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Effect of coiled-coil peptides on the function of the type III secretion system-dependent activity of enterohemorragic *Escherichia coli* O157:H7 and *Citrobacter rodentium*

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ABSTRACT

Many animal and human pathogenic Gram-negative bacteria such as *Salmonella*, *Yersinia*, enterohemorrhagic *Escherichia coli* (EHEC), and enteropathogenic *Escherichia coli* (EPEC) possess a type III secretion system (TTSS) that is used to deliver virulence proteins directly into the host cell. Recent evidence has suggested that CoilA and CoilB, two synthetic peptides corresponding to coiled-coil domains of the translocator protein EspA, are effective in inhibiting the action of TTSS from EPEC. In the current study, the action of these coiled-coil peptides on the TTSS of EHEC O157:H7 and *Citrobacter rodentium* was examined. CoilA and CoilB showed to be effective in reducing the red blood cell lysis mediated by EHEC O157:H7 and the in vitro secretion of translocator proteins EspB and EspD by EHEC O157:H7 and EspD by *C. rodentium*. Treatment of mice with CoilA and CoilB peptides prevented colon damage when the animals were inoculated with *C. rodentium*. Colon samples of the non-treated group showed areas with loss of superficial epithelium, damaged cells, and endoluminal mononuclear inflammatory infiltrate, consistent with histological lesions induced by *C. rodentium*, whereas mice treated with the synthetic peptides displayed normal surface epithelium showing a similar structure as the uninfected control group. These encouraging results prompt us to test coiled-coil peptides as treatment or vaccines in other models of bacterial infections in future work.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are two categories of pathogenic *E. coli* associated with diarrhea in humans. EPEC is a major cause of infantile diarrhea in developing countries (Nataro and Kaper, 1998), and EHEC is responsible for diseases whose clinical spectrum includes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), the leading cause of renal failure in children in Argentina and several other countries (Karch et al., 2005; Miliwebsky et al., 2007; Repetto, 2005).

Both categories of *E. coli* (EPEC and EHEC) are known to carry the locus of enterocyte effacement (LEE) pathogenicity island (Elliott et al., 1998; McDaniel et al., 1995; Wieler et al., 1997). The LEE is comprised of 5 operons encoding a type III secretion system (TTSS) (Ritchie and Waldor, 2005) that injects effector proteins into enterocytes, some of which alter signaling pathways. This process results in the formation of 'attaching and effacing' (A/E) lesions on intestinal epithelia, which are characterized by the intimate adhesion of bacteria to actin-rich pedestals and a localized destruction of microvilli. The TTSS is a complex structure of more than 20 proteins, which form a 'needle and syringe' apparatus that allows effector proteins to be injected directly into the host cell (Sekiya et al., 2001; Vallance and Finlay, 2000).

EspA (Kenny et al., 1996), EspB, and EspD (Wainwright and Kaper, 1998) are some of the proteins encoded by the LEE4 operon that make up the translocon portion of the TTSS (Knutton et al., 1998). EspA makes hollow, filamentous appendages surrounding the bacteria, which are present in a transient manner (Knutton et al., 1998), whereas EspB and EspD are involved in pore formation on the membranes of the infected cells (Ide et al., 2001). Tir is the receptor of the bacterial outer membrane adhesin intimin, responsible for the intimate attachment of the bacteria to host enterocytes. This receptor is translocated through the TTSS into the host cell surface and encoded in the LEE, as well as intimin (Kenny et al., 1997).

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The ability of EPEC and EHEC to form A/E lesions is shared with other pathogens such as *Citrobacter rodentium* (Schauer and Falkow, 1993), which possesses the pathogenicity island LEE that encodes the genes responsible for the A/E lesion. Neither EPEC nor EHEC are able to colonize mice. In contrast, *C. rodentium* is a natural highly infectious A/E pathogen of mice, which spreads through contaminated feces (Borenshtein et al., 2008) and causes a disease spectrum ranging from self-limiting subclinical epithelial hyperplasia in the colon to clinical diarrhea and colitis and even death, depending on the age, genetic background, and health status of the host. Mundy et al. (2005) have demonstrated the importance of A/E formation in the colonization and survival of *C. rodentium* in the mouse gastrointestinal tract. Mutants of *C. rodentium* in the *espD*, *escD*, or *eae* genes with a complete inability to colonize the colon or cause hyperplasia (as measured by colon weight) have been obtained by transposon insertion (Mundy et al., 2005). Similarly to what is observed in EPEC and EHEC, the pathogenesis strategy of *C. rodentium* is to colonize the intestinal mucosa through formation of A/E lesions on the apical surface of enterocytes. These lesions are essential to the pathogenicity of *C. rodentium* (Wiles et al., 2005).

Previously, we have shown that synthetic peptides designed to correspond to the coiled-coil domains of EspA are effective in inhibiting the action of the TTSS of EPEC (Larzábal et al., 2010). Considering that there is high homology between EspA proteins identified in EPEC, EHEC, and *C. rodentium*, we tested these peptides against EHEC and *C. rodentium* in vitro and in a mouse model of *C. rodentium* infection.

