Subtractive hybridization and identification of putative adhesins in a Shiga toxin-producing *eae*-negative *Escherichia coli*

Maricel Vidal[1], Valeria Prado[1], Gregory C. Whitlock[2,3], Aldo Solari[5], Alfredo G. Torres[2,4] and Roberto M. Vidal[1]

[1] Programa de Microbiología y Micologia, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile
[2] Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555-1070, USA
[3] Department of Clinical Laboratory Sciences, University of Texas Medical Branch, Galveston, TX 77555-1070, USA
[4] Department of Pathology and Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, TX 77555-1070, USA
[5] Programa de Biologia Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile

Correspondence
Roberto M. VidalE-mail:
rvidal@med.uchile.cl
or
Alfredo G. Torres
altorres@utmb.edu

ABSTRACT

Adherence to epithelial cells by specific adhesins is a characteristic of Shiga toxin-producing *Escherichia coli* (STEC) strains. The *eae*-encoded protein intimin is the main adhesin implicated in intestinal colonization *in vivo*. We recently showed that STEC strains isolated in Chile displayed a wide variety of adhesins; here we demonstrate that some of these STEC strains are *eae*-negative and still adhere to epithelial cells at a level 100-fold higher than enterohaemorrhagic *E. coli* (EHEC) O157 : H7 prototype strain EDL933. This phenotype is associated with the presence of adherence factors different from the intimin protein. Subtractive hybridization between EHEC EDL933 and STEC *eae*-negative strain 472-1 was used to identify regions implicated in adhesion. In addition to the *saa* gene, we identified 18 specific genes in STEC 472-1, 16 of which had nucleotide identity to *Salmonella* ST46 phage genes; the two remaining ones shared identity to a gene encoding a hypothetical protein of uropathogenic *E. coli*. The DNA sequence of the STEC 472-1 *psu-int* region identified five open reading frames with homology to phage genes. We constructed mutant strains in the *saa* gene and the *psu-int* region to study the participation of these genes in the adherence to epithelial cells and our results demonstrated that STECΔ*saa* and STECΔ*psu-int* mutants displayed a 10-fold decrease in adherence as compared to the STEC 472-1 wild-type strain. Overall, our results suggest that STEC strain 472-1 adheres to epithelial cells in an *eae*-independent matter and that *saa* and *psu-int* participate in this adhesion process.

Abbreviations: A/E, attaching and effacing; EHEC, enterohaemorrhagic *E. coli*; HC, haemorrhagic colitis; HUS, haemolytic uraemic syndrome; LEE, locus of enterocyte effacement; STEC, Shiga toxin-producing *Escherichia coli*

Supplementary material is available with the online version of this paper.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) strains of different serotypes have been increasingly isolated from humans with enteric disease and from healthy domestic animals (Beutin *et al.*, 1993; Caprioli *et al.*, 1993). In the human host, STEC may cause diarrhoea, and certain STEC strains, designated enterohaemorrhagic *E. coli* (EHEC), cause life-threatening haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Verweyen *et al.*, 2000). Various virulence factors have been identified in STEC strains, including Shiga toxins (Stx1 and Stx2) encoded by the stx_1 and stx_2 genes, respectively, and several surface-associated proteins (Nicholls *et al.*, 2000; Tarr *et al.*, 2000; Brunder *et al.*, 2001; Torres *et al.*, 2002). Most EHEC strains can tightly attach to intestinal epithelial cells through intimin, which is encoded by the *eae* gene, located in the locus of enterocyte effacement (LEE) pathogenicity island. This protein is associated with the formation of attaching and effacing (A/E) lesions on the infected cells (Kaper *et al.*, 1998). Interestingly, *eae* and stx_2 genes were previously associated with isolates from other serotypes implicated in severe human disease (Boerlin *et al.*, 1999) and it was proposed that clinical isolates of EHEC must harbour both *stx* and *eae* genes. However, some *eae*-negative STEC strains, such as those of serotypes O91 : H21 and O113 : H21, that cause bloody diarrhoea and HUS in humans, have been described (Paton *et al.*, 1999; Caprioli *et al.*, 2005). Furthermore, STEC O113 : H21 is the most common *eae*-negative, LEE-negative STEC serotype associated with human disease (Karmali, 1989).

Because adherence and colonization to intestinal epithelium by STEC strains is a key component of the pathogenic process, most studies have focused on the characterization of the adhesins in STEC *eae*-negative strains. The adherence phenotypes of several *eae*-negative STEC strains have been examined and information exists concerning the global mechanisms and/or the proteins involved in the adherence to tissue culture cells (De Azavedo *et al.*, 1994; Dytoc *et al.*, 1994; Scotland *et al.*, 1994). We recently showed that STEC strains isolated in Chile carried a wide variety of genes encoding adhesins (Vidal *et al.*, 2007). In the present study, we demonstrate that some of these *eae*-negative STEC (non-EHEC) strains adhere to tissue-cultured cells (HEp-2 cells) better than or similarly to the *eae*-positive reference EHEC strain EDL933 and that this adhesion phenotype is due to the presence of both novel and previously established adherence factors.

METHODS

Bacterial strains and culture conditions.

Bacterial strains used in this study are listed in Table 1. *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) broth, or on LB agar supplemented with appropriate antibiotics or substrates for screening at the following final concentrations: ampicillin (Ap), 100 µg ml^{-1}; chloramphenicol (Cm), 30 µg ml^{-1}; streptomycin (Sm), 100 µg ml^{-1}; kanamycin (Km), 50 µg ml^{-1}; 0.2 mM IPTG; X-Gal, 80 µg ml^{-1}. Plasmid pGEM-T Easy (Promega) was used to construct the library for the subtractive hybridization. *E. coli* strain 472-1 (STEC O125 *eae*-negative, referred to as tester) and *E. coli* EDL933 (STEC O157 : H7 prototype strain, referred to as driver), were used for subtractive hybridization.

Table 1. Bacterial strains used in this study

DNA extraction.

Genomic DNA was isolated using the Qiagen Genomic-tip 100/G. Plasmid DNA extraction for pGEM-T Easy was performed according to the manufacturer's guidelines (Wizard Plus Minipreps, Promega).

Tissue-cultured cell assays.

HEp-2 cells were cultured in disposable Petri plates (100 mmx20 mm) (Falcon), at $2.5x10^5$ cells ml^{-1} in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % (v/v) fetal bovine serum and 1 % (v/v) penicillin/streptomycin (complete DMEM) at 37 °C in an atmosphere of 5 % (v/v) CO_2. The culture medium of each plate was changed every 24 h; cells grew to 95 % semi-confluence in about 3 days. A solution of 0.25 % (v/v) trypsin and 0.53 mM EDTA was added to harvest the cells and the incubation was performed at 37 °C for 10 min. The concentrated cell suspensions were transferred to sterile tubes and centrifuged at 1000 ***g*** for 10 min. The cellular pellet was washed with sterile PBS and resuspended in 1 ml fresh complete DMEM. The quantity of cells present in each sample was estimated using a Neubauer haemocytometer. Finally, the concentration of cells in suspension was adjusted with complete DMEM and placed in 24-well microplates (Nunc) at a concentration of $5x10^4$ cells per plate. Microplates were incubated at 37 °C in an atmosphere of 5 % (v/v) CO_2 for 24 h before each adherence test was performed. When microplates were not used immediately, cells were stored at –80 °C in 70 % (v/v) DMEM, 20 % (v/v) fetal bovine serum, and 10 % (v/v) DMSO.

Adherence assays on epithelial cells.

Adherence assays were performed using the method described by Torres *et al.* (2002). HEp-2 cells were grown to semi-confluence in 24-well microplates at 37 °C in 5 % (v/v) CO_2. Before being infected, cells were washed three times with PBS (pH 7.4) and the wells were filled with DMEM supplemented with 1 % w/v mannose. Bacterial strains grown in LB broth overnight were diluted to give $1x10^7$ cells per well and incubation was continued for 3 h at 37 °C in 5 % (v/v) CO_2. The culture medium was removed from the wells and cells were washed three times with PBS to remove non-adherent bacteria, followed by the addition of 200 µl of 0.1 % (v/v) Triton X-100 in PBS buffer and incubated for 30 min at room temperature to detach the HEp-2 cells from the wells. Finally, 100 µl of this mixture

was transferred to 1.5 ml tubes with 900 µl LB broth and serial dilutions were grown on LB agar plates to get an indirect count of the adhered bacteria.

For qualitative analysis, we placed a coverslip in the microplate wells for each sample tested, and after 3 h incubation, the coverslip was washed three times with PBS, fixed with methanol for 10 min and stained with Giemsa for 30 min, to visualize adherent bacteria under the light microscope. All the experiments were performed in triplicate and the data represent the results of at least two independent experiments. Results are expressed as the mean±SD and statistical significance was established by a Student's *t*-test.

Subtractive hybridization, DNA cloning and differential screening of the subtracted DNA library.

The Clontech PCR-Select bacterial genome subtraction kit (Clontech Laboratories) was used to identify DNA sequences present in the genome of one strain but absent in the genome of the other one. Genomic DNA of STEC strain 472-1 was used as tester and subtracted from the genomic DNA of EHEC strain EDL933 (driver). Subtractive hybridization was performed according to the manufacturer's instructions. Briefly, the tester and driver DNA samples were digested with a four-base cutting restriction enzyme (*Rsa*I) that yields blunt ends. The tester fragments were divided into two portions, each of which was ligated with a different adaptor that facilitates amplification of the tester DNA by PCR. The driver fragments and the adaptor-ligated tester fragments then underwent a two-step round of hybridization, followed by PCR to enrich tester-specific DNA fragments. Later, these fragments were cloned into a pGEM-T Easy cloning vector (Promega).

PCR-select subtraction was performed using STEC strain 472-1 as tester and EHEC strain EDL933 as driver. The subtracted genomic DNA library was T/A cloned. Clones were picked and their inserts were amplified and arrayed in duplicate on nylon membranes (Bio-Dot Microfiltration System, Bio-Rad). Membranes were screened by hybridization with genomic DNA from tester and driver; each genomic DNA sample was labelled by random priming with 5 µl [α-^{32}P]dCTP (1.85 MBq, 1.11×10^{14} Bq mmol^{-1}, aqueous solution, Promega). The true tester-specific clones were sequenced by PolyScience Medica.

Preparation of electrocompetent cells.

