σE pathway is involved in biofilm formation by Crohn’s disease-associated adherent-invasive Escherichia coli

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Abbreviations used in this paper: CD, Crohn’s disease; AIEC, adherent-invasive Escherichia coli.

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Abstract

Ileal lesions of patients with Crohn’s disease are colonized by adherent-invasive *Escherichia coli* (AIEC), able to adhere to and to invade intestinal epithelial cells (IEC), to replicate within macrophages and to form biofilm. Clinical observations showed that bacterial biofilms were associated with the mucosa of IBD patients. In the present study we analyzed the relationship between AIEC colonization of the gut and the formation of biofilm focusing on the involvement of $\sigma^E$ pathway in AIEC-intestinal epithelial cell interaction. We observed that $\sigma^E$ pathway inhibition in AIEC reference strain LF82 led to impaired ability to adhere to and to invade IEC, but also induced a high decrease ability to colonize the intestinal mucosa and to form biofilm. This indicated that targeting $\sigma^E$ pathway could be very potent therapeutic strategies to interfere with ability of AIEC to form biofilm on the gut mucosa of Crohn's disease patients.
Introduction

Crohn’s disease (CD) is an inflammatory bowel disease occurring in individuals with a genetic predisposition in whom an environmental or infectious trigger causes an abnormal immune response (1-3). Several lines of evidence suggest that bacteria play a role in the onset and perpetuation of inflammatory bowel disease (IBD) (4), and clinical observations showed that bacterial biofilms were associated with the mucosa of IBD patients, since the mean density of the mucosal biofilm was 2-fold higher in IBD patients than in patients with IBS or controls, and that the bacteria were mostly adherent (5) *Escherichia coli* has been assigned a putative role in CD. These bacteria are abnormally predominant in early and chronic ileal lesions of CD, and most *E. coli* strains isolated from the ileal mucosa of CD patients adhere to intestinal epithelial cells (6-8). In addition to their ability to adhere, *E. coli* cells are able to invade intestinal epithelial cells, and belong to the pathogenic group of adherent-invasive *E. coli* (AIEC) (9). Many independent studies have reported the abnormal presence of AIEC bacteria associated with the ileal mucosa of CD patients (8, 10-14) owing to increased ileal expression of CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6), which acts as a receptor for AIEC binding to the intestinal mucosa (15, 16). The adhesion and invasion process of reference AIEC strain LF82 involves, in addition to type 1 pili, flagella, outer membrane proteins and outer membrane vesicles (17-21). The LF82 invasion process occurs via the interaction between the endoplasmic reticulum (ER)-localized stress response chaperone Gp96 and the outer membrane protein OmpA expressed at the surface of outer membrane vesicles (OMV), allowing OMVs to fuse with intestinal epithelial cells and to deliver vesicle components and virulence factors to or into host cells (21).

The $\sigma^E$ factor, also called RpoE, is activated by stresses that interfere with the folding of outer membrane proteins (OMPs) such as heat shock, overexpression of OMP genes, and mutations in genes encoding chaperones required for OMP folding (22-24). As expected from its role in the stress response, the $\sigma^E$ regulon includes genes encoding periplasmic foldases, proteases, and chaperones that aid in OMP folding. In addition, $\sigma^E$ transcribes an array of biosynthetic enzymes that are involved in phospholipid, fatty acid, LPS, and membrane-derived oligosaccharide synthesis and transport, and a number of other cell envelope proteins including lipoproteins, inner membrane proteins, and envelope proteins of unknown function (25-27). In AIEC strains, a model proposed by Rolhion and collaborators indicated that, at high osmolarity similar to that of the gastrointestinal tract, increased expression of OmpC in...
AIEC LF82 bacteria led to the activation of the $\sigma^E$ regulatory pathway, pathway that could regulate type 1 pili and flagella expression (19).

The aim of the present study was to investigate the activation of the $\sigma^E$ regulatory pathway in AIEC bacteria, and its involvement in the AIEC virulence. We report here that the $\sigma^E$-mediated pathway is directly involved in the ability of AIEC strains to adhere to and to invade intestinal epithelial cells, and we interestingly demonstrate that the $\sigma^E$ pathway is fully required for the biofilm formation and the colonization of the intestinal mucosa by AIEC strains. We also demonstrate that the $\sigma^E$-mediated pathway is activated during adhesion and biofilm formation processes, bringing into light an original mechanism by which $\sigma^E$-mediated pathway is induced by and required for adhesion and biofilm formation in AIEC strains.
**Materials and methods**

**Ethics Statement**

Animal protocols were approved by the committee for ethical issues, CEMEA Auvergne (Permit Number: CEMEAA, CE16-0927-2956), and all animals were used in accordance with the European Community guiding in the care and use of animals (86/609/CEE).

**Reference bacterial strains, plasmids, and cell lines**

The bacterial strains and plasmids used in this study are listed in table S1. Bacteria were grown routinely in LB broth overnight at 37°C and without shaking. Antibiotics were added to media at the following concentrations: ampicillin (50 mg.mL⁻¹), kanamycin (50 mg.mL⁻¹) and chloramphenicol (25 mg.mL⁻¹).

Intestine-407 cells (derived from human intestinal embryonic jejunum and ileum) were purchased from Flow Laboratories, Inc., McLean, VA. Cultured cells were maintained in an atmosphere containing 5% CO₂ at 37°C in modified Eagle medium (Seromed; Biochrom KG, Berlin, Germany) supplemented with 10% (vol/vol) fetal bovine serum (Lonza), 1% nonessential amino acids (Lonza), 1% L-glutamine (Lonza), 200 U of penicillin, 50 mg of streptomycin, and 0.25 mg of amphotericin B per liter and with 1% of minimal essential medium (MEM) vitamin mix X-100 (Lonza).

**Bacterial growth**

Each strain was tested for its ability to grow under static conditions in in cell culture medium (MEM supplemented with 10% heat-inactivated FCS) at 37°C for at least 10 h. Growth was monitored by measuring the OD₆₂₀.

**Promoter expression assay**

To generate promoter fusion constructs, rpoE and rpoH promoters of strains LF82 were amplified by PCR using BamHI-rpoE / EcoRI-rpoE and BamHI-rpoH / EcoRI-rpoH primers, respectively (Table S2). The resulting fragments contain the entire RpoE binding site previously described to be present in these promoters (24, 28). These PCR fragments were ligated into the plasmid vector pRS550 (29), and designated pRS550-rpoE and pRS550-rpoH (Table S1). β-galactosidase activity was analyzed using a β-galactosidase assay kit (QIAGEN) with strains harboring the constructs pRS550-rpoE or pRS550-rpoH grown in LB culture medium containing arabinose (0, 0.02, 0.2, 1.0 and 2.0% L-arabinose, Sigma Aldrich).
The β-galactosidase activity of each sample was determined by measuring the OD420 at 24h, and the number of bacteria in each sample was calculated by the OD620 measurement for Miller unit determination.

Adhesion and invasion assay
The bacterial adhesion assay was performed as described previously (9). Briefly, Intestine-407 cells were seeded in 24-well tissue culture plates with 4 x 10^5 cells per well. Monolayers were then infected at a multiplicity of infection of 10 bacteria per cell in 1 ml of the cell culture medium without antibiotics and with heat-inactivated fetal calf serum (FCS, PAA). When needed, adhesion and invasion assays were performed after centrifugation for 8 min at 1,000 g. After a 3 h incubation period at 37°C, monolayers were washed three times in phosphate-buffered saline (PBS, pH 7.2). The epithelial cells were then lysed with 1% Triton X-100 (Euromedex) in deionized water. Samples were diluted and plated onto Muller-Hinton agar plates to determine the number of colony-forming units (cfu) corresponding to the total number of cell-associated bacteria (adherent and intracellular bacteria). To determine the number of intracellular bacteria, fresh cell culture medium containing 100 mg.ml^-1 gentamicin was added for 1 h to kill extracellular bacteria. Monolayers were then lysed with 1% Triton X-100, and bacteria were quantified as described above. Inhibition invasion assays were performed after a 30 min pretreatment of cells at 37°C using anti-Gp96 (H-212, Santa Cruz Biotech).

Construction of isogenic mutants and transcomplementation assays
Isogenic mutants were generated with a PCR product using the method described by Datsenko et al. (30) and modified by Chaveroche et al. (31). Primers used are listed in Table S2. For transcomplementation assays, a PCR product containing the entire 1,614 bp rseAB operon or the entire 575 bp rpoE gene were cloned into the pBAD24 and pBAD30 vectors, respectively (32) (Table S1 and S2).

RNA manipulations, reverse transcription and RT-PCR
Cultures were grown at 37°C in LB with or without NaCl, in Eagle medium supplemented with 10% (vol/vol) of heat inactivated fetal bovine serum at normal pH (pH = 7.0 - 7.5), at pH = 6, or with 2% bile salts (Sodium choleate, Sigma Aldrich). At OD 0.2 at 620 nm and when needed, L-arabinose was added to induce the overexpression of RseAB.
Total RNAs were extracted from overnight-cultured bacteria and treated with DNase (Roche Diagnostics) to remove any contaminating genomic DNA.

For RNA isolation from bacteria associated with epithelial Intestine-407 cells, 4h post-infection, I-407 epithelial cells monolayer were washed 2 times and were lysed by exposure to 0.1% (wt/vol) sodium dodecyl sulfate, 0.1% (vol/vol) acidic phenol and 19% (vol/vol) ethanol in water for 30 min on ice (33). Bacteria were not lysed by this procedure, and mRNA were stabilized and protected from degradation. After centrifugation (10 min, 6000g), pellet of bacteria were subjected to RNA extraction.

For RNA extraction of biofilm-associated bacteria, strains were grown overnight in Luria-Bertani broth with 5 g.L⁻¹ of glucose (Euromedex) at 35.5°C, after which 1/100 dilutions were made in M63 minimal medium (US Biological) supplemented with 8 g.L⁻¹ (0.8%) glucose. 15 mL aliquots were then placed in wells of non-cell-treated polystyrene petri plates and incubated at 30°C without shaking. At different time points, plates were washed once, bacteria were harvested using a scraper and RNA were extracted as previously described.

The RNAs were reverse transcribed and amplified using specific primers to *rseA*, *rpoE* or 16S rRNA (Table S2). Amplification of a single expected PCR product was confirmed by electrophoresis on a 2% agarose gel. RT-PCR was performed using an Eppendorf Realplex and the RNA levels were quantified using RNA master SYBR Green I (Roche Diagnostic) with 0.25 mg of total RNA.

**Motility assay**

Bacterial strains were grown overnight at 37°C without agitation in LB broth, and 2µl portions of the culture were inoculated into the center of 0.3% LB agar plates. The plates were incubated at 37°C, and motility was assessed quantitatively by examining the circular swimming motion formed by the growing motile bacterial cells every other hour.

**Biofilm formation assays**

Biofilm formation assays were performed using a previously described method (34). Strains were grown overnight in Luria-Bertani broth with 5 g.L⁻¹ of glucose (Euromedex) at 35.5°C, after which 1/100 dilutions were made in M63 minimal medium (US Biological) supplemented with 8 g.L⁻¹ (0.8%) glucose. When required, arabinose was added to the medium. 130-µl aliquots were then placed in wells of non-cell-treated polystyrene microtiter plates and incubated overnight at 30°C without shaking. Afterwards, growth optical densities...
(OD) were read at 630 nm. The wells were washed once, and adhered bacteria were stained with 1% crystal violet solubilised in ethanol, and ODs read at 570 nm. Biofilm measurements were calculated using the formula \( \text{SBF} = \frac{\text{AB}-\text{CW}}{\text{G}} \), in which SBF is the specific biofilm formation, AB is the OD570nm of the attached and stained bacteria, CW is the OD570nm of the stained control wells containing only bacteria-free medium (to eliminate unspecific or abiotic OD values), and G is the OD630nm of cell growth in broth (35, 36). Assays were performed in triplicate.

Biofilm formation assays were also performed using PFA-fixed intestinal epithelial cells I-407 monolayers. Briefly, confluent I-407 monolayers were fixed for 15 min in 4% PFA, and, after washing, bacterial strains expressing GFP (37) were applied in M63 minimal medium as previously described and incubated overnight at 30ºC without shaking. For visualization, infected epithelial monolayers were fixed for 15 min in 4% PFA, phalloidin-TRITC were used to visualize actin and Hoechst strains were used to visualize nuclei. The slides were examined with a Zeiss LSM 510 Meta confocal microscope.

**Mouse ileal loop experiments**

Six-week-old FVB wild-type male mice were starved for 24h before surgery, with water available ad libitum. They were anesthetized, and their intestines exteriorized through a midline incision (38). Two or three intestinal segments (about 1 cm) without PP were ligated and inoculated by mixed inoculums comprising equivalent numbers (5.10^7 CFU) of two bacterial strains (LF82 / LF82, LF82 / LF82 + pBAD24, and LF82 / LF82 + pBAD24-rseAB) in the presence of 2% arabinose, and the number of each bacterial strain associated with the mucosa of ligated loops was determined to establish competitive index (CI), which provides a sensitive measurement of the relative degree of attenuation (39). Animal protocols were approved by the committee for ethical issues, CEMEA Auvergne (Permit Number: CEMEAA, CE16-0927-2956), and all animals were used in accordance with the European Community guiding in the care and use of animals (86/609/CEE). Surgery was performed under Ketamine - Xylasine anesthesia, and all efforts were made to minimize suffering. Mice were killed by cervical dislocation according to animal care procedure.