Materials and methods

Peptides, bacterial strains and growth media

CoilA and CoilB peptides were synthesized commercially (Genbiotech, Buenos Aires, Argentina), and then solubilized with PBS (pH 12.0). CoilB is soluble at pH 12.0. Due to the alkalinity of the peptide solution, a similar amount of PBS pH 12.0 was used as a mock control in the hemolysis and fluorescent actin staining (FAS) assays to rule out the influence of the pH on the effects observed. Dr. Marta Rivas (INEI-ANLIS-Instituto Nacional de Microbiología Dr. Carlos G. Malbrán, Buenos Aires) provided EPEC E2348/69 (Levine et al., 1978) and *Escherichia coli* O157:H7 125/99. *C. rodentium* biotype 4280 was kindly provided by Dr. Fernando Navarro García (CINVESTAV, Mexico). Bacteria were grown in Luria Bertani broth (LB) medium or Dulbecco's modified Eagle medium (DMEM) lacking phenol red (Gibco-BRL), without antibiotics and bovine fetal serum at 37 °C without shaking. A *C. rodentium* strain resistant to nalidixic acid was obtained through successive passages on LB agar plates with 25 μg/ml of nalidixic acid and then selected from feces from infected mice. The presence of *espA*, *espB*, *eae*, and *tir* genes was determined by PCR.

Red blood cell lysis assay

The possible inhibitory effect of CoilA and CoilB peptides on the hemolytic activity exhibited by TTSS-encoding *E. coli* strains (Warawa et al., 1999) was evaluated. The EHEC O157:H7 125/99 strain was grown in LB broth overnight at 37 °C without shaking. OD_{600} was used to measure the number of bacteria, and then the bacterial culture diluted 1:100 into Dulbecco's modified Eagle medium (DMEM) lacking phenol red (Gibco-BRL) with 0.56 mg/ml of peptides into 12-well plates. The bacterial suspensions in the 12-well plates were incubated for 1 h under a 5% CO_2 atmosphere to allow the interaction between the peptides and bacteria. In turn, red blood cells (RBCs) were separated by centrifugation from fresh defibrinated sheep or bovine blood, washed 3 times with 10 mM PBS (pH 7.4) and resuspended at 5% in PBS. Then, 2 ml of the 5% suspension of RBCs in PBS was added to the cultures in the plates and incubated for 4 h at 37 °C under a 5% CO_2 atmosphere. The suspension was removed from the plates and centrifuged at 12,000 × *g* for 1 min. The supernatants were monitored for the presence of released hemoglobin by measuring OD_{543}. In order to eliminate any possible action of other virulence factors of *E. coli* 125/99 on the RBCs lysis, such as secretory enterohemolysin (Ehly) or Shiga toxins (Stx), the bacteria were incubated in DMEM for 5 h to allow secretion of these factors. The culture supernatant was filtered (0.22 μm filter) to have secretory virulence factors free of bacteria. The supernatant was incubated with a 5% suspension of RBCs in PBS pH 7.4.

Immunofluorescence staining

HEp-2 cells (5×10^4) were seeded in 8-well chamber slides (NUNC, Lab-Tek, USA) and incubated at 37 °C under a 5% CO_2 atmosphere up to 70–90% confluence. LB cultures of EHEC O157:H7 125/99 and *C. rodentium* biotype 4280 were grown overnight without shaking and then diluted in 400 μl of DMEM. The OD_{600} of bacterial suspension was adjusted to a multiplicity of infection (MOI) of 50:1. CoilA and CoilB peptides were added to the bacterial cultures at a 0.56 mg/ml concentration, allowing the interaction with bacteria for 1 h at 37 °C in an atmosphere of 5% CO_2. Then, HEp-2 cells were infected with peptide-treated bacteria and incubated for 4 h with EHEC O157:H7 or *C. rodentium* at 37 °C in 5% CO_2. Parallel cultures of HEp-2 were infected with non-treated bacteria. Cells were stained as described by Knutton et al. (1998), with minor modifications. Briefly, the monolayers were gently washed 3 times with PBS to eliminate the non-adherent bacteria, fixed in 4% paraformaldehyde in PBS for 20 min and permeabilized for 5 min in 0.15% Triton X-100 in PBS. After 3 washes with PBS, the cells were stained by FAS with fluorescein isothiocyanate (FITC) conjugated with phalloidin (Sigma Chemical Co., St. Louis, MO, USA) to stain actin. TO-PRO-3 staining was used to detect eukaryotic nuclei and bacterial DNA (Invitrogen).