To obtain electrocompetent cells, we used 50 µl of a bacterial culture grown overnight to inoculate 5 ml LB broth, which was then incubated for 2 h at 37 °C. The culture was aliquoted into four previously chilled 1.5 ml tubes, centrifuged for 10 min at 3000 ***g*** at 4 °C, and resuspended with 150 µl cold sterile water. The samples were centrifuged again for 10 min at 3000 ***g***, and a final wash was performed with 125 µl of a cold solution of 10 % (v/v) glycerol in water. The contents of the four tubes were combined, centrifuged again and the bacterial pellet resuspended in 100 µl cold 10 % (v/v) glycerol in water, and used immediately for electroporation.

In silico analysis of STEC 472-1-specific sequences and confirmation by molecular techniques.

BLASTN searches with the putative STEC 472-1-specific genes were performed against DNA sequences deposited in the National Center for Biotechnology Information (NCBI) website to identify the genes from the subtractive library. When the unique DNA sequences from STEC 472-1 were identified from the subtracted library, their absence in EDL933 was confirmed by PCR and Southern hybridization.

Deletion of the *saa* gene.

The mutation of the *saa* gene followed the protocol described by Paton *et al.* (2001). The plasmid pΔ*saa* : : km was electroporated into SM10 (λ*pir*), and this strain was conjugated with STEC 472-1S (STEC 472-1 streptomycin-resistant strain) for 8 h at 30 °C. (See Supplementary Table S1 for details of plasmids used in this study.) The selection of the transconjugant colonies was performed after overnight growth in LB agar supplemented with Km, Cm and Sm and incubating at 30 °C. The next day, the transformed colonies were seeded in LB broth supplemented with Km, Cm and Sm and incubated at 30 °C overnight. Subsequently, the colonies grown in LB broth were plated in LB agar and incubated at 42 °C [non-permissive temperature for pCACTUS (parent plasmid of pΔ*saa* : : km)] to obtain the loss of the plasmid. After 24 h the colonies grown at 42 °C were diluted and plated on LB agar without NaCl, supplemented with 5 % (v/v) sucrose, Km and Sm, and incubated at 37 °C. Finally, the mutant colonies were plated on LB agar containing different antibiotics, and selection was performed by identifying Cm^s, Km^r and Sm^r colonies. To confirm the mutation in strain 472-1Δ*saa*, PCR was used to screen for the absence of a 1508 bp fragment using *saa* specific primers: saa-F (5'-CCC CCA TAA TGG AGT GAC TT-3') and saaR (5'-CGC CTG TTC CAT GTT GTG TA-3'). The sequence of the *saa* gene can be found in GenBank under accession number AY258503 (*E. coli* strain EH41 plasmid pO113).

Mutagenesis of the hypervariable region of phage P4 (*psu-int* fragment).

The mutation of the fragment *psu-int* was performed using the methodology described by Datsenko & Wanner (2000). The primer pair rhF (5'-TGC GCG CAA GGC TGA TAA CAT AAA TAA AAT ACA ATG AAC TGT GTA GGC TGG AGC TGC TTC-3') and rhR (5'-GCG ATA TAG CGT TCT GGT TAT ATG GGA TGT GAA ATA GTC CCA TAT GAA TAT CCT CCT TAG-3') containing a 40 bp sequence homologous to the *psu-int* hypervariable region (underlined sequence) was used for PCR utilizing plasmid pKD3 as a template, which contains the Cm antibiotic resistance cassette. Eight microlitres of the purified PCR product was mixed with 50 µl of STEC 472-1(pKD46) electrocompetent cells (previously induced with IPTG), and subjected to electorporation at 1800 V. The electroporation mixture was then transferred to 1 ml LB broth and incubated for 1 h at 37 °C. The bacterial culture was centrifuged, resuspended in 100 µl LB broth, and plated on LB agar supplemented with Cm. Finally, the Cm^r colonies were confirmed by PCR. To confirm the mutation in strain 472-1Δ*psu-int*, PCR was used to screen for the absence of approximately 2500 bp fragment generated by PCR using primer pair psu-F (5'-CCC CCA TAA TGG AGT GAC TT-3') and intR (5'-CGC CTG TTC CAT GTT GTG TA-3').

Complementation of the 472-1 Δ*saa* and 472-1 Δ*psu-int* mutants.

The *saa* gene and the hypervariable region harbouring the *psu* and *int* genes were amplified from the STEC 472-1 strain with primers for the *saa* gene: F (5'-CCA TGG TCA AAA GAA ATT GTG TGC ATT CGA-3'; *Nco*I site underlined) and R (5'-GTC GAC TTA CCA TCC AAT GGA CAT GCC TG-3'; *Sal*I site underlined); and for the hypervariable *psu-int* region: F (5'-CCG AAT TCG AAC TCC CCG AAA ATC CGC CCG TT-3'; *Eco*RI site underlined) and R (5'-GTC GAC TTC AAA AGT TGA TTT TTA TAA AC-3'; *Sal*I site underlined). The PCR products were cloned into pGEM-T easy vector (Promega). The fragments were excised using *Nco*I/*Sal*I and *Eco*RI/*Sal*I restriction digestion and inserted into the respective restriction sites of the linearized pTrc99A plasmid, resulting in plasmids

pTrc99A-*saa* and pTrc99A-*psu-int*. These constructs were used to complement strains 472-1 Δ*saa* and 472-1 Δ*psu-int*. The plasmids used for complementation were induced with 1 mM IPTG for 3 h in LB broth prior to the adherence assays on epithelial HEp-2cells.