**Statistical analysis**

Numerical values were expressed as means with SEM. Statistical comparisons were performed using 2-tailed student \( t \) test, unless the variables required a 2-tailed Fisher exact
test. A $P$ value less than 0.05 was considered statistically significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 

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Results

\( \sigma^E \) pathway activation during adhesion process of AIEC strain LF82

\( \sigma^E \) pathway activation was analyzed by quantification of rpoE mRNA, since it is well established that \( \sigma^E \) upregulates its own transcription (25). After growth of AIEC strain LF82 or E. coli K-12 strain in an acid culture medium or in the presence of 2% bile salts, the rpoE mRNA levels were similar to those observed in bacteria grown in cell culture medium. In contrast, 2.7- and 2.3-fold increases in the rpoE mRNA levels were observed after growth of LF82 and MG1655 bacteria at high osmolarity, respectively (Figure 1A and B). Interestingly, when we analyzed \( \sigma^E \) pathway activation during the adhesion process, we also observed a 1.9-fold increase \( \sigma^E \) pathway activation in AIEC LF82 bacteria associated with intestinal epithelial cells (IEC). No such increase was observed for MG1655 bacteria associated with IEC, although MG1655 is also able to adhere to I-407 cells as shown in comparison with the non-adherent LF82-ΔfimA isogenic mutant (Figure 1C). To address whether the \( \sigma^E \) pathway was activated during the adhesion or the invasion steps of AIEC LF82 bacteria, we pretreated IEC monolayer with anti-Gp96 antibodies, since it was previously reported that Gp96 molecule expressed at the surface of IEC is required for the invasion process of AIEC strain LF82 and that such a pretreatment highly decreased the invasion ability of strain LF82 (21). After Gp96 blockade, we still observed a high, 2.4-fold, increase in rpoE mRNA level in AIEC LF82 bacteria associated with intestinal epithelial cells (Figure 1D). Altogether, these findings show for both pathogenic LF82 and non-pathogenic MG1655 E. coli strains that high osmolarity activates \( \sigma^E \) pathway but that the adhesion process activates \( \sigma^E \) pathway in AIEC strain LF82 but not in E. coli K-12 strain MG1655.

Involvement of the \( \sigma^E \) pathway in the ability of LF82 to interact with host cells

We next addressed the involvement of \( \sigma^E \) pathway in the adhesion and invasion process of AIEC strain LF82. For this purpose, we decided to create a mutant of AIEC strain LF82 deleted for the \( \sigma^E \) encoding gene. We obtained no mutant, which suggests that such a mutation is probably lethal in this strain. To counteract this methodology problem, we generated strains LF82 and MG1655 transformed with plasmid pBAD24-\( \text{rseAB} \), that allows the expression of the anti-sigma factors RseA and RseB, which prevents \( \sigma^E \) interaction with RNA polymerase (40, 41). As controls, we analyzed growth curves of these constructs, and this showed that overexpression of RseAB led to no significant difference in growth curves (Figure S1). AIEC LF82 bacteria with \( \text{rseAB} \) cloned into the arabinose inducible pBAD24
vector were grown in the presence of various arabinose concentrations and the levels of \textit{rseA} mRNA were analyzed by quantitative RT-PCR. We observed an arabinose dose-dependent expression of \textit{rseA} (Figure 2A). In parallel we observed an arabinose dose-dependent decrease in \textit{rpoE} mRNA levels (Figure 2B). In addition, since a RpoE binding site was previously described to be present in \textit{rpoE} and \textit{rpoH} promoters (24, 28), we analyzed the activity of these promoters by generating constructs using pRS550 plasmid to obtain a β-galactosidase expression driven by \textit{rpoE} or \textit{rpoH} promoters. In the presence of increased arabinose concentrations, we observed decreased activities of both \textit{rpoE} and \textit{rpoH} promoters (Figure 2C and D). When a 2% dose of arabinose was used, the overexpression of RseAB led to a significant 7.81 and 5.38-fold decrease in \textit{rpoE} and \textit{rpoH} promoter activity, respectively. These data confirm that overexpression of RseAB leads to a significant decrease in \textit{σ}^{E} pathway activity, and an arabinose concentration of 2% was used in all experiments requiring overexpression of RseAB to allow \textit{σ}^{E} pathway inhibition.