Immunoblotting

In order to determine the effect of synthetic peptides on the production and excretion of TTSS proteins by *C. rodentium* and *E. coli* O157:H7, cellular and supernatant culture extracts of peptide-treated bacteria were subjected to Western blot analysis with specific mice antisera. The *C. rodentium* strain grown overnight at 37 °C in LB was diluted 1:100 into 2 ml of DMEM containing CoilA or CoilB peptides at a concentration of 0.56 mg/ml and cultured for another 4 h in the presence of 5% CO_2. Bacteria were collected by centrifugation and then resuspended in 2× SDS sample buffer and boiled for 5 min. The proteins present in the culture supernatants were precipitated by the addition of trichloroacetic acid at 20% (w/v) and incubated overnight at 4 °C. The protein precipitates were subsequently collected by centrifugation at 14,000 × *g* for 25 min, washed twice with 200 μl of ice-cold acetone, and resuspended in 20 μl Tris–HCl 10 mM pH 8.8. Whole-cell extracts and culture supernatants were then analyzed by Western blotting using polyclonal rabbit antibodies against EspA, EspB, and EspD. Primary antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) diluted 1:10,000. The immunoblots were revealed with 4-Cl-1-naphthol (Pierce, Rockford, IL).

Mice infection

All the experiments were approved by the Ethical Committee on Animal Welfare of the Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria. Female C57BL/6J mice (6–8 weeks of age) were obtained at the

laboratory animal center of the School of Veterinary of La Plata University, La Plata, Argentina. During the course of the experiment, sentinel animals were used to detect any murine pathogens. All animals were housed in individually ventilated cages with filtered air (HEPA) and free access to food and sterile water. Two groups of 5 female C57BL/6J mice were used. One group was treated daily with 100 μl of a solution of 1.12 mg/ml of both peptides (CoilA and CoilB) dissolved in PBS pH 7.4, for 8 days prior to a challenge with *C. rodentium* and for 6 additional days after infection (total time elapsed: 14 days). The inoculation of the solution containing the peptide was administered via a gastric tube. The other group was non-treated with peptides. For challenge, the inoculum was prepared with fresh *C. rodentium* from a 1:100 dilution in LB medium, which was grown for 16 h at 37 °C in LB medium containing 25 μg/ml of nalidixic acid. The bacteria were grown with shaking at 37 °C until reaching an $OD_{600} \sim 1$. The culture was then centrifuged and the bacteria resuspended at a concentration of 5×10^9 CFU in 200 μl of PBS. The mice of the 2 groups were inoculated orally using a stomach tube with 200 μl of the bacterial suspension. The viable bacterial count of the inoculum was determined by plating on LB agar containing 25 μg/ml of nalidixic acid. Stool samples were aseptically recovered from individual mice from days 9–17 at different times post inoculation, and the number of viable bacteria (CFU) per gram of feces was determined by plating serial dilutions on LB agar containing 25 μg/ml of nalidixic acid. Mice were sacrificed on day 21 (13 days post challenge), and samples of large intestine were analyzed by histology.

Histological studies

Mice colonic tissues were fixed for at least 24 h in 10% formol in PBS buffer (145 mM NaCl/10 mM NaH_2PO_4, pH 7.2). The tissue sections were dehydrated and included in paraffin. After fixation, longitudinal 6–8 μm thick sections were cut, dehydrated, and carefully embedded in paraffin to obtain sections perpendicular to the mucosa. Cuts (5 μm) were made with a microtome (Leica RM 2125, Wetzlar, Germany) and mounted on 2% silane-coated slides. The slides were stained with hematoxylin–eosin and observed by light microscopy (Nikon Eclipse 200, N.Y.). Each tissue section was examined by an observer unaware of the treatment status. The following pathological score was used: 0, no changes from control; 1, mild lesion corresponding to loss of superficial epithelium; 2, moderate lesion corresponding to loss of superficial epithelium with a mononuclear inflammatory infiltrate; 3, severe lesion corresponding to destruction of the crypts with mononuclear inflammatory infiltrate.

Results

Sequence analysis of EspA of EPEC, EHEC, and C. rodentium

TTSS functions and components are preserved among EPEC, EHEC, and *C. rodentium*. An alignment of EspA orthologs of these species was performed (Fig. 1). The results showed that between EspA orthologs of EPEC and EHEC there is 81% identity and 91% similarity, whereas between those of EPEC and *C. rodentium* there is 79% identity and 90% similarity.

Coiled-coil peptides inhibit the red blood cell hemolysis produced by the TTSS of EHEC O157:H7

When O157:H7 125/99 was pre-incubated with CoilA or CoilB peptides for 1 h, CoilA peptide inhibited hemolysis of RBCs by 70%, while CoilB inhibited it by 74% at 0.2 mg/ml (Fig. 2). At 0.4 mg/ml, CoilA and CoilB were almost as affective as the dose selected for most of the experiments (0.56 mg/ml). The difference between hemolysis caused by O157:H7 125/99 treated with peptides to that produced by non-treated bacteria was significant starting from