RESULTS

Detection of STEC *eae*-negative strains from clinical samples

We have previously characterized the distribution of three adhesin-encoding genes (*efa-1*, *lpfA* and *saa*) in a large collection of Chilean STEC strains (Vidal *et al.*, 2007). We reported that these adhesins, in addition to *eae* (encoding the intimin protein), are present in a large proportion of the strains analysed, suggesting that they might be playing a role in pathogenesis. Interestingly, we also noticed that some of these strains did not carry the *eae* gene. To confirm that some of the STEC strains were in fact *eae*-negative, we designed a primer pair set to include all the intimin variants currently described. Using PCR analysis, we found that three strains, isolated from patients with different clinical syndromes, were *eae*-negative (Table 1). The absence of the *eae* gene in these strains was further confirmed using a set of oligonucleotides previously described to identify all the intimin variants (Blanco *et al.*, 2005). Furthermore, we performed PCR analysis of two other LEE-encoded genes (*tir* and *cesT*), located next to the *eae* gene (*tir*, *cesT* and *eae* are part of a LEE operon known as *LEE5*). Our PCR results indicated that these two genes could not be amplified from the *eae*-negative strains, suggesting that these strains lack the *LEE5* operon (data not shown). Finally, we tested whether the STEC *eae*-negative strain 472-1 expressed the Tir protein using an anti-Tir antibody. Western blotting showed that Tir is not expressed under type-III-inducible conditions in STEC 472-1 as compared with the prototype EHEC strain EDL933, a result that confirmed our PCR analysis.

Adherence of *eae*-negative STEC strains to HEp-2 cells

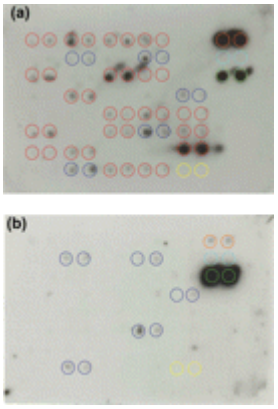
We analysed the ability of *eae*-negative STEC strains to adhere to tissue-cultured cells. Because intimin is the main adhesin of STEC strains, we hypothesized that the *eae*-negative strains would adhere less efficiently than the EHEC O157 : H7 prototype strain. Therefore, HEp-2 cells were infected with EHEC strain EDL933 and the three STEC *eae*-negative strains; the results showed that STEC strain 472-1 adhered better ($1.3\text{x}10^5 \pm 3\text{x}10^4$ c.f.u. ml^{-1}) than EHEC strain EDL933 ($7.9\text{x}10^3 \pm 3.2\text{x}10^2$ c.f.u. ml^{-1}). The other two STEC strains adhered slightly less than EDL933 (STEC B2P2-2, $3.5\text{x}10^3 \pm 1.7\text{x}10^3$ c.f.u. ml^{-1} and B2P5-2, $7.7\text{x}10^3 \pm 1.2\text{x}10^3$ c.f.u. ml^{-1}). Scanning electron microscopy was used to visualize the A/E lesions associated with expression of the LEE-encoded proteins. Only strain EDL933 formed the classical A/E lesion while the *eae*-negative strains adhered in a diffuse adherence pattern and did not produce A/E lesions (see Supplementary Fig. S1, available with the online version of this paper). Our data showed that the three STEC *eae*-negative strains adhere to HEp-2 cells but strain 472-1 adhered better than EDL933, suggesting that this STEC strain might be expressing additional factors associated with the adherence phenotype.

Identification of adhesin-associated genes in *eae*-negative STEC strains

To determine whether the STEC *eae*-negative strains possess any of the adherence factors previously described in other pathogenic *E. coli* strains, we selected four genes encoding known adhesion factors: *iha*, encoding a putative adhesin of EHEC O157 : H7; *efa-1*, encoding an adhesin found in EHEC O111; *lpfA*, encoding the fimbrial adhesin of EHEC O157 : H7; and *saa*, a plasmid-encoded gene required for the production of the agglutinating adhesin of LEE-negative STEC strain serogroup O91. As displayed in Table 2, two of the four genes analysed by PCR (*iha* and *saa*) were amplified in the three *eae*-negative STEC strains. Interestingly, the *saa* gene was present in these three *eae*-negative STEC strains regardless of the serogroup, and was absent from EHEC EDL933, suggesting that the protein product encoded by this gene could be playing a role in adhesion. As expected, strain EDL933 possesses three of the genes analysed, but not *saa* (Table 2). To determine whether the genes analysed were expressed in the *eae*-negative STEC strains, we performed RT-PCR using RNA extracted from the test and control strains. Although STEC strain 472-1 possesses *iha* and *saa*, the RT-PCR results indicated that the strain only expressed the *saa* gene. The two other outbreak isolates (B2P2-2 and B2P5-2) expressed the *iha* and *saa* genes under the laboratory conditions tested. The control EDL933 strain induced expression of the three analysed genes under the conditions tested. Because the three *eae*-negative STEC strains expressed the *saa* gene, it was difficult to conclude that the Saa adhesin was the only protein responsible for the enhanced adherence phenotype observed with STEC 472-1; therefore, we hypothesized that this strain might carry an additional adhesion factor responsible for this phenotype, which is absent in EHEC EDL933.