AIEC LF82 bacteria over-expressing the \textit{σ}^{E} inhibitory complex RseAB had significantly decreased ability to adhere and to invade IEC (Figure 3A and B), with 20.51 and 66.99 fold decreases in adhesion and invasion, respectively, compared to LF82 wild type strain. Interestingly, the over-expression of the \textit{σ}^{E} inhibitory complex RseAB had no effect on the ability of non pathogenic \textit{E. coli} strain MG1655 to interact with IEC. These findings demonstrate for the first time that the \textit{σ}^{E} pathway is directly involved in the adhesion and invasion process of AIEC strain LF82. To further address the mechanism causing the decreased interaction of AIEC LF82 bacteria over-expressing the \textit{σ}^{E} inhibitory complex RseAB with IECs, we analyzed the expression of flagella and type 1 pili. Flagella expression was determined by analyzing motility of bacteria on 0.3% LB agar, and we observed that motility was only slightly decreased in the AIEC strain LF82 over-expressing the \textit{σ}^{E} inhibitory complex RseAB, compared to wild type strain (Figure 4A). Type 1 pili expression was determined by analyzing type 1 pili phase variation. This showed that 50% of bacteria expressed the ON phase of the \textit{fim} promoter when RseAB was over-expressed compared to 78% and 69% in the wild-type strain or the wild-type strain complemented with the empty vector, respectively (Figure 4B). Following these observations, we performed adhesion and invasion experiments with a LF82 + pBAD24-\textit{rseAB} strain transformed with the cloned \textit{fim} operon in order to force the bacteria to express type 1 pili. A centrifugation step was preformed to establish close contact between bacteria and epithelial cells in order to abrogate
any defect in bacterial motility. Interestingly, even with constitutive type 1 pili expression and with a centrifugation step, the adhesion and invasion levels of the LF82 + pBAD24- rseAB construct were only partially restored compared to LF82 wild-type strain (Figure 4 C and D). These results demonstrate that type 1 pili and flagella are involved in the decreased adhesion and invasion abilities observed during σE pathway inhibition.

Involvement of the σE pathway in the ability of LF82 to form biofilm

While we demonstrated that σE-mediated pathway was involved in the interaction of AIEC strain LF82 with host cells (Figure 3A and B), and since bacterial biofilms were associated with the mucosa of IBD patients (5) and biofilm formation capacity is a novel pathogenic feature of the AIEC pathovar (34), we analyzed whether σE-mediated pathway is involved in AIEC biofilm formation. For this purpose, we compared biofilm production on the plastic surface of wild-type strain LF82 and of strain LF82 overexpressing RseA and B. Inhibition of the σE pathway led to a significant decrease in the ability of strain LF82 to form biofilm, since LF82 + pBAD24- rseAB strain had a mean specific biofilm formation (SBF) index of 0.79 ± 0.14 compared to 3.23 ± 0.14 for wild type strain LF82 (P=0.00013) (Figure 5A). Interestingly, inhibition of the σE pathway in E. coli K-12 strain MG1655 had no effect on its ability to form biofilm even if overexpression of the σE pathway inhibitory complex RseAB led to similar decreased expression of rpoE mRNA in both AIEC strain LF82 and E. coli K-12 strain MG1655. In addition, we observed that strain MG1655 had a similar SBF index (1.14 ± 0.11) to that of strain LF82 overexpressing RseAB (0.79 ± 0.14). To confirm these data, and to counteract the lethality due to σE deletion, we generated the σE deletion in strain LF82 already transformed with plasmid pBAD30- rpoE. This construction reveals that, in the absence of arabinose, a significant decrease in biofilm formation was observed, with a 35.60 % ± 7.39 % residual level compared to wild type strain LF82 (Figure 5B). Interestingly, when arabinose concentration increased, the biofilm formation increased in a dose-response manner, and in the presence of 5 and 10 g.L-1 of arabinose, no difference was observed between LF82-ΔrpoE + pBAD30- rpoE and wild type strain. These data suggest that the σE pathway is involved in the ability of AIEC strain LF82 to form biofilm.

The ability of strain LF82 and strain LF82 overexpressing RseAB to form biofilm was also analyzed using an original method that we developed for this study with PFA-fixed intestinal epithelial cells on glass coverslips. Results presented in figure 6A show that strain
LF82 was able to form biofilm at the surface of fixed intestinal epithelial cells. In contrast, with strain LF82 + pBAD24-rseAB and the K-12 strain MG1655, only a few diffusely adhering bacteria were observed at the surface of the intestinal epithelial cells.