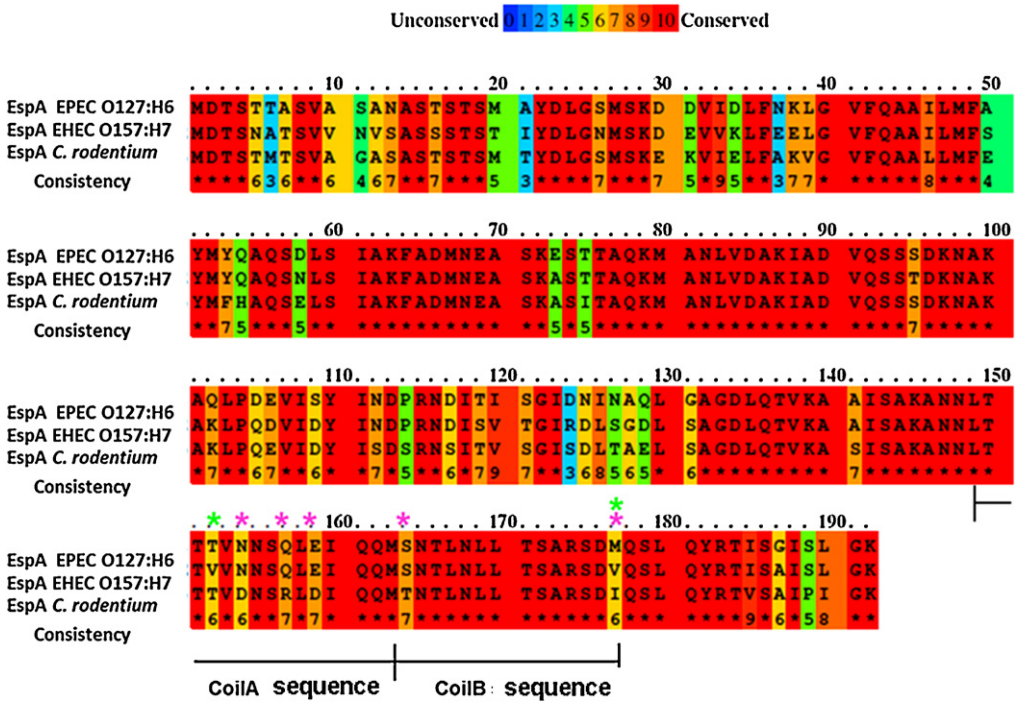

Fig. 1. Sequence alignment of EspA in EPEC, EHEC, and *C. rodentium*. The coiled-coil domain, corresponding to the C-terminal region of EspA, is located between amino acids 149 and 177 (underlined). The color represents consistency, indicating the degree of conservation between the amino acids of the coiled-coil domain for EPEC, EHEC, and *C. rodentium*. The asterisks indicate a lower conservation for amino acids of coiled-coil region of EPEC-EHEC (green color) and EPEC-*C. rodentium* (pink color). Alignments were produced using PRALINE (http://www.ibi.vu.nl/programs/pralinewww/). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

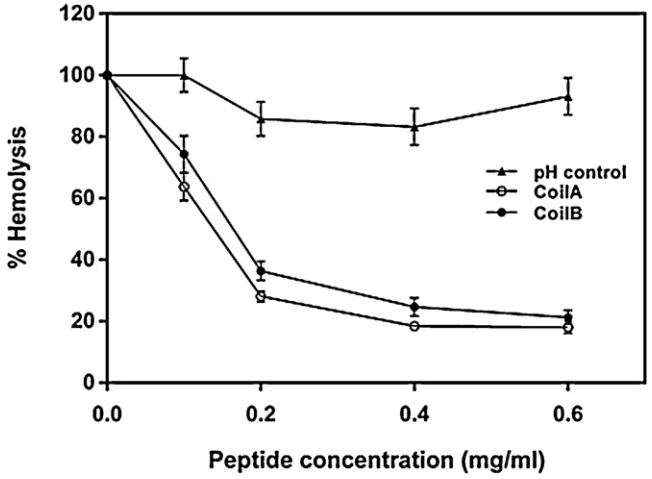

Fig. 2. Coiled-coil peptides effectively reduce red blood cell (RBC) hemolysis promoted by the TTSS of EHEC O157:H7 125/99. A decrease in hemolysis of sheep RBCs was observed when EHEC O157:H7 bacteria were pre-incubated with CoilA or CoilB peptides. NT (non-treated) is a control to rule out the biological influence of alkaline pH in which the peptides are dissolved; PBS in alkaline pH was used in a volume identical to those in which peptides were added to bacterial cultures. Results are expressed as percentage relative to hemolysis (±standard deviation) observed upon incubation of RBCs with EHEC O157:H7 without peptides (NT). $^{*}p < 0.0001$.

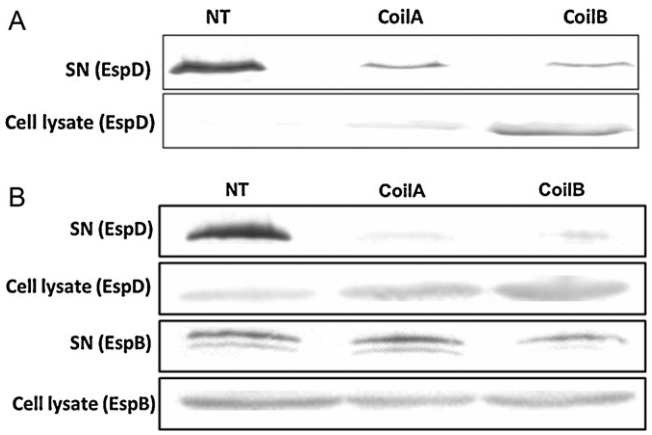

Fig. 3. Effect of coiled-coil peptides on the secretion of TTSS proteins of *C. rodentium* and EHEC O157:H7 125/99. Supernatants (SN) and bacterial extracts (lysate) in vitro culture of *C. rodentium* (A) or EHEC O157:H7 125/99 (B) pre-incubated with CoilA or CoilB peptides were analyzed by Western blot using antiserum directed against EspD and EspB. Coiled-coil peptides affected the secretion of EspD in *C. rodentium* (A SN EspD) and EHEC O157:H7 125/99 (B SN EspD) and EspB in EHEC O157:H7 125/99 (B SN EspB). Equal amounts of bacteria were loaded in every lane. NT (non-treated) is a control to rule out the biological influence of alkaline pH in which the peptides are dissolved; PBS in alkaline pH was used in a volume identical to those in which peptides were added to bacterial cultures.

concentrations of 0.2 mg/ml. Strain O157:H7 125/99 was previously selected because it produced the highest rate of RBC lysis among the isolates analyzed (data not shown).

Supernatants of the bacteria incubated with a suspension of RBC failed to release hemoglobin to the medium, concluding that RBC lysis by *E. coli* 125/99 strain was mediated by the TTSS and not by secreted virulence factors such as the enterohemolysin (Ehly) and Shiga toxin (Stx).

Despite the homology between the TTSS of *C. rodentium*, EPEC, and EHEC, *C. rodentium* biotype 4280 was unable to induce lysis of sheep and bovine red blood cells through its TTSS. Therefore, it was not possible to detect RBC lysis with *C. rodentium* in spite of several attempts (data not shown).

Effect of coiled-coil peptides on the secretion of TTSS proteins in C. rodentium and EHEC O157:H7

To determine whether the pre-incubation of *C. rodentium* with CoilA and CoilB peptides would have any implication on TTSS secretory proteins, peptides and bacteria were incubated together. CoilA and CoilB peptides decreased the amount of EspD present in the culture supernatant (Fig. 3 A), which was correlated with an increase in cytoplasmic EspD. No significant differences between secreted and cytoplasmic EspA and EspB were observed (data not shown). The fact that the intracellular content of EspA and EspB remains unchanged or that EspD cytoplasmic content increased in a similar proportion compared to the reduction of its secreted form upon coiled-coil peptides treatment suggests that the gene expression of these proteins is not affected.

We observed that pre-incubation with CoilA and CoilB of EHEC O157:H7 greatly reduced the secretion of EspD and caused an accumulation in bacteria. A slight decrease in EspB secretion was observed upon incubation with CoilB, but without concomitant intrabacterial accumulation (Fig. 3B). No effect on EspA was observed (data not shown).

Coiled-coil peptides in the formation of pedestal lesion by EHEC 125/99

The FAS test was performed to determine whether the coiled-coil peptides could inhibit the pedestal lesion in HEp-2 cells infected with EHEC O157:H7. The FAS test was performed with a MOI 50:1 for 4 h, and a reduced pedestal formation was observed when EHEC O157:H7 was pre-incubated with 0.56 mg/ml of CoilA or CoilB (Fig. 4B and C) in comparison with bacteria without treatment (Fig. 4A). The difference between pedestals caused by O157:H7 125/99 treated with peptides to that produced by non-treated bacteria was significant. A 50:1 MOI was used because otherwise no pedestals were observed with EHEC O157:H7 (data not shown), contrasting with the ability of EPEC strains to form pedestals. These results showed that coiled-coil peptides inhibit RBC lysis, secretion of EspD and EspB, and pedestal formation by EHEC, in concordance with previous results (Larzábal et al., 2010). Furthermore, we can deduce that there is also a reduction of Tir translocation.

Next, we investigated the effect of coiled-coil peptides on *C. rodentium* capability of pedestal formation. For these tests, it was necessary to use a 4-fold higher MOI (MOI 20) than that used for EPEC (Larzábal et al., 2010) and an increase of time of infection of 4 h to observe pedestals. However, an inhibitory effect of coiled-coil peptides on pedestal formation was not observed in bacteria pre-incubated with 0.56 mg/ml CoilB or CoilA (Supplementary figure) when compared to the non-treated control.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2012.12.001.

Coiled-coil peptides in protection assays against infection with C. rodentium

The susceptibility of mice to *C. rodentium* in contrast to EPEC and EHEC makes *C. rodentium* a model to study pathogenesis in vivo. Therefore, this animal model was used to determine whether coiled-coil peptides can protect mice from *C. rodentium* infection. At day 2 post infection (2 dpi), mice treated with peptides excreted a lower amount of viable bacteria than non-treated mice (where a sustained excretion of around 10^6 CFU/g feces was observed) (Fig. 5). The *C. rodentium* excretion level around the curve showed no statistical differences (data not shown). Besides, no loss of weight or hair or alteration in water consumption or listlessness was observed in mice.

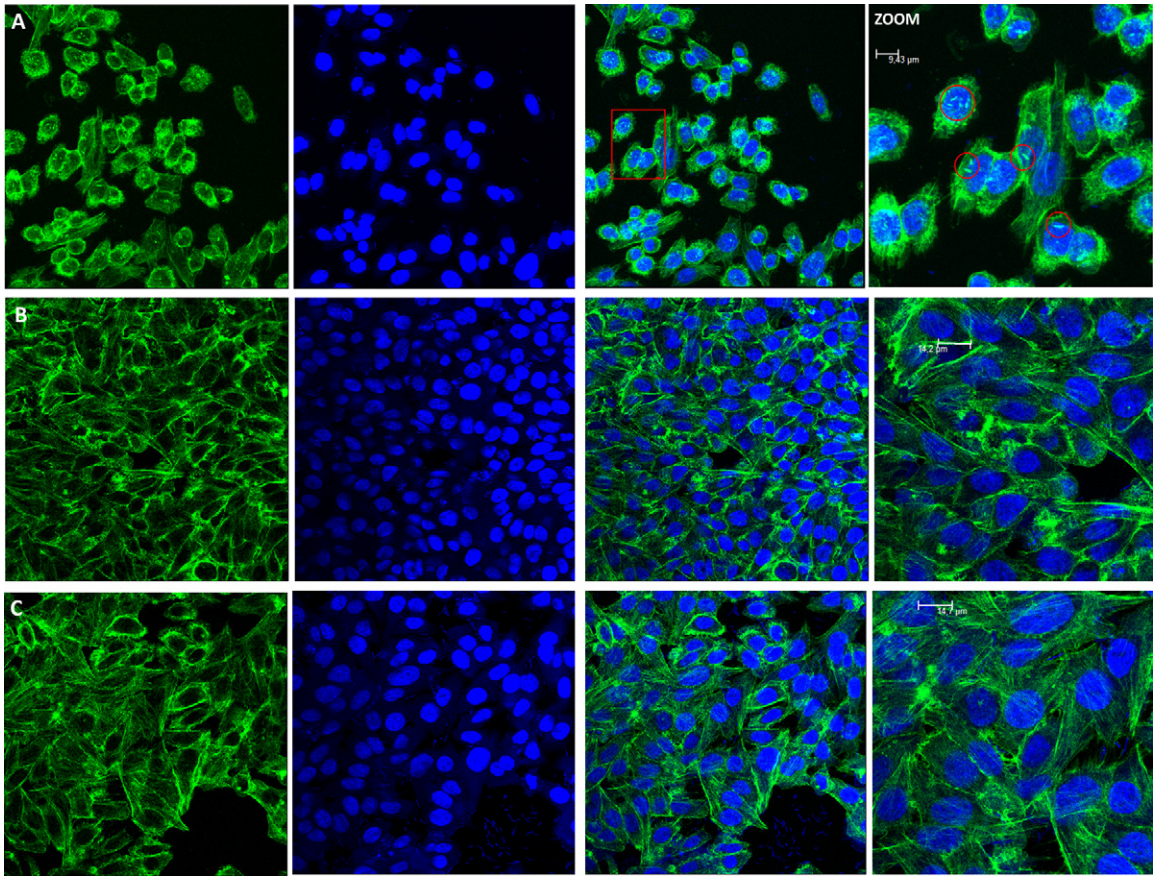

Fig. 4. Pedestal lesions caused by EHEC O157:H7. HEp-2 cells were infected with non-treated (PBS pH 12.0 used as a mock control) EHEC O157:H7 125/99 (MOI 50) (A) or pre-incubated with CoilA (B) and CoilB (C). The polymerization of actin was revealed through the technique of FAS (fluorescent actin staining) using FITC-conjugated phalloidin (green) and TO-PRO-3 to stain double-stranded DNA of both bacteria and the cell nucleus (blue) and photographed with a confocal microscopy at Zoom 1.0. Red circles show the pedestal lesions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Coiled-coil peptides inhibit the in vivo intestinal damage by C. rodentium

The 2 groups of mice were sacrificed on day 21 (13 dpi), and large intestine samples were taken for histological analysis. The results of histological studies of the colon from mice of the non-treated group (Fig. 6A and B) showed areas with loss of superficial epithelium (white arrows), damaged cells, and endoluminal mononuclear inflammatory infiltrate (black arrows). On the other hand, mice treated with CoilA and CoilB peptides kept a normal surface epithelium (Fig. 6C and D), showing a structure similar to that of the uninfected control group (Fig. 6E and F). The average severity grade of lesions evaluated in 5 fields × 200 for each mouse shows that mice untreated with peptides and infected with *C. rodentium* had an average severity grade of 2.25 ± 0.25, while those animals pretreated with CoilA and CoilB peptide or uninfected with *C. rodentium* showed an average severity grade of 0.25 ± 0.16 and 0.13 ± 0.16, respectively ($p < 0.001$).

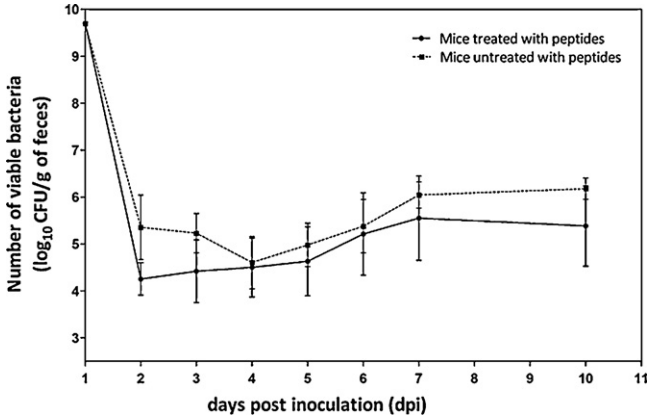

Fig. 5. *C. rodentium* excretion levels of mice treated with CoilA and CoilB peptides and non-treated with peptides. Excretion levels of viable bacteria (mean $\log_{10}$ CFU/g of feces) as a function of time.

Discussion

Several TTSS functions and components are preserved between different enteric pathogens, suggesting that an inhibitor directed against a component of EPEC might be effective against a wide range of Gram-negative pathogens such as EHEC and murine *C. rodentium*. We previously showed that peptides based on coiled-coil structures of EspA block the virulence action of the EPEC TTSS (Larzábal et al., 2010). The CoilA and CoilB peptides inhibited the EPEC-mediated hemolysis of RBC and the formation of pedestals in epithelial cells infected by EPEC in vitro (Larzábal et al., 2010).

In the current study, CoilA and CoilB peptides inhibited the TTSS-mediated hemolysis of RBC produced by EHEC O157:H7 by 70% and 74%, respectively. The conservation of coiled-coil regions of EspA in EPEC and EHEC allows us to infer that the decrease in hemoglobin released by EHEC O157:H7 occurred as a result of the failure to produce the complete EspA filament and the formation of cellular pore over the RBC membrane in the presence of the coiled-coil peptides. The inhibitory effect of CoilA and CoilB on the EHEC-TTSS function

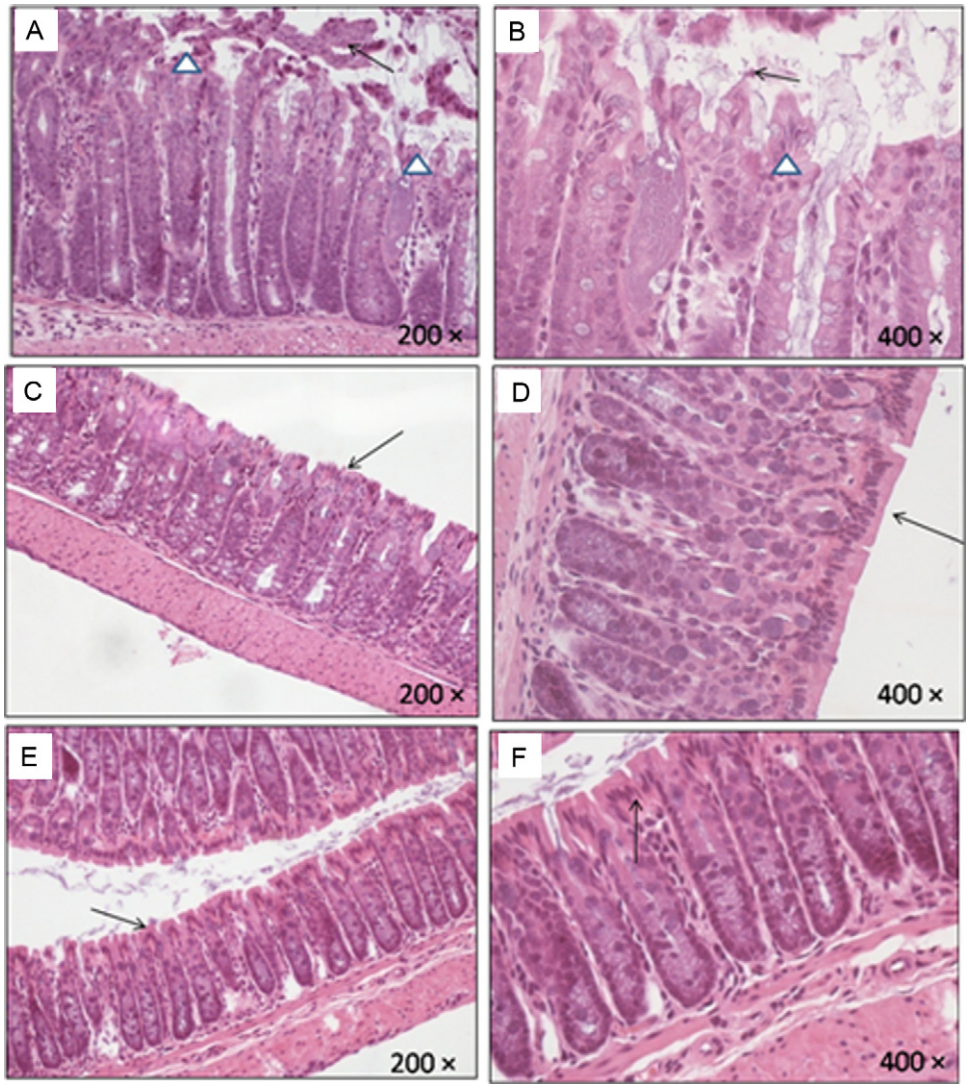

Fig. 6. Histological studies of the colon mice. Hematoxylin and eosin-stained colonic sections of mice infected by *C. rodentium* with non-treatment (A and B), pretreated with CoilA and CoilB peptides and infected by *C. rodentium* (C and D), or not infected (E and F). A, C, and E 200× magnification and B, D, and F 400× magnification. An image representative of 20 fields is shown. Group 2 shows areas with a loss of superficial epithelium (white arrows), sloughed cells, and mononuclear inflammatory infiltrate endoluminal (black arrows), consistent with histological lesions induced by *C. rodentium*.

was also demonstrated by the reduction of EspD and EspB secretion and Tir translocation when the bacteria were co-incubated with any of both peptides. We can hypothetize that Tir translocation by *C. rodentium* and EHEC is also affected. This effect correlates with the inhibition of pedestal formation, as established by means of the FAS test which has already been observed with EPEC. Impact on part of the machinery of colonization of EHEC may be effective in preventing the onset of the infection of bacteria and the release of Shiga toxins.

C. rodentium treated with coiled-coil peptides was impaired in the secretion of EspD, similar to that observed in EPEC (Larzábal et al., 2010) and EHEC in this study; which could involve a limitation in the formation of the A/E lesion. The EspA protein of *C. rodentium* has a lower percentage of identity in the coiled-coil region with $EPEC_{EspA}$ than that observed for EHEC and despite this, the effects were similar to those observed with EPEC E2348/69 regarding the inhibition of the secretion of EspD. Despite the presence of a TTSS in *C. rodentium*, we could not observe hemolytic activity mediated by the bacteria. It suggests that either the efficiency of *C. rodentium* to express its TTSS in vitro or that the adherence to the red blood cell surface is low and that these trials were not suitable to determine the effect of peptides in the functionality of the TTSS of *C. rodentium*. The lack of hemolytic activity by *C. rodentium* was also observed by other researchers (Kimura et al., 2011, and Gad Frankel, Imperial College, UK, and Akio Abe Kitasato University, Japan, personal communication).

The observed blocking of *C. rodentium* TTSS secretion led us to develop a test for protection against *C. rodentium* in vivo to evaluate if the peptides have inhibitory activity in *C. rodentium* mouse colonization mediated by TTSS. Oral treatment of mice with a combination of peptides CoilA and CoilB prior and during the infection with *C. rodentium* prevented colonic lesions, without producing significant differences in average excreted viable bacteria. Reduction of colonic damage in spite of no reduction in bacterial excretion was observed when mice were infected with *C. rodentium* and treated with probiotics (Gardiner et al., 2009; Johnson-Henry et al., 2005; Varcoe et al., 2003). The high burden of inoculum used and/or the ability of *C. rodentium* to persist in the gastrointestinal tract of mice by a TTSS-independent adherence mechanism would explain the colonization and shedding of *C. rodentium* without colonic damage in the peptides-treated mice. Contrasting to our results, Uren et al. (2005) and Maaser et al. (2004) observed that mice control the infection and *C. rodentium* is cleared in 4–6 weeks.

The protein Ler positively regulates transcription of LEE operons and counteracts the repression by the H-NS global regulator in response to environmental signals (Laaberki et al., 2006), which are encountered by bacteria in the establishment of infection and act as markers of niches that support the growth of the pathogen. Therefore, TTSS components are expressed only transiently during a particular phase of the infection and not constitutively, indicating that there is an opportunity for their blockade before the infection. The results presented here suggest that the effects of coiled-coil peptides are related to their availability during the formation of the TTSS, i.e. during the polymerization of EspA and prior to the secretion of the translocator proteins EspD and EspB (Tree et al., 2009), as indicated by the fact that the peptides are effective only when added to the bacteria during TTSS expression and not once the TTSS has already been formed (Larzábal et al., 2010).

Based on these results, we might consider peptides to persist in the intestine of mice long enough to block the A/E lesion. It can be expected that coiled-coil peptides could protect against EHEC and EPEC in infection models in vivo similar to that observed in *C. rodentium*, considering the high levels of inhibition observed in experiments of TTSS functions for these bacteria.

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