View this table**:** Table 2. PCR and RT-PCR analysis of putative adhesins of STEC *eae*-negative strains

To test our hypothesis, we performed a subtractive hybridization analysis using EHEC EDL933 (driver) and STEC 472-1 (tester). We obtained 39 positive subtractive hybridization clones, indicative of DNA sequences specific to STEC 472-1. To confirm our hybridization results, we prepared radiolabelled DNA probes from both strains, and two membranes containing the 39 PCR clones were used. Eighteen out of 39 clones analysed hybridized only with the STEC 472-1 probe (Fig. 1a, b), while the remaining 21 clones hybridized also with the EHEC EDL933 probe.



View larger version (68K):

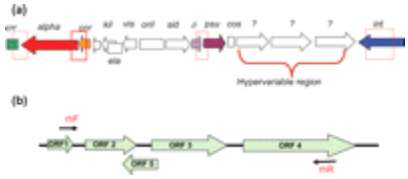
Fig. 1. Subtractive hybridization of the clones obtained with ^{32}P-labelled probes from (a) STEC 472-1 and (b) STEC EDL933. Clones that hybridized with only the 472-1 probe are enclosed by red circles; those that hybridized with the 472-1 and EDL933 probes are enclosed by dark blue circles. Controls for STEC 472-1 and STEC EDL933 strains are enclosed by orange and green circles, respectively. The unsubtracted control of STEC 472-1 strain is enclosed by yellow circles and the primer controls used in this protocol are enclosed by light blue circles. All clones were analysed on duplicate spots.

Identification of the specific genes found in STEC 472-1

The 18 clones specific to STEC strain 472-1 were sequenced and the DNA was analysed using the BLASTN and Omiga software (OMIGA 2.0, Oxford Molecular). Sixteen of the clones had identity with ORFs found in three enterobacterial pathogens, *Salmonella enterica* serovar Typhi CT18, *S.* Typhi Ty2 and enteroaggregative *E. coli* O42 (Table 3). All these genes had identity with genes found in a phage of the P4 family first identified in *S.* Typhi (*cnr*, *crr*, *alpha*, *psu*, and *int*) (see the supplementary sequence data available with the online version of this paper). The other two clones had identity to genes encoding a hypothetical protein in uropathogenic *E. coli* and to a region located in plasmid pO113 of *E. coli* O113 : H21.

View this table**:** Table 3. BLASTN analysis of the DNA sequences of the clones specific to STEC 472-1

The distribution of the phage genes in STEC 472-1 showed a high similarity to that in ST46 phage from *S.* Typhi CT18 (Thomson *et al.*, 2004). The clones displayed more than 95 % identity to the P4 phage of ST46 and in addition to the structural phage genes, there was a region defined as hypervariable (Fig. 2a). In phages of the P4 family, the hypervariable region is located between the *cos* and the *int* (integrase) genes. In some enterobacterial organisms, genes associated with virulence are associated with this region (Pierson & Kahn, 1987). Our interest was to locate novel genes associated with adherence in STEC 472-1 and we therefore designed oligonucleotides within the *psu* and *int* genes, flanking the hypervariable region, to amplify and determine its DNA sequence. Further, we also designed oligonucleotides to amplify the fimbrial major subunit SefA gene from *Salmonella*, which is located next to phage ST46 in this strain, but unknown in STEC 472-1.



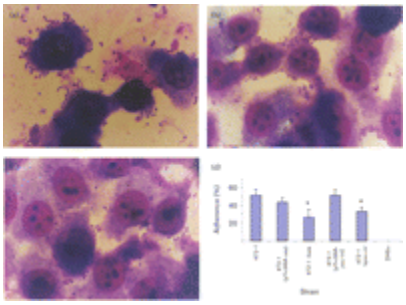
View larger version (12K):

Fig. 2. (a) Map of bacteriophage ST46, located within the SPI-10 in the *S.* Typhi CT18 genome (similar to the P4 family of phages). The regions enclosed in red rectangles showed significant nucleotide sequence identity with clones selected by subtractive hybridization (≥98 %). Question marks indicate the hypervariable region with unknown ORFs. The sequences of the specific clones (1 to 16) of STEC 472-1 obtained for subtractive hybridization corresponded to the *cnr*, *crr*, *alpha*, *psu*, *cos* and *int* genes. (b) Organization of the predicted ORFs within the hypervariable region of the P4 phage located in STEC 472-1. The small black arrows indicate the location of the oligonucleotides used to construct the mutant.

PCR analysis of the STEC 472-1 and the *Salmonella* control strains showed that *sefA* was present in all *S.* Typhi tested and absent in strain 472-1 (data not shown). In contrast, we obtained an approximately 3000 bp fragment containing the *psu-int* region in STEC 472-1. DNA sequence analysis indicated that the fragment was 2393 bp in length and contained five ORFs (Fig. 2b). BLAST analysis of the putative proteins encoded in the five ORFs showed that they shared homology with hypothetical proteins from *Brucella abortus*, *Yarrowia lipolytica* and *Photorhabdus luminescens*, but no obvious association with adhesion (Fig. 2b).

Construction of mutant strains and characterization of the STEC Δ*saa* and STEC Δ*psu-int* adherence phenotypes

To determine whether the *psu-int* region had any role in adherence of STEC 472-1, we constructed a mutant strain deleting the hypervariable region. We also created a *saa* mutant, to define the role in adhesion to HEp-2 cells of the agglutinating adhesin encoded in this gene. We observed a difference in adhesion when comparing the STEC Δ*saa* to wild-type strain 472-1 (Fig. 3a, b). Furthermore, an evident difference in adhesion was obtained with the STEC Δ*psu-int* strain (Fig. 3c). Interestingly, both mutant strains displayed a statistically significant reduction of ~20 % in adhesion to HEp-2 cells when compared with the wild-type strain (Fig. 3d). The growth rate of both mutants and wild-type strains was compared and their doubling time was comparable (data not shown). Although the difference in adhesion was statistically significant, the trend suggested that these two factors participate in the adherence of STEC 472-1. Complemented strains re-established the adherence phenotype to values that were not significantly different from the adherence of the wild-type strain but displayed statistical significance compared to the mutant strains (Fig. 3d). Overall, our results suggest that these two systems are either redundant and complement each other, or possibly other adhesion factors, also present in EHEC EDL933, and which were not identified in our subtractive hybridization analysis, are responsible for the high adherence phenotype observed in STEC 472-1.



View larger version (96K):

Fig. 3. Adherence of STEC strains quantified in HEp-2 cell assays. (a–c) Giemsa staining of STEC 472-1 (a); STECΔ*saa* (b) and STECΔ*psu-int* (c). (d) Percentages of STEC 472-1, STECΔ*saa*, STECΔ*psu-int*, complemented strains STECΔ*saa*(pTrc99A-*saa*) and STECΔ*psu-int*(pTrc99A-*psu-int*), and DH5α adherent to cultured HEp-2 cells after 3 h incubation. The error bars indicate standard deviation. **P*<0.05.

DISCUSSION

The principal virulence characteristic of the most prevalent STEC strains in clinical practice, in addition to production of Shiga toxins, is the presence of the LEE. These virulence markers define whether the strains are capable of producing illness in humans and, in general, confer their zoonotic character. The *eae* gene (encoding intimin) contained in the LEE was described as the only important adhesin factor for *in vivo* intestinal colonization (Donnenberg *et al.*, 1993). However, the subsequent isolation of STEC strains without the *eae* gene, from patients suffering from HC and HUS have motivated the study of new adherence factors different from intimin, that might be associated with intestinal colonization (Dytoc *et al.*, 1994). Our initial results are in agreement with other studies indicating a high prevalence of the *eae* gene in strains isolated from humans (Blanco *et al.*, 2003). The search for the intimin gene using PCR and universal *eae* primers allowed the detection of three *eae*-negative strains (STEC 472-1, STEC B2P2-2 and STEC B2P5-2; Tables 1 and 2). Furthermore, the molecular and antigenic characterization of these three strains concurred with the properties of other *eae*-negative strains previously characterized: (1) their serogroups (O125, O91 and O174) are not related to other *eae*-positive strains (Blanco *et al.*, 2005); (2) presence of the *saa* gene, which is a characteristic of STEC strains that do not have the LEE pathogenicity island (Paton *et al.*, 2001; Lucchesi *et al.*, 2005); (3) inability to amplify by PCR the *tir* and *cesT* genes, indicative of absence of the *LEE5* operon; and finally, (4) PCR amplification of the tRNAs described to date as insertion sites for the LEE (Bertin *et al.*, 2004) confirmed the absence of this pathogenicity island in our *eae*-negative STEC strains (data not shown).

The *eae*-negative strains STEC 472-1, B2P2-2 and B2P5-2 adhered to HEp-2 cells; however, they displayed qualitative and quantitative differences. Strain STEC 472-1 showed 100-fold higher adherence capacity than the other *eae*-negative strains and the *eae*-positive prototype EDL933 strain. Electron microscopy examination of the *eae*-negative STEC strains revealed a semi-localized adherence pattern, similar to that described by Paton *et al.* (2001) for the *eae*-negative strain 98NK2. This pattern was clearly different from that observed in the *E. coli* O157 : H7 EDL933 strain, which displayed a diffuse adherence with some areas of localized adherence, similar to the phenotype reported by

Torres *et al.* (2002). Scanning electron microscopy showed that the *E. coli* O157 : H7 EDL933 strain formed actin pedestals, typical of bacteria that produce A/E lesions. These data further confirmed that the 472-1, STEC B2P2-2 and STEC B2P5-2 strains lacked the intimin protein and, therefore, did not display the A/E lesion formation phenotype (see Fig. S1).

Currently, there is limited knowledge about the mechanisms of adherence to the gastrointestinal epithelium by STEC *eae*-negative strains. However, it has been reported that they efficiently adhere to tissue-cultured cells (Paton & Paton, 1998), which is in agreement with the results obtained with strain STEC 472-1. We initially characterized the presence of previously described adherence factors in *E. coli*, such as *efa-1*, *iha*, *lpf* and *saa* (Nicholls *et al.*, 2000; Tarr *et al.*, 2000; Paton *et al.*, 2001; Torres *et al.*, 2002). The amplification of the *efa-1* gene was positive only in the control EHEC EDL933 strain, similar to the study by Nicholls *et al.* (2000), who analysed the presence of this gene in 207 strains, 116 able and 91 unable to produce A/E lesions. The *efa-1* gene was found in all A/E-producing strains, but was absent in those strains that did not produce this lesion. According to these results, our three *eae*-negative STEC strains lacked the *efa-1* gene. We also found that the *lpf* gene was amplified only in the EHEC EDL933 control strain and not in the three *eae*-negative strains analysed. However, we must consider that the presence of the *lpf* gene has not been associated only with serogroup O157; therefore it cannot be used as a definitive marker to identify *eae*-negative strains. The gene *iha*, which was first described in a STEC O157 : H7 strain, was amplified in all the strains tested in this study, which leads us to conclude that this gene is not specifically related to serogroup O157. Results published by Toma *et al.* (2004) showed that *iha* was the most prevalent adhesin gene in all the strains analysed (127 of 139 strains were positive for *iha*) and the distribution was not related to a specific serogroup or serotype. Finally, the *saa* gene, which encodes an auto-agglutinating adhesin, was amplified in the three *eae*-negative strains but not in the STEC EDL933 control strain. This gene has been specifically associated with non-O157 LEE-negative strains (Paton *et al.*, 2001). However, in the study by Toma *et al.* (2004), the *saa* gene was present only in strains of three seropathotypes that were defined as LEE-negative, and interestingly, these three seropathotypes are formed by serotypes isolated from food and from animals, and less frequently associated with human infections.

The gene expression experiments were all performed using RT-PCR and although this is not a direct measure of protein expression, our data suggested that it may be translated into a functional protein. Using this method, we determined that the STEC B2P2-2 and B2P5-2 strains expressed the *iha* and *saa* genes. In contrast, strain STEC 472-1 only expressed the *saa* gene. Our control EDL933 strain expressed all three genes (*efa-1*, *iha* and *lpf*), which were also positive by PCR analysis. We are aware that these results are dependent on the conditions in which the experiments were performed; however, it is plausible to speculate that the *saa* gene was not expressed when *iha* expression was induced, which may indicate some type of negative regulatory relationship between these two genes. The results of gene expression obtained for strain STEC 472-1 were interesting because this strain showed the ability to adhere to HEp-2 cells and we could hypothesize that this phenotype was due to the Saa adhesin. However, the other two *eae*-negative STEC strains also expressed the *saa* gene but did not show the same capacity to adhere to HEp-2 cells. One possible explanation for these results is that both strains expressed the *iha* and *saa* genes, and if our previous

observation is correct, expression of the *iha* gene might negatively regulate the expression of the *saa* gene, preventing the two strains from displaying all their adherence capabilities (Table 2). This possibility is currently being investigated in our laboratory.

Another possibility is that in STEC strain 472-1, the *saa* gene is not the only gene encoding a protein participating in adherence and perhaps there are some unidentified adherence factors that are absent in the other *eae*-negative STEC strains. Therefore, we investigated this possibility using subtractive hybridization (Calia *et al.*, 1998; Zhang *et al.*, 2000). The hybridization of the membranes with probes of the tester and the driver strains produced 18 clones which hybridized specifically with STEC 472-1-derived probes, and such clones were confirmed by differential screening as being present only in strain 472-1. These clones represented 46 % of the total number of clones obtained, which is in agreement with the manufacturer's description (about 50 %). Sixteen of the 18 STEC 472-1 clones sequenced showed identity with genes of a bacteriophage of the P4 family described in *S.* Typhi CT18 (*cnr*, *crr*, *alpha*, *δ*, *psu* and *int*). Thomson *et al.* (2004) described the genetic organization of a number of different phages of the P4 family that are found in enterobacteria and according to their description the DNA sequences identified in STEC 472-1 corresponded to phage ST46 of *S.* Typhi CT18. This finding suggests that there has been horizontal gene transfer among different species of bacteria and that perhaps the phage was recently acquired by STEC 472-1 due to the high identity (over 95 %) to phage ST46.

There appear to be no previous reports indicating that phage genes of the P4 family are directly related to virulence properties, and in STEC strain 472-1 they do not seem to encode an adherence factor. However, the genetic organization of the phage includes a region described as hypervariable, located between the *cos* and *int* genes (Fig. 2a), and it has been suggested that these hypervariable regions may contain genes associated with the virulence of the strain (Thomson *et al.*, 2004). DNA sequencing and BLAST analysis of this region did not find homology with any known adherence factor; instead, five ORFs encoding potential proteins of unknown function were identified (Table 3). The best amino acid sequence identity hits were to hypothetical proteins of *Photorhabdus luminescens*, which is an enteric pathogen of insects. Recently, a set of factors shared by *Yersinia enterocolitica* and *P. luminescens* were described, including those that are involved in the host infection process, persistence within the insect, and in host exploitation. Some of these genes might be selected during the association with insects and then adapted to pathogenesis in mammalian hosts (Heermann & Fuchs, 2008). Interestingly, the adhesion to HEp-2 cells of the *psu-int* mutant strain was one order of magnitude less than that of the wild-type strain and this phenotype can be complemented with the plasmid pTrc99A-*psu-int*. Therefore, the difference in adhesion of the mutant strain compared with the wild-type and the complemented strains was significant. In previous studies, in which we analysed adherence properties of strains containing multiple adherence factors, the reduction in adherence was not significant; although we were able to identify other phenotypes related to adherence patterns or to elucidate alternative adherence factors (Torres *et al.*, 2002). Therefore, our laboratory is currently investigating what is the exact contribution of the *psu-int* hypervariable region in the adherence phenotype of STEC 472-1 and whether one or more of the ORFs in this region are directly associated with the reduction in the adhesion phenotype.

ACKNOWLEDGEMENTS

The laboratory of R. M. V. was supported by grant FONDECYT 1061088 and the laboratory of A. G. T. was funded in part by institutional funds from the UTMB John Sealy Memorial Endowment Fund for Biomedical Research. M. V. received a fellowship from Conicyt, Chile and a travel grant from the Program in Biomedical Sciences, School of Medicine, University of Chile.

REFERENCES

Bertin, Y., Boukhors, K., Livrelli, V. & Martin, Ch. (2004). Localization of the insertion site and pathotype determination of the locus of enterocyte effacement of Shiga toxin-producing *Escherichia coli* strains. *Appl Environ Microbiol* **70**, 61–68.

Beutin, L., Geier, D., Steinrück, H., Zimmerman, S. & Scheutz, F. J. (1993). Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *J Clin Microbiol* **31**, 2483–2488.

Blanco, J., Blanco, M., Blanco, J. E., Mora, A., González, E., Bernárdez, M., Alonso, M., Coira, A., Rodríguez, A. & other authors (2003). Verotoxin-producing *Escherichia coli* in Spain: prevalence, serotypes, and virulence genes of O157 : H7 and non-O157 VTEC in ruminants, raw beef products, and humans. *Exp Biol Med (Maywood)* **228**, 345–351.

Blanco, M., Schumacher, S., Tasara, T., Zweifel, C., Blanco, J. E., Dahbi, G., Blanco, J. & Stephan, R. (2005). Serotypes, intimin variants and other virulence factors of *eae* positive *Escherichia coli* strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (*eae*-2). *BMC Microbiol* **5**, 23–33.

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

Brunder, W., Khan, A. S., Hacker, J. & Karch, H. (2001). Novel type of fimbriae encoded by the large plasmid of sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157 : H–. *Infect Immun* **69**, 4447–4457.

Calia, K. E., Waldor, M. K. & Calderwood, S. B. (1998). Use of representational difference analysis to identify genomic differences between pathogenic strains of *Vibrio cholerae*. *Infect Immun* **66**, 849–852.

Caprioli, A., Nigrelli, A., Gatti, R., Zavanella, M., Blando, A. M., Minelli, F. & Donelli, G. (1993). Characterisation of verocytotoxin-producing *Escherichia coli* isolated from pigs and cattle in northern Italy. *Vet Rec* **133**, 323–324.

Caprioli, A., Morabito, S., Brugère, H. & Oswald, E. (2005). Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. *Vet Res* **36**, 289–311

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

De Azavedo, J., McWhirter, E., Louie, M. & Brunton, J. (1994). Eae-negative verotoxin-producing *Escherichia coli* associated with haemolytic uremic syndrome and hemorrhagic colitis. In *Recent Advances in Verocytotoxin-Producing Escherichia coli Infections*, pp. 265–268. Edited by M. A. Karmali & A. G. Goglio. Amsterdam, The Netherlands: Elsevier Science B.V.

Donnenberg, M. S., Tacket, C. O., James, S. P., Losonky, G., Nataro, J. P., Wasserman, S. S., Kaper, J. B. & Levine, M. M. (1993). Role of the *eaeA* gene in experimental enteropathogenic *Escherichia coli* infection. *J Clin Invest* **92**, 1412–1417

Dytoc, M. T., Ismaili, A., Philpott, D. J., Soni, R., Brunton, J. L. & Sherman, P. M. (1994). Distinct binding properties of *eae* negative verocytotoxin-producing *Escherichia coli* of serotype O113 : H21. *Infect Immun* **62**, 3494–3505.

Heermann, R. & Fuchs, T. M. (2008). Comparative analysis of the *Photorhabdus luminescens* and the *Yersinia enterocolitica* genomes: uncovering candidate genes involved in insect pathogenicity. *BMC Genomics* **9**, 40

Kaper, J. B., Gansheroff, L. J., Wachtel, M. R. & O'Brien, A. D. (1998). Intimin-mediated adherence of Shiga toxin-producing *Escherichia coli* and attaching-and-effacing pathogens. In *Escherichia coli O157 : H7 and Other Shiga Toxin-Producing E. coli Strains*, pp. 148–156. Edited by J. B. Kaper & A. D. O'Brien. Washington, DC: American Society for Microbiology.

Karmali, M. A. (1989). Infection by verocytotoxin-producing *Escherichia coli*. *Clin Microbiol Rev* **2**, 15–38.

Lucchesi, P. M., Kruger, A. & Parma, A. E. (2005). Distribution of *saa* gene variants in verocytotoxigenic *Escherichia coli* isolated from cattle and food. *Res Microbiol* **157**, 263–266.

Nicholls, L., Grant, T. H. & Robins-Browne, R. M. (2000). Identification of a novel genetic locus that is required for *in vitro* adhesion of a clinical isolate of enterohemorrhagic *Escherichia coli* to epithelial cells. *Mol Microbiol* **35**, 275–288.

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

Paton, A. W., Woodrow, M. C., Doyle, R. M., Lanser, J. A. & Paton, J. C. (1999). Molecular characterization of a shiga toxigenic *Escherichia coli* O113 : H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. *J Clin Microbiol* **37**, 3357–3361.

Paton, A. W., Srimanote, P., Woodrow, M. C. & Paton, J. C. (2001). Characterization of Saa, a novel autoagglutinating adhesin produced by locus enterocyte effacement-negative shiga-toxigenic *Escherichia coli* strains that are virulent for