Finally, the phenotype of strain LF82 + pBAD24-rseAB was analyzed in another approach using intestinal ileal loop assay as an *in vivo* model to assess the interaction of bacteria with intestinal mucosa. The intestinal ileal loops were inoculated with a mixed inoculum comprising equivalent numbers of wild-type LF82 and LF82 + pBAD24-rseAB bacteria, and their presence was compared by competitive index (CI) analysis, which provided a sensitive measurement of the relative degree of attenuation (39). The LF82 strain overexpressing RseAB had a mean CI of 0.27 ± 0.033, indicating that its ability to interact with the surface of intestinal ileal biopsy was greatly impaired compared to that of wild-type strain (*P*<0.001) (Figure 6B). The analyses of *in-vitro* co-cultures of LF82 wild-type bacteria and LF82 + pBAD24-rseAB bacteria revealed that both strains remained stable over time (Figure S2). Altogether, these data indicate that the inhibition of the σE pathway lead to a decreased ability of AIEC strain LF82 to form biofilm at the surface of intestinal epithelial cells.

σE pathway was activated in AIEC strain LF82 during biofilm formation

Since we demonstrate in the present study that the σE pathway is involved in biofilm formation process, we analysed whether σE pathway was activated during biofilm formation in AIEC strain LF82 compared to planktonic bacteria. Results, presented in figure 7, revealed that σE pathway was highly activated during biofilm formation in AIEC strain LF82, with a 1.71 and 5.79 fold increase, compared to planktonic bacteria at 4h post inoculation, taken as 1. This activation of the σE pathway during biofilm formation process was similar to the one previously observed during the adhesion process as shown in Figure 1.
Discussion

Clinical observations showed that bacterial biofilms were associated with the mucosa of IBD patients, since the mean density of the mucosal biofilm was 2-fold higher in IBD patients than in patients with IBS or controls, and that the bacteria were mostly adherent (5). Among bacteria highly adherent to the ileal mucosa of CD patients adherent-invasive E. coli has been observed (10), in which we have identified type 1 pili and flagella as important virulence factors mediating the interaction of the bacteria with intestinal epithelial cells (17, 18). In AIEC strain LF82, a model elaborated by Rolhion et al. proposed that, at high osmolarity similar to that of the gastrointestinal tract, the activation of the \( \sigma^E \) regulatory pathway could modulates expression of genes involved in AIEC interactions with host cells (19). Such involvement of the \( \sigma^E \) pathway has been reported in various pathogens. For example, it is involved in the virulence of Salmonella enterica serovar Typhimurium, since after \( \sigma^E \) inactivation, Salmonella were no longer able to survive inside macrophages and were highly attenuated in their virulence in mice (42). In Vibrio cholerae, a \( \Delta rpoE \) mutant was highly attenuated in its ability to colonize the intestine and in its lethal effect on mice (43).

The role of \( \sigma^E \) pathway in the ability of E. coli to interact with intestinal epithelial cells was observed for AIEC strain LF82 but not for the non pathogenic E. coli K-12 MG1655. In the present study, comparison of \( \sigma^E \)-pathway activation in AIEC strain LF82 and in non pathogenic E. coli K-12 strain MG1655 showed that, when the bacteria interact with intestinal epithelial cells, \( \sigma^E \) mRNA level increased in AIEC strain LF82, but not in strain K-12. Since it is well established that \( \sigma^E \) upregulates its own transcription, (25), this indicates that AIEC adhesion to host cells leads to activation of the \( \sigma^E \) pathway. This is the first report indicating such an activation of \( \sigma^E \) pathway in bacteria interacting with host cells and is in contrast with a previous report concerning Neisseria gonorrhoeae in which the analysis of global gene expression during the interaction of the bacteria with intestinal epithelial cells showed that there was no activation of the \( \sigma^E \) pathway during the adhesion process (44).

Analysis of the relationship between AIEC phenotype and activation of the \( \sigma^E \)-mediated pathway indicated that \( \sigma^E \) plays a crucial and direct role in AIEC LF82 since inhibition of this pathway greatly decreased the adhesion and invasion process. The analysis of the bacterial factors involved in this phenotype revealed that type 1 pili and flagella, previously reported to play a key role in the adhesion and invasion processes (17, 18), were
partially involved in the decreased adhesion and invasion abilities observed during $\sigma^E$ pathway inhibition. This indicates that other bacterial factor(s), regulated by the $\sigma^E$ pathway, is(are) involved in these phenotypes. In addition, when we analyzed another phenotypic characteristic of AIEC strains, i.e. the ability to form bacterial biofilm, as described by Martinez-Medina et al (34), we observed that inhibition of the $\sigma^E$ pathway led to greatly decreased ability of AIEC strains LF82 to form biofilm on plastic surfaces and on fixed intestinal epithelial cells. In addition, when we analyzed another phenotypic characteristic of AIEC strains, i.e. the ability to form bacterial biofilm, as described by Martinez-Medina et al (34), we observed that inhibition of the $\sigma^E$ pathway led to greatly decreased ability of AIEC strains LF82 to form biofilm on plastic surfaces and on fixed intestinal epithelial cells. In addition, we developed in vivo intestinal ileal loop assay model to perform competitive index analysis between wild-type strain LF82 and LF82 overexpressing RseAB. Competitive index determination provides a sensitive measurement of the relative degree of attenuation of mutants compared to wild-type strains, and such analysis was very helpful for the identification of virulence factors in Salmonella spp. (39) and Listeria monocytogenes (45). With AIEC strain LF82, results revealed that, after inhibition of the $\sigma^E$ pathway, the presence of LF82 bacteria at the surface of murine intestinal mucosa was highly impaired. Such a phenotype was not observed with K-12 strain MG1655. These findings indicate that some genes whose transcription is under the control of $\sigma^E$ should be involved in biofilm formation by AIEC strains and that such factors or their $\sigma^E$-dependent expression are absent in K-12 strain MG1655.

In conclusion, our findings demonstrate for the first time a specific activation of the $\sigma^E$-mediated pathway during the adhesion process of AIEC strain LF82, that results in an increased ability of bacteria to adhere to and to invade intestinal epithelial cells and to form biofilm. These results also bring into light an original mechanism by which $\sigma^E$-mediated pathway is induced in bacteria during biofilm formation, and will control the expression of gene(s) required for this process. Altogether, these data suggest that both adhesion and biofilm formation process i) conduct to the activation of the $\sigma^E$ pathway and ii) are $\sigma^E$-dependent mechanisms. This circle could explain the high capacity of AIEC strains to colonize intestinal mucosa and to form biofilm, as previously described in patients with Crohn’s disease (10, 34). Finally, our study indicate that targeting $\sigma^E$ pathway could be very potent therapeutic strategies to interfere with ability of AIEC to form biofilm on the gut mucosa of Crohn’s disease patients and to prevent subsequent intestinal chronic inflammation.
Acknowledgments

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Author contributions

B.C. and A.D-M. designed research, performed experiments, analyzed the data and wrote the manuscript; A.D-M. obtained funding.

Conflict of interest

The authors have no financial conflicts of interest.
References


Figures legends

Figure 1: A-B. Activation of σE pathway in AIEC strain LF82 and non pathogenic E. coli strain MG1655. Fold variation of rpoE mRNA levels in wild-type strains LF82 (A) and MG1655 (B) grown in media at pH = 6, in media with 2% of bile salts, in media with 20 g.L⁻¹ of NaCl, or adherent to I-407 epithelial cells, relative to that of wild-type strains grown in classic media. 16S rRNA levels were measured as controls. Data are mean ± SEM of three separate experiments. * P < 0.05. C. Adhesion of AIEC strain LF82, non-pathogenic E. coli K-12 strain MG1655 and LF82-ΔfimA isogenic mutant to Intestine-407 cells. Cell-associated bacteria were quantified after a 3 h infection period. Each value is the mean number of CFU ± SEM of at least four separate experiments. D. Activation of σE pathway in AIEC strain LF82 associated with I-407 cells after anti-Gp96 antibody pretreatment. Fold variation of rpoE mRNA levels in I-407 epithelial cell-adhering bacteria of wild-type strain LF82 with or without a 30 min pretreatment of cell monolayer with anti-Gp96 antibody.

Figure 2: A-B. Fold variation of rseA (A) and rpoE (B) mRNAs levels in strains LF82, LF82 + pBAD24 and LF82 + pBAD24-rseAB in the presence of various doses of arabinose. Results are expressed as relative expression compared to that of the wild-type LF82 strain in the absence of arabinose. 16S rRNA levels were measured as controls. Data are mean ± SEM of three separate experiments. C-D. Activation of the rpoE (C) and rpoH (D) promoters in strains LF82, LF82 + pBAD24 and LF82 + pBAD24-rseAB in the presence of various doses of arabinose. β-Galactosidase activity per OD620 unit resulting from the expression of lacZ driven by the DNA sequence upstream of the rpoE or rpoH gene. Data are mean ± SEM of four separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3: A-B. Adhesion (A) and invasion (B) abilities of strains LF82, LF82 + pBAD24-rseAB, MG1655 and MG1655 + pBAD24-rseAB, with intestinal epithelial cells I-407. Each value is the mean ± SEM of at least four separate experiments. **P < 0.01.

Figure 4: A. Motility assay on 0.3% agar at 37°C for wild-type strain LF82 and strain LF82 + pBAD24-rseAB. B. Regulation of type 1 pili in the LF82 + pBAD24-rseAB strain. Determination by PCR analysis of the invertible element orientation of the fim operon in strains LF82, LF82 + pBAD24 and LF82 + pBAD24-rseAB. A 450 bp product revealed ON orientation and a 750 bp product revealed OFF orientation of the invertible element. C-D.
Adhesion (C) and invasion (D) abilities of strains LF82 + pHSG575-fim, LF82 + pBAD24-rseAB and LF82 + pBAD24-rseAB + pHSG575-fim, with intestinal epithelial cells I-407. A centrifugation was performed to force contact between bacteria and Intestine-407 epithelial cells. Each value is the mean ± SEM of at least four separate experiments. * P<0.05, ** P<0.01.

Figure 5: A. Specific biofilm formation (SBF) index of AIEC strain LF82 and non-pathogenic MG1655 E. coli strain, with or without RseAB overexpression. Data are mean ± SEM of three separate experiments. B. Specific biofilm formation (SBF) index of LF82-ΔrpoE isogenic mutant transcomplemented with pBAD30-rpoE grown in the presence of 0.00, 0.31, 0.63, 1.25, 2.50, 5.00 or 10.00 g.L⁻¹ of arabinose. Data are mean ± SEM of three separate experiments. ** P<0.01, *** P<0.001.

Figure 6: A. Confocal analysis of LF82, LF82 + pBAD24, LF82 + pBAD24-rseAB and MG1655 biofilm formation at the surface of PFA-fixed monolayer of intestinal epithelial cells I-407. Bacteria expressing GFP were used, actin is labeled in red with phalloidin-TRITC and nuclei are labeled with Hoescht in blue. Representative z-sections were visualized under each confocal slice. Bar, 50 µm. B. Competitive index of LF82 + pBAD24-rseAB strain compared to that of LF82 wild type strain. Intestinal ileal loops were inoculated by mixed inoculums comprising equivalent numbers of wild-type and LF82 pBAD24-rseAB strain, and their presence was compared by competitive index (CI) analysis, which provides a sensitive measurement of the relative degree of attenuation. ** P<0.01, *** P<0.001.

Figure 7: Activation of σE pathway in AIEC strain LF82 during biofilm formation process. Fold variation of rpoE mRNA levels in wild-type strains LF82 during biofilm formation (4h, 16h and 24h), relative to those of wild-type strains grown 4h in classic media. 16S rRNA levels were measured as controls. Data are mean ± SEM of three separate experiments. *P<0.05, **P<0.01.

Figure S1: Growth curves of wild-type strains LF82 and MG1655, and of strains LF82 + pBAD24-rseAB and MG1655 + pBAD24-rseAB in cell culture medium (MEM supplemented with 10% heat-inactivated FCS). Data are mean ± SEM of three separate experiments.
Figure 1.

A. Control media, pH 6, 20 g.L⁻¹ NaCl, 2% bile, associated bacteria 3.5, 4.0

B. rpoE mRNA levels 1.5, 2.0, 2.5, 3.0

C. CFU of associated bacteria 1.0, 1.5, 2.0

D. RpoE mRNA level after LF82 interaction with I-407 c 1.0, 2.0

Figure 1.
Figure 2.
Figure 3.

MG1655 + rseAB

CFU of associated bacteria (x10^7)

MG1655 + rseAB

CFU of internalized bacteria (x10^5)

MG1655 + rseAB

N.S.
Figure 4.
Figure 5.
Figure 6.

A

LF82  
vs LF82 
LF82 + rseAB 
vs LF82 
LF82 
LF82 + rseAB MG1655 
LF82 + pBAD24 

B

**

LF82 + 
pBAD24 
vs LF82
Figure 7. Relative psf mRNA levels in planktonic and biofilm-forming bacteria.