The serine protease Pic as a virulence factor of atypical enteropathogenic Escherichia coli

Afonso G. Abreu, Cecilia M. Abe, Kamila O. Nunes, Claudia T. P. Moraes, Lucia Chavez-Dueñas, Fernando Navarro-García, Angela S. Barbosa, Roxane M. F. Piazza, and Waldir P. Elias

Laboratory of Bacteriology, Butantan Institute, São Paulo, Brazil; Programa de Pós-Graduação em Ciências da Saúde, Federal University of Maranhão, São Luís, Brazil; Laboratory of Cell Biology, Butantan Institute, São Paulo, Brazil; Department of Cell Biology, Centro de Investigación y Estudios Avanzados del IPN (CINVESTAV), Mexico DF, Mexico

ABSTRACT
Autotransporter proteins (AT) are associated with bacterial virulence attributes. Originally identified in enteroaggregative Escherichia coli (EAEC), Shigella flexneri 2a and uropathogenic E. coli, the serine protease Pic is one of these AT. We have previously detected one atypical enteropathogenic E. coli strain (BA589) carrying the pic gene. In the present study, we characterized the biological activities of Pic produced by BA589 both in vitro and in vivo. Contrarily to other Pic-producers bacteria, pic in BA589 is located on a high molecular weight plasmid. PicBA589 was able to agglutinate rabbit erythrocytes, cleave mucin and degrade complement system molecules. BA589 was able to colonize mice intestines, and an intense mucus production was observed. The BA589 Δpic mutant lost the capacity to colonize as well as the above-mentioned in vitro activities. Thus, Pic represents an additional virulence factor in aEPEC strain BAS89, associated with adherence, colonization and evasion from the innate immune system.

KEYWORDS
atypical enteropathogenic Escherichia coli; colonization; complement system; Pic; SPATE

Introduction
The serine protease autotransporters of the Enterobacteriaceae (SPATE) represent a large family of virulence factors, which members resemble those belonging to the trypsin-like superfamily of serine proteases. They are secreted through the type V secretion system, also known as the autotransporter pathway, and are often associated with virulence functions, such as adhesion, aggregation, invasion, biofilm formation and toxicity. SPATE members can be divided into class 1 (cytotoxins) and class 2 (lectin-like immunomodulators) groups. One important member of the class 2 is the protein involved in intestinal colonization (Pic), originally identified in cultures of enteroaggregative Escherichia coli (EAEC) and Shigella flexneri 2a. Uropathogenic E. coli (UPEC), and the hybrid EAEC/Shiga toxin-producing E. coli (serotype O104:H4) also produce Pic.

Pic presents proteolytic activity on mucin and induces mucus hypersecretion, contributing to the mucosal colonization by EAEC. Additional roles of Pic include serum resistance, hemagglutination, degradation of coagulation factor V, and cleavage of leukocyte surface glycoproteins.

We have recently shown that Pic from EAEC 042 mediates immune evasion by the direct cleavage of complement molecules, significantly reducing complement activation by all 3 pathways. Pic is able to cleave C3, a central molecule of the complement cascade, as well as C3b and proteins from the classic and lectin pathways, such as C4 and C2. Degradation of complement components was observed to purified protein and supernatant of HB101 E. coli cultures that express Pic. Additional proteolytic assays using human serum as a source of complement proteins indicated that Pic is also active in a more physiological milieu, being able to cleave C3, C4 and C2.

Recent epidemiological studies have demonstrated that atypical EPEC (aEPEC) are more prevalent than typical EPEC (tEPEC) both in developing and industrialized countries. These strains are associated with endemic diarrhea in children and diarrhea...
outbreaks. The classification of the EPEC into tEPEC and aEPEC subgroups is based on the presence and absence of plasmid pEAF, respectively. Both subgroups share a common mechanism of pathogenesis consisting in the induction of a characteristic alteration on enterocytes denominated attaching and effacing (A/E) lesion.

Since the original definition of EPEC in subgroups, additional distinