Stx) 2 and intimin [3]. Moreover, it has been recently demonstrated that the chromosomal insertion site of Stx-encoding bacteriophage in O157:H7 bacteria might determine its virulence or transmissibility to humans [4]. Nonetheless, in LEE-negative non-O157:H7 strains, it is difficult to predict whether an isolate could represent a risk to human health.

STEC possess numerous virulence factors that allow colonization and pathogenesis. Genes in the locus of enterocyte effacement (LEE) code for proteins implicated in the intimate adhesion of the bacteria to eukaryotic cells, i.e. intimin encoded by *eae* and its receptor Tir [5,6], and for a type III secretion system (TTSS). EspA is the main component of the pilus-like appendage, which forms the needle of the TTSS [7]. EspD protein is essential for the formation of surface appendages and is integrated in the cytoplasmic membranes of eukaryotic cells [8]. Two additional proteins, SepL and EscD, also called Pas, are involved in the translocation of Tir and secretion of Esp proteins [9,10]. TTSS-secreted proteins interact with the host signal transduction, leading to actin polymerization and microvilli effacement [1].

However, it is not known whether attachment/effacement is a prerequisite for the development of severe diseases, notably because LEE-negative EHEC strains are often isolated from cases of HUS [1]. Additionally, the EHEC virulence factors associated with severe human diseases are Stx1 and Stx2; these toxins are produced in the lower intestine, translocate across intestinal epithelium, and induce necrosis or apoptosis of vascular endothelial cells by inhibiting protein synthesis [11]. Stx may also modulate the innate immune response of human enterocytes [12,13] and favor the attachment of the bacteria to the colonic epithelium [14].

Clinical studies highlight that the host inflammatory response is induced during the course of EHEC infection and correlates with the development of HUS [15,16]. This inflammation depends on the EHEC-induced mucosal innate immune response. Activated enterocytes synthesize chemokines and cytokines in response to pathogen-associated molecular patterns to attract and activate leukocytes to the site of infection. In fact, histological analysis of gut tissues harvested from O157:H7-infected patients has shown neutrophils infiltrating the lamina propria and crypts [17]. It has been proposed that the colonic mucosa damaged by the inflammation represents a way for Stx to cross the epithelial barrier. In vitro studies have shown that STEC strains induce the expression in intestinal epithelial cells of IL-8 and CCL20 [13,18], which can recruit neutrophils and dendritic cells, respectively. In this context, the ability of STEC strains to induce the host innate immune response could be a reliable marker of bacterial virulence.

Thus, studies were performed to evaluate the induction of IL-8 and CCL20 in human epithelial cells in response to STEC strains isolated from either the animal reservoir or HUS patients and belonging to O157:H7, O91:H21, O91:H10, O113:H21, or O6:H10 serotypes. In addition, we analysed the potential contribution of different bacterial factors to the stimulation of the innate immune response using mutant strains lacking the main STEC virulence factors.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are described in Table 1 [19-22]. Bacteria were isolated on Luria-Bertani (LB) agar plates. One clone of each strain was grown overnight in LB broth with agitation at 37°C, then diluted ($OD_{600\ nm} \approx 0.03$) and grown for 2 h until the exponential growth phase ($OD_{600\ nm} \approx 0.3$) in DMEM (Invitrogen). Media were supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), and/or IPTG (see Table 2) when required. For the motility experiments, LB agar was overlaid by LB soft agar. Bacterial concentration was estimated to be 5×10^8 bacteria per ml per OD unit at 600 nm, as calculated by plating.

2.2. Mutagenesis and complementation

The mutant and complemented STEC strains used in this study are presented in Table 2. Deletion of the *eae* and *fliC* genes of EDL933 and of the *fliC* genes of CHO14, CHO13, and NV268, were obtained by allelic exchange with a kanamycin cassette using the one-step PCR-based method of Datsenko and Wanner [23]. For complementation of the mutants, the genes of interest were cloned under the control of the IPTG-inducible *trc* promoter in the expression vector pTrc99A.

Mutations and complementations of *fliC* mutants were checked by measuring the motility of the strains in soft agar (Table 2). For the *eae* gene, wild-type (WT), mutant, and complemented strains were grown in DMEM to the early exponential phase; subsequently, intimin protein in crude bacterial lysates was analyzed by Western blotting using polyclonal anti-intimin antibody provided by Josée Harel (Groupe de Recherche sur les Maladies

Infectieuses du Porc, Université de Montréal, Sainte-Hyacinthe, Canada). Intimin was detected in the strains EDL933 and EDL933 Δ*eae* tC, and was completely absent in the strain EDL933 Δ*eae* (data not shown).

2.3. Cell culture and infections

The human colon adenocarcinoma cell line T84 was maintained in 50% DMEM with glutamax, 50% Ham's F12, 10% FCS, 1% sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. Of importance is that human colonic epithelial cells and T84 cells do not express the Stx receptor glycolipid globotriaosylceramide-3. Cells (5 X 10^5 per well in 1 ml) were plated on 24-well plates and cultured for 7-10 days at 37°C under 5% CO_2. Bacteria were added to the cells at a multiplicity of infection of 10 in complete medium devoid of antibiotics for 3 h. In some experiments, cocultures were washed three times and a fresh complete medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin was added for 21 h. Subsequently, the filtered supernatants were used for measurement of chemokine concentrations.

2.4. Analysis of chemokine induction

The expression levels of IL-8 and CCL20 mRNAs was analyzed by real-time PCR as previously described [13]. CCL20 and IL-8 concentrations were measured using the Duo Set ELISA kit (R&D Systems) according to the manufacturer's protocol.

2.5. FAS test

T84 cells were infected for 3 h with EHEC strains on Lab-tek slides (Nunc). Monolayers were washed, fixed in paraformaldehyde in PBS, and permeabilized for 5 min in 0.1% saponin. Cells were stained with FITC-phalloidin (Molecular Probes Europe) that labels F-actin filaments. Cells were examined by fluorescence microscopy. The enteropathogenic *E. coli* strain E2348/69 was used as a positive control for the FAS test.

2.6. Statistics

Student's *t* test was used to determine significant difference between two groups. ANOVA with the Games–Howell post hoc test was used to identify significant differences among multiple test groups; this test was two-tailed, and $P = 0.05$ was considered significant.

3. Results

3.1. Differential expression of chemokine mRNA in T84 cells infected with O157:H7 and non-O157:H7 STEC isolates

The expression of IL-8 and CCL20 genes in human intestinal epithelial T84 cells in response to O157:H7 EHEC was analyzed by real-time RT-PCR. As depicted in Fig. 1, all the O157:H7 strains induced a significant increment in the levels of IL-8 and CCL20 mRNA when compared to cells infected with *E. coli* DH5α (Table 3). Similar IL-8 and CCL20 induction levels were observed when bovine, HC, HUS, or outbreak O157:H7 isolates were used to stimulate the cells. But intriguingly, IL-8 and CCL20 mRNAs were expressed significantly less by the cells infected with the three O157:H7 strains that possess *stx1* and *stx2* genes, namely the strains Sakaï, EDL933 and NV95, in comparison with the three other O157:H7 isolates harboring only the gene *stx2* ($P = 0.015$ and $P = 0.026$ for CCL20 and IL-8 by unpaired *t* test, respectively).

We also investigated the chemokine response of T84 cells infected with non-O157:H7 strains belonging to different serotypes and isolated from human clinical samples or from healthy bovines. Variable responses were observed according to the origin of the isolates or to the strain serotype (Fig. 1; Table 3). Chemokine mRNA expression was significantly increased when compared to DH5α-infected cells when O91:H21 and O113:H21 strains, but not O91:H10 or O6:H10 strains, were used to stimulate the cells. However, in the serotype O113:H21, chemokine mRNA expression was significantly higher in cells stimulated with strains obtained from HUS patients than in cells infected with bovine isolates. Nonetheless, it should be noted that a strong discrepancy was observed between the O113:H21 HUS strains tested; the highest levels of IL-8 and CCL20 mRNA were induced by the strain CL3, whereas

the levels of mRNA induced by the strains CL15 and 87-307 were comparable to those induced by the three bovine strains. In the serotypes O91:H21 and O91:H10, for which strains of different origin were used, similar mRNA expression was observed when enterocytes were activated with bovine or HUS isolates.

Interestingly, we observed that IL-8 and CCL20 genes were more expressed in cells stimulated with strains harboring the flagellin H21 or H7 when compared to the stimulation with isolates belonging to the H10 serogroup (Fig. 1 and Table 3). No significant differences were observed when the different serogroup O were compared.

These results were confirmed by the measurement of CCL20 and IL-8 concentrations in culture supernatant (Fig. 2); chemokine production was increased in cells stimulated with STEC O157:H7, O91:H21, or O113:H21 in comparison to control cells (data not shown), DH5α-activated T84 cells, or cells infected with O91:H10 or O6:H10 isolates (Fig. 2).

3.2. Implication of O157:H7 LEE-encoded proteins

To evaluate the importance of the LEE in the induction of chemokine gene expression, CCL20 mRNA levels induced by the EDL933 strain were compared to those obtained after infection with the isogenic derivatives carrying in-frame deletions in the LEE genes.

CCL20 was significantly more upregulated in enterocytes co-cultured with the *eae* mutant strain compared to the WT strain (Fig. 3), whereas the levels of CCL20 mRNAs in cells infected with the complemented *eae* mutant strain were similar to those obtained with the WT strain. Expression of CCL20 mRNA in T84 cells stimulated by the mutant strains *espA*$^{-}$, *espD*$^{-}$, *sepL*$^{-}$, or *escD*$^{-}$ decreased by ~ 97, 88, 86, and 82% compared with the WT strain, respectively (Fig. 3). Introduction in the mutant strains of the corresponding WT allele in multicopy resulted in a total, even higher, complementation of the mutant phenotype (Fig.

3). Moreover, this inflammatory response was not related to the capacity of the WT strains to induce attaching/effacing lesions since none of them presented a positive FAS test on T84 cells (data not shown).

Similar results were obtained when the expression of IL-8 was analyzed (data not shown).

These results indicate that the TTSS of O157:H7 EHEC, but not the intimate adherence, plays a crucial role in the induction of pro-inflammatory chemokine gene expression.

3.3. The role of flagellin in LEE positive and negative EHEC strains

The results presented in Fig. 1 underline that strains belonging to the serogroups H7 or H21 induce more CCL20 and IL-8 than STEC expressing H10. To determine whether chemokine gene expression was related to flagellin, T84 cells were infected with *fliC* mutants and complemented strains constructed in different serotypes. The *fliC* deletion in the strain CHO14, belonging to the serotype O91:H21, resulted in a ~ 60% decrease in CCL20 mRNA expression when compared to the WT strain (Fig. 4). However, in the serotypes O91:H10, O6:H10, and O157:H7 the absence of flagellin did not result in a significant change in gene expression compared to WT strains. In T84 cells infected with complemented CHO14 ΔH21, CHO13 ΔH10, NV268 ΔH10, and EDL933 ΔH7 strains, the levels of CCL20 mRNA were 3.3-, 8.3-, 4.8-, and 5.3-fold higher than in cells stimulated with WT strains, respectively (Fig. 4). Similar results were obtained with the gene encoding IL-8 (data not shown).

Therefore, we suggest that flagellin of O91:H21 bacteria is partially responsible for the induction of chemokine gene expression in human enterocytes. In addition, H7, H10, and H21 flagellins are potent activators when synthesized in large amount.

4. Discussion

In this study we analyzed in this study the possible relationship between the pathogenicity of STEC strains and their capacity to induce IL-8 and CCL20 gene expression in human enterocytes. Our results indicate that there is no correlation between the origin of the isolates (bovine or HUS) and IL-8/CCL20 gene expression in the serotypes O157:H7, O91:H21, and O91:H10. In the serotype O113:H21, we found a significant difference in chemokine gene expression between the cells infected with bovine or human isolates; although this difference in induction was significant, it was probably due to the high levels of chemokine mRNA in T84 cells infected with the strain CL3. On the other hand, we determined that the induction of cell chemokine production was dependent on different bacterial factors according to the serotype of the tested strains.

Our results indicate that LEE genes of O157:H7 EHEC modulate the innate immune response of human enterocytes. The genes of this pathogenicity island are expressed in the conditions in which the bacteria were co-cultured with T84 cells, i.e. exponential growth phase in DMEM [24]; thus it is likely that they may play a role in the activation of epithelial cells. We demonstrated for the first time that EHEC TTSS is necessary to induce the activation of T84 cells. Similarly, it has been reported that enteropathogenic *E. coli* lacking a functional TTSS induce lower levels of MAPK-dependent IL-8 gene expression than the parental strains [25]. However, we established that the LEE-dependent intimate attachment of the bacteria to the cell is not required to induce the production of chemokine as previously described in Caco-2 cells [26]. Together these results suggest that translocated bacterial factors are required for the initiation of the pro-inflammatory response. However, the intimin/Tir association-induced signal transduction [27] is not implicated in chemokine gene expression. Further, these molecular events may be responsible for the inhibition of the innate

immune response because a significant increase of CCL20 and IL-8 mRNA expression was observed in cells infected with the *eae*-deficient strain in comparison with WT bacteria.

Moreover, we observed that the cells stimulated with the O157:H7 strains expressing the *stx1* and *stx2* genes produced fewer chemokines than the isolates only harboring *stx2*. We have recently shown that EHEC-derived Stx inhibits NF-κB signaling and chemokine gene expression in T84 cells [13]. Thus, we hypothesize that IL-8 and CCL20 mRNA expression were more inhibited by the strains producing both toxins in comparison with the strains that only synthesize Stx2. In support of this hypothesis, we have established that the single deletion of either the *stx1* or *stx2* gene in the EDL933 strain resulted in a partial increase in chemokine gene expression in T84 cells when compared to the WT strain (data not shown); nonetheless, the higher enhancement of IL-8 and CCL20 mRNA expression was observed with the double mutant [13]. However, this observation deserves further investigations with increased number of strains.

In LEE-negative EHEC, we found that strains harboring the H21 flagellin were more potent to induce chemokine production than the H10 isolates. Accordingly, it has been reported that purified H21 flagellin induces IL-8 and MIP-2α synthesis in Hct-8 cells [28]. This led us to investigate the role of the flagellin in cell activation by constructing strains invalided for the *fliC* genes and the complemented corresponding strains. In O91:H21 EHEC, the absence of flagellin resulted in a partial decrease of chemokine gene expression in T84 cells in comparison with the WT strain. Unfortunately, we have not succeeded in constructing a *fliC* mutant in the O113:H21 background in order to confirm the results obtained with O91:H21 bacteria. Nonetheless, we suggest that bacteria expressing H21 flagellin induce a cellular response using flagellin and other unidentified factor(s). Oppositely, strains harboring the H10 flagellin do not stimulate T84 cells, suggesting that in our experimental conditions, these bacteria do not express an inducing factor. On the other hand, we showed that H7, H21,

and H10 flagellin over-expressed in complemented strains are potent activators of chemokine gene expression. This effect was probably due to the increase of flagellin/flagella released by the complemented mutant strain, knowing that the polymerized form of flagellin is less effective on Toll-like receptor (TLR)-5 than the monomer [29]. In this context, the question raised is, why didn't the isolates belonging to the H10 serogroups activate T84 cells when the H21 bacteria did? Although the amino acid sequence recognized by TLR-5 is highly conserved in H7 (accession number: AM228903.1), H10 (accession number: AY249995.1), and H21 (accession number: DQ862122.1) flagellins, there is a global heterogeneity between them that might explain the differential cellular responses. We postulate that *i*) the H21 flagellin is produced/released to a greater extent than the flagellin H10, *ii*) the flagellin H21 is more efficient in activating the cells than the flagellin H10 when used at the same concentrations, or *iii*) the strains belonging to the serogroup H10 inhibit the immune response more efficiently than the other strains. In fact, our culture conditions were not favorable for *fliC* expression and the synthesis of flagellin was probably very low, except when the gene was over-expressed in complemented strains. Additionally, it should be noted that human colonic epithelial cells and T84 cells express high levels of TLR-5 [30], the innate immune receptor that binds flagellin. These cells, however, express low levels of TLR-2 and -4 [31,32], suggesting that bacterial lipoproteins and lipopolysaccharides do not play a major function in eliciting chemokine gene expression. Moreover, the nucleotide oligomerization domain-1 [33], but not the nucleotide oligomerization domain-2 [34], is expressed in T84 cells, and may thus sense bacterial peptidoglycan. Thus, EHEC peptidoglycan could play a role in the stimulation of human enterocyte, as previously described for the other extracellular pathogen *Helicobacter pylori*, which use a type IV secretion system to deliver peptidoglycan to host cells [35].

Although there is no clear correlation between the pathogenicity of the strain and the chemokine gene expression in human enterocytes, our results highlight that EHEC have elaborated numerous strategies to initiate the host inflammatory response according to their genetic background. The O157:H7 strains induce chemokine mRNA expression using the TTSS and flagellin when expressed in large amount. In non-O157:H7 LEE-negative isolates, those that spontaneously induce the innate immune response, i.e. strains harboring the flagellin H21, stimulate the cells using flagellin and other unknown factors. Lastly, the strains belonging to the serogroup H10 can stimulate the cells with high concentrations of flagellin. Interestingly, the O91:H21 and O113:H21 isolates belongs to the same STEC 1 clonal group (see http://www.shigatox.net/cgi-bin/stec/clonal), whereas O6:H10 and O91:H10 are genetically distant from this group. The control of the expression of EHEC virulence and metabolic factors by the external environment, i.e. gut microbiota or host mediators, may thus have a critical role in bacteria-induced mucosal inflammation.

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References

[1] J.C. Paton, A.W. Paton, Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections, Clin. Microbiol. Rev. 11 (1998) 450-479.

[2] P.I. Tarr, C.A. Gordon, W.L. Chandler, Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome, Lancet 365 (2005) 1073-1086.

[3] P. Boerlin, S.A. McEwen, F. Boerlin-Petzold, J.B. Wilson, R.P. Johnson, C.L. Gyles, Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans, J. Clin. Microbiol. 37 (1999) 497-503.

[4] T.E. Besser, N. Shaikh, N.J. Holt, P.I. Tarr, M.E. Konkel, P. Malik-Kale, C.W. Walsh, T.S. Whittam, J.L. Bono, Greater diversity of Shiga toxin-encoding bacteriophage insertion sites among *Escherichia coli* O157:H7 isolates from cattle than in those from humans, Appl. Environ. Microbiol. 73 (2007) 671-679.

[5] B. Kenny, B.B. Finlay, Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase C-gamma1, Infect. Immun. 65 (1997) 2528-2536.

[6] A.E. Jerse, J. Yu, B.D. Tall, J.B. Kaper, A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells, Proc. Natl. Acad. Sci. USA 87 (1990) 7839-7843.

[7] S. Knutton, I. Rosenshine, M.J. Pallen, I. Nisan, B.C. Neves, C. Bain, C. Wolff, G. Dougan, G. Frankel, A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells, EMBO J. 17 (1998) 2166-2176.

[8] A.U. Kresse, M. Rohde, C.A. Guzman, The EspD protein of enterohemorrhagic *Escherichia coli* is required for the formation of bacterial surface appendages and is incorporated in the cytoplasmic membranes of target cells, Infect. Immun. 67 (1999)

4834-4842.

[9] A.U. Kresse, F. Beltrametti, A. Muller, F. Ebel, C.A. Guzman, Characterization of SepL of enterohemorrhagic *Escherichia coli*, J. Bacteriol. 182 (2000) 6490-6498.

[10] A.U. Kresse, K. Schulze, C. Deibel, F. Ebel, M. Rohde, T. Chakraborty, C.A. Guzman, Pas, a novel protein required for protein secretion and attaching and effacing activities of enterohemorrhagic *Escherichia coli*, J. Bacteriol. 180 (1998) 4370-4379.

[11] Y. Endo, K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, K. Igarashi, Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins, Eur. J. Biochem. 171 (1988) 45-50.

[12] W.E. Smith, A.V. Kane, S.T. Campbell, D.W. Acheson, B.H. Cochran, C.M. Thorpe, Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells, Infect. Immun. 71 (2003) 1497-1504.

[13] A.P. Gobert, M. Vareille, A.L. Glasser, T. Hindre, T. de Sablet, C. Martin, Shiga-toxin produced by enterohemorrhagic *Escherichia coli* inhibits PI3K/NF-κB signaling pathway in globotriaosylceramide-3-negative human intestinal epithelial cells, J. Immunol. 178 (2007) 8168-8174.

[14] C.M. Robinson, J.F. Sinclair, M.J. Smith, A.D. O'Brien, Shiga toxin of enterohemorrhagic *Escherichia coli* type O157:H7 promotes intestinal colonization, Proc. Natl. Acad. Sci. USA 103 (2006) 9667-9672.

[15] M.M. Fitzpatrick, V. Shah, R.S. Trompeter, M.J. Dillon, T.M. Barratt, Interleukin-8 and polymorphoneutrophil leucocyte activation in hemolytic uremic syndrome of childhood, Kidney Int. 42 (1992) 951-956.

[16] A.P. Gobert, K.T. Wilson, C. Martin, Cellular responses to attaching and effacing bacteria: activation and implication of the innate immune system, Arch. Immunol. Ther.

Exp. 53 (2005) 234-244.

[17] P.M. Griffin, L.C. Olmstead, R.E. Petras, *Escherichia coli* O157:H7-associated colitis. A clinical and histological study of 11 cases, Gastroenterology 99 (1990) 142-149.

[18] S. Dahan, V. Busuttil, V. Imbert, J.F. Peyron, P. Rampal, D. Czerucka, Enterohemorrhagic *Escherichia coli* infection induces interleukin-8 production via activation of mitogen-activated protein kinases and the transcription factors NF-κB and AP-1 in T84 cells, Infect. Immun. 70 (2002) 2304-2310.

[19] S.W. Lindgren, A.R. Melton, A.D. O'Brien, Virulence of enterohemorrhagic *Escherichia coli* O91:H21 clinical isolates in an orally infected mouse model, Infect. Immun. 61 (1993) 3832-3842.

[20] N. Pradel, V. Livrelli, C. De Champs, J.B. Palcoux, A. Reynaud, F. Scheutz, J. Sirot, B. Joly, C. Forestier, Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food, and children during a one-year prospective study in France, J. Clin. Microbiol. 38 (2000) 1023-1031.

[21] N.T. Perna, G. Plunkett 3rd, V. Burland, B. Mau, J.D. Glasner, D.J. Rose, G.F. Mayhew, P.S. Evans, J. Gregor, H.A. Kirkpatrick, G. Posfai, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E.J. Grotbeck, N.W. Davis, A. Lim, E.T. Dimalanta, K.D. Potamousis, J. Apodaca, T.S. Anantharaman, J. Lin, G. Yen, D.C. Schwartz, R.A. Welch, F.R. Blattner, Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7, Nature 409 (2001) 529-533.

[22] T. Hayashi, K. Makino, M. Ohnishi, K. Kurokawa, K. Ishii, K. Yokoyama, C.G. Han, E. Ohtsubo, K. Nakayama, T. Murata, M. Tanaka, T. Tobe, T. Iida, H. Takami, T. Honda, C. Sasakawa, N. Ogasawara, T. Yasunaga, S. Kuhara, T. Shiba, M. Hattori, H. Shinagawa, Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12, DNA Res. 8 (2001)

11-22.

[23] K.A. Datsenko, B.L. Wanner, One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products, Proc. Natl. Acad. Sci. USA, 97 (2000) 6640-6645.

[24] F. Beltrametti, A.U. Kresse, C.A. Guzman, Transcriptional regulation of the *esp* genes of enterohemorrhagic *Escherichia coli*, J. Bacteriol. 181 (1999) 3409-3418.

[25] D. Czerucka, S. Dahan, B. Mograbi, B. Rossi, P. Rampal, Implication of mitogen-activated protein kinases in T84 cell responses to enteropathogenic *Escherichia coli* infection, Infect. Immun. 69 (2001) 1298-1305.

[26] M.C. Berin, A. Darfeuille-Michaud, L.J. Egan, Y. Miyamoto, M.F. Kagnoff, Role of EHEC O157:H7 virulence factors in the activation of intestinal epithelial cell NF-κB and MAP kinase pathways and the upregulated expression of interleukin 8, Cell. Microbiol. 4 (2002) 635-648.

[27] J. Garmendia, A.D. Phillips, M.F. Carlier, Y. Chong, S. Schüller, O. Marches, S. Dahan, E. Oswald, R.K. Shaw, S. Knutton, G. Frankel, TccP is an enterohaemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton, Cell. Microbiol. 6 (2004) 1167-1183.

[28] T.J. Rogers, A.W. Paton, S.R. McColl, J.C. Paton, Enhanced CXC chemokine responses of human colonic epithelial cells to locus of enterocyte effacement-negative shiga-toxigenic *Escherichia coli*, Infect. Immun. 71 (2003) 5623-5632.

[29] K.D. Smith, E. Andersen-Nissen, F. Hayashi, K. Strobe, M.A. Bergman, S.L. Barrett, B.T. Cookson, A. Aderem, Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility, Nat. Immunol. 4 (2003) 1247-1253.

[30] A.T. Gewirtz, T.A. Navas, S. Lyons, P.J. Godowski, J.L. Madara, Cutting edge: bacterial

flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression, J. Immunol. 167 (2001) 1882-1885.

[31] M.T. Abreu, P. Vora, E. Faure, L.S. Thomas, E.T. Arnold, M. Arditi, Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide, J. Immunol. 167 (2001) 1609-1616.

[32] G. Melmed, L.S. Thomas, N. Lee, S.Y. Tesfay, K. Lukasek, K.S. Michelsen, Y. Zhou, B. Hu, M. Arditi, M.T. Abreu, Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut, J. Immunol. 170 (2003) 1406-1415.

[33] T. Hisamatsu, M. Suzuki, D.K. Podolsky, Interferon-gamma augments CARD4/NOD1 gene and protein expression through interferon regulatory factor-1 in intestinal epithelial cells, J. Biol. Chem. 278 (2003) 32962-32968.

[34] N. Barnich, J.E. Aguirre, H.C. Reinecker, R. Xavier, D.K. Podolsky, Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-κB activation in muramyl dipeptide recognition, J. Cell. Biol. 170 (2005) 21-26.

[35] J. Viala, C. Chaput, I.G. Boneca, A. Cardona, S.E. Girardin, A.P. Moran, R. Athman, S. Memet, M.R. Huerre, A.J. Coyle, P.S. DiStefano, P.J. Sansonetti, A. Labigne, J. Bertin, D.J. Philpott, R.L. Ferrero, Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island, Nat. Immunol. 5 (2004) 1166-1174.

Figure Legends

Fig. 1. Expression of IL-8 and CCL20 mRNAs in T84 cells. Epithelial cells were exposed for 3 h to *E. coli* DH5α or STEC strains from bovine (b) or HUS origin, belonging to the serogroup O157:H7, O91:H21, O91:H10, O113:H21, or O6:H10. Chemokine gene expression was semi-quantified by real-time PCR. Each point represents the mean ± SEM of 3-5 independent experiments made in duplicate; for the strain DH5α, we present values obtained in 3 different experiments. The name of several particular strains has been added to the figure.

Fig. 2. Synthesis of chemokines. Epithelial cells were infected for 3 h with *E. coli* DH5α or with STEC strains from bovine (b) or HUS origin, belonging to various serogroups. Cells were then washed and a fresh medium containing antibiotics was added for 21 h. CCL20 and IL-8 concentrations were measured in the culture supernatants. CCL20 and IL-8 concentrations in the supernatant of uninfected cells were 2.8 ± 1.3 ng/ml and 0.8 ± 0.1 ng/ml, respectively. Each point represents the mean ± SEM of 4-6 independent experiments; for the strain DH5α, we present values obtained in 3 different experiments.

Fig. 3. CCL20 mRNA expression in T84 cells upon infection with EDL933 and LEE gene mutants. The strain EDL933, the isogenic mutant Δ*eae*, Δ*espA*, Δ*espD*, Δ*sepL*, or Δ*escD*, and the corresponding trans-complemented (tC) strains were used to infect the cells for 3 h. Subsequently, the levels of CCL20 mRNA were assessed by real-time PCR. Data represent the mean ± SEM of 3 experiments performed in duplicate. *, $p < 0.05$ and **, $p < 0.01$ versus cells infected with EDL933.

Fig. 4. The role of EHEC flagellin in CCL20 mRNA expression of human enterocytes. T84 cells were infected for 3 h with the isolates CHO14 (O91:H21), CHO13 (O91:H10), NV268 (O6:H10), and EDL933 (O157:H7), the corresponding *fliC* mutants, or the trans-complemented (tC) strains. CCL20 gene expression was analyzed by real-time PCR and the results depicted are the mean ± SEM of 3 duplicate experiments. *, $p < 0.05$ and **, $p < 0.01$ versus cells infected with WT bacteria; §§, $p < 0.01$ in comparison with T84 cells infected with *fliC* mutant strains.

Table 1
Bacterial strains

Strain	Serotype	Origin	Genetic Characteristic			Reference
			LEE	*stx1*	*stx2*	
EDL933	O157:H7	HC/Outbreak	+	+	+	[21]
Sakaï	O157:H7	HUS/Outbreak	+	+	+	[22]
86-24	O157:H7	HC/Outbreak	+	–	+	T.S. Whittam [a]
ChVi-1	O157:H7	HUS	+	–	+	[20]
Ch1898	O157:H7	HUS	+	–	+	[20]
NV95	O157:H7	Bovine	+	+	+	[20]
CHO14	O91:H21	HUS	–	–	+	[20]
B2F1	O91:H21	HUS	–	–	+	[19]
VTH13	O91:H21	HC	–	+	+	J. Blanco [b]
NV127	O91:H21	Bovine	–	–	+	[20]
NV197	O91:H21	Bovine	–	–	+	[20]
NV20	O91:H21	Bovine	–	–	+	[20]
CHO13	O91:H10	HUS	–	–	+	[20]
CB67-74	O91:H10	HUS	–	+	–	L. Beutin [c]
CB67-75	O91:H10	HUS	–	–	+	L. Beutin [c]
NV130	O91:H10	Bovine	–	–	+	[20]
NV148	O91:H10	Bovine	–	–	+	[20]
NV280	O91:H10	Bovine	–	–	+	[20]
CL3	O113:H21	HUS	–	–	+	T.S. Whittam [a]
CL15	O113:H21	HUS	–	–	+	T.S. Whittam [a]
87-307	O113:H21	HUS	–	–	+	T.S. Whittam [a]
NV254	O113:H21	Bovine	–	–	+	[20]
NV298	O113:H21	Bovine	–	–	+	[20]
NV299	O113:H21	Bovine	–	–	+	[20]
NV106	O6:H10	Bovine	–	–	+	[20]
NV183	O6:H10	Bovine	–	–	+	[20]
NV268	O6:H10	Bovine	–	–	+	[20]
DH5α	NA	NA	NA	NA	NA	Invitrogen

[a] STEC center: http://shigatox.net
[b] Laboratorio de Referencia de *E. coli*, Universidade de Santiago de Compostela, Lugo, Spain.
[c] National Reference Laboratory for *Escherichia coli*, Federal Institute for Risk Assessment, Berlin, Germany.

Table 2
Mutant and complemented strains

Strain	Resistance [a]/Phenotype	Reference
EDL933 Δ*eae*	Kn^r/no expression of intimin	This work
EDL933 Δ*eae* tC [b]	Kn^r, Amp^r/intimin synthesis with 0.5 mM IPTG	This work
EDL933 Δ*espA*	Kn^r/no functional TTSS	[24]
EDL933 Δ*espA* tC	Kn^r, Amp^r/functional TTSS	[24]
EDL933 Δ*espD*	Kn^r/no functional TTSS	[8]
EDL933 Δ*espD* tC	Kn^r, Amp^r/functional TTSS	[8]
EDL933 Δ*sepL*	Kn^r/no functional TTSS	[9]
EDL933 Δ*sepL* tC	Kn^r, Amp^r/functional TTSS	[9]
EDL933 Δ*escD*	Kn^r/no functional TTSS	[10]
EDL933 Δ*escD* tC	Kn^r, Amp^r/functional TTSS	[10]
EDL933 Δ*fliC*	Kn^r/no motility (0.4 cm) [c]	This work
EDL933 Δ*fliC* tC	Kn^r, Amp^r/motility restored with 0.01 mM IPTG (9 cm) [c]	This work
CHO14 Δ*fliC*	Kn^r/no motility (0.4 cm) [c]	This work
CHO14 Δ*fliC* tC	Kn^r, Amp^r/motility restored with 0.01 mM IPTG (4.8 cm) [c]	This work
CHO13 Δ*fliC*	Kn^r/no motility (0.5 cm) [c]	This work
CHO13 Δ*fliC* tC	Kn^r, Amp^r/motility restored without IPTG (3.8 cm) [c]	This work
NV268 Δ*fliC*	Kn^r/no motility (0.4 cm) [c]	This work
NV268 Δ*fliC* tC	Kn^r, Amp^r/motility restored with 0.1 mM IPTG (9 cm) [c]	This work

[a] Kn^r, kanamycin resistant; Amp^r, ampicillin resistant
[b] tC, trans complemented.
[c] Measurement after 6 h of the growth spread. Values were 4.5, 1.5, 1.5, and 5.5 cm for the WT strains EDL933, CHO14, CHO13, and NV268, respectively (representative data of three independent experiments).

Table 3
Statistical analysis of the Fig. 1

Analysis	Comparison	*P* (ANOVA)	
		CCL20	IL-8
Pathogenicity	O157:H7 *vs* DH5α	0.05	0.01
	O91:H21 *vs* DH5α	0.05	0.05
	O113:H21 *vs* DH5α	0.05	0.05
Origin	O113:H21 HUS *vs* O113:H21 B [a]	0.05	0.05
Serotype	O91:H21 *vs* O91:H10	0.01	0.01
	O91:H21 *vs* O6:H10	0.01	0.01
	O113:H21 *vs* O91:H10	0.05	0.05
	O157:H7 *vs* O91:H10	0.01	0.01
	O157:H7 *vs* O6:H10	0.01	0.01
Serogroup H	O91:H21 B *vs* O91:H10 B	0.05	0.05
	O91:H21 B *vs* O91:H10 HUS	0.05	0.05
	O91:H21 HUS *vs* O91:H10 B	0.05	0.01
	O91:H21 HUS *vs* O91:H10 HUS	0.05	0.01
	O157:H7 *vs* all H10 strains	0.01	0.05
	all H21 strains *vs* all H10 strains	0.01	0.05

[a] B, Bovine

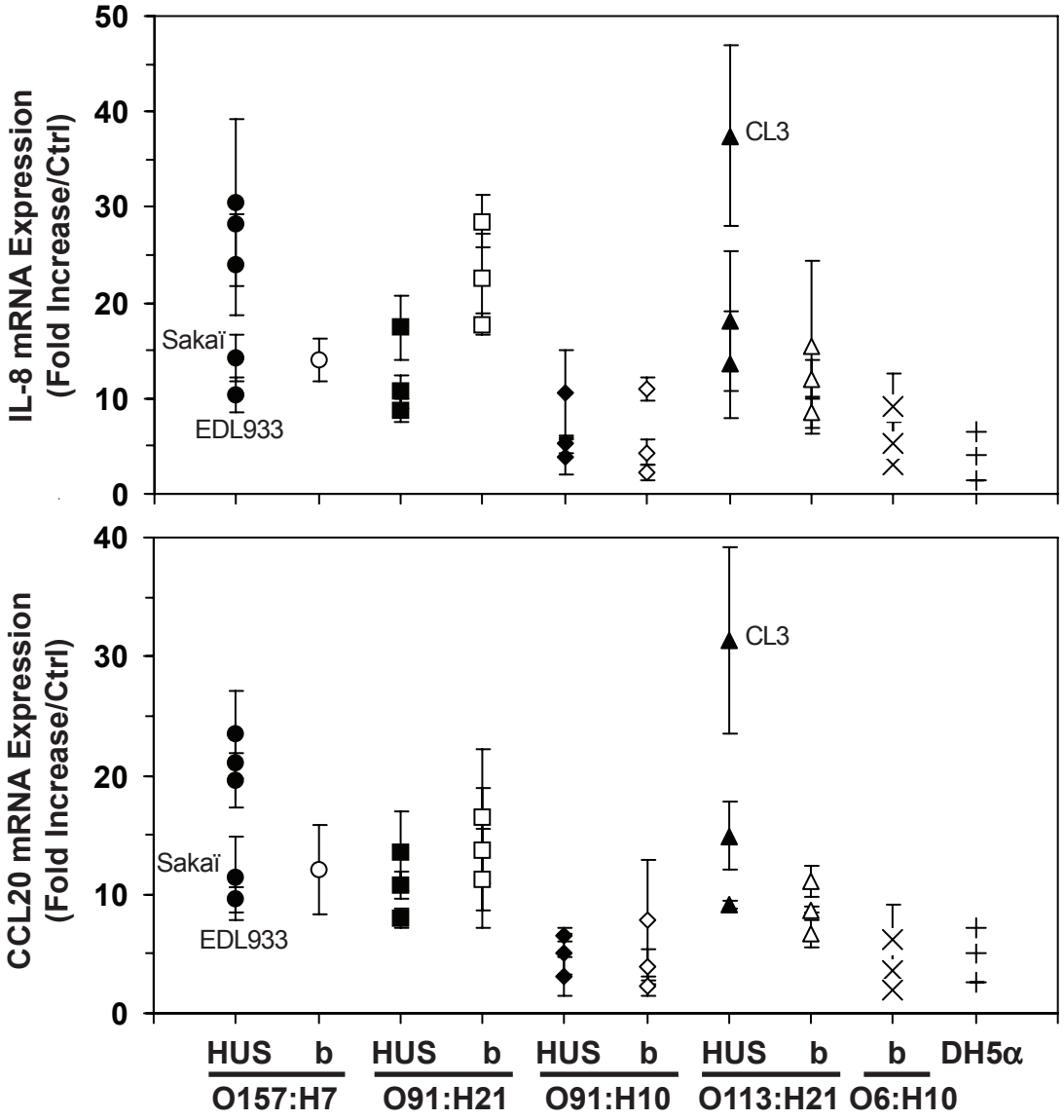
IL-8 mRNA Expression
(Fold Increase/Ctrl)
50
40
30
20
10
0
CL3
Sakaï
EDL933
CCL20 mRNA Expression
(Fold Increase/Ctrl)
40
30
20
10
0
CL3
Sakaï
EDL933
HUS
b
HUS
b
HUS
b
HUS
b
b
DH5α
O157:H7
O91:H21
O91:H10
O113:H21
O6:H10

Figure 1

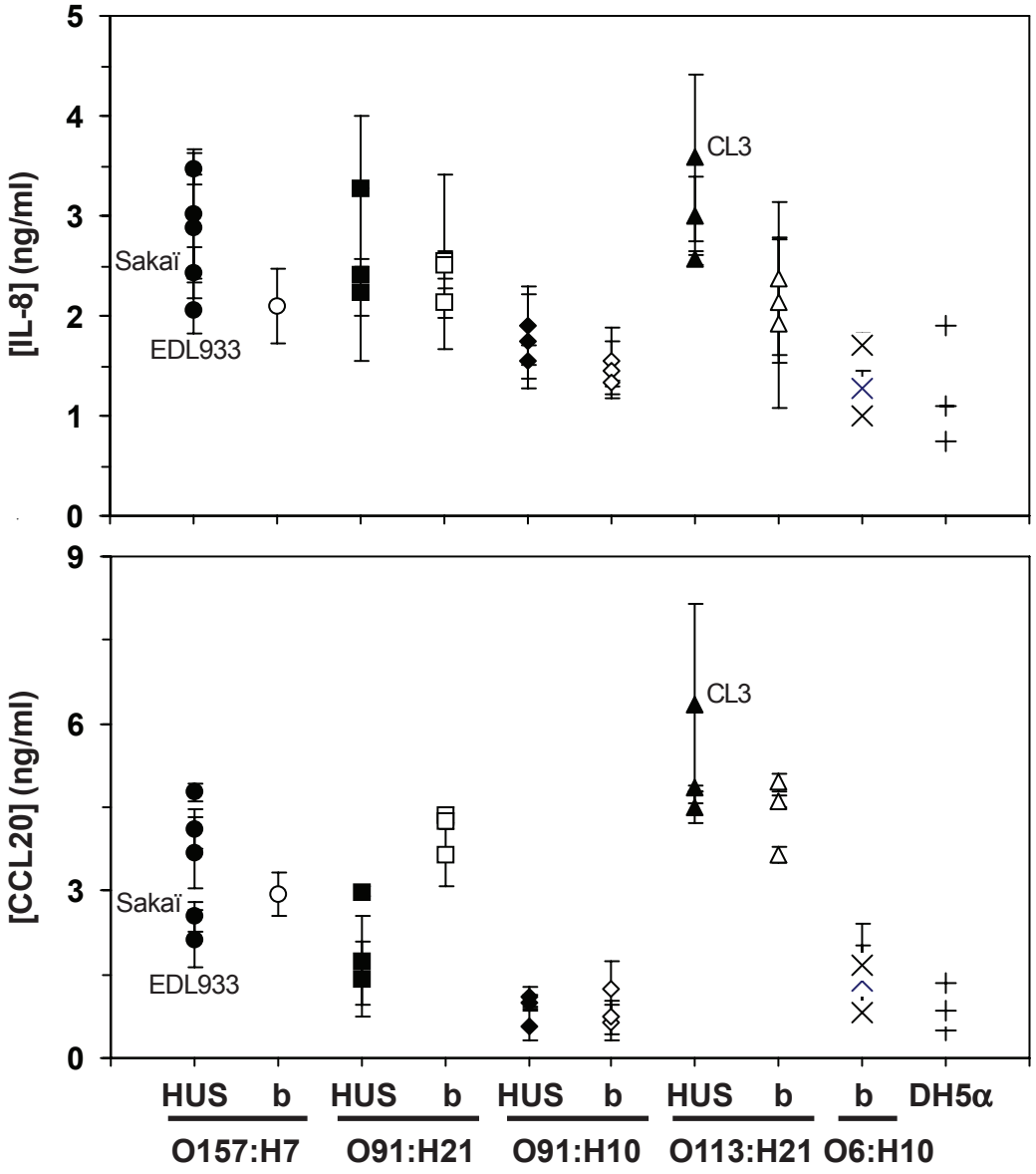
[IL-8] (ng/ml)
5
4
3
2
1
0
Sakaï
EDL933
CL3
[CCL20] (ng/ml)
9
6
3
0
Sakaï
EDL933
CL3
HUS
b
HUS
b
HUS
b
HUS
b
b
DH5α
O157:H7
O91:H21
O91:H10
O113:H21
O6:H10

Figure 2

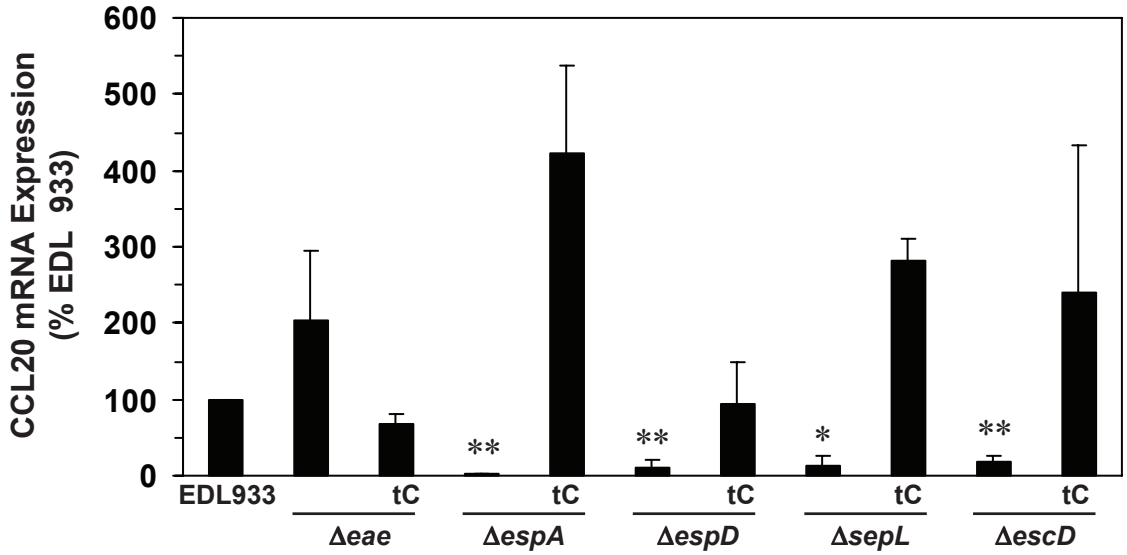
CCL20 mRNA Expression
(% EDL 933)
600
500
400
300
200
100
0
**
**
*
**
EDL933
tC
tC
tC
tC
tC
Δeae
ΔespA
ΔespD
ΔsepL
ΔescD

Figure 3

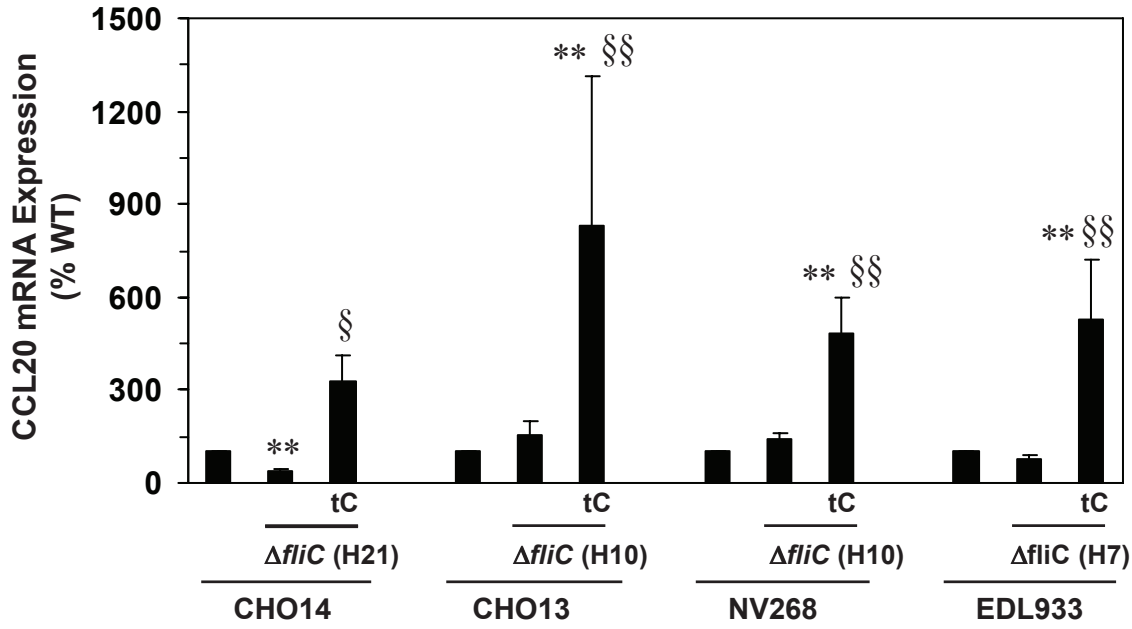
CCL20 mRNA Expression (% WT)
1500
1200
900
600
300
0
**
§
** §§
** §§
** §§
tC
tC
tC
tC
ΔfliC (H21)
ΔfliC (H10)
ΔfliC (H10)
ΔfliC (H7)
CHO14
CHO13
NV268
EDL933

Figure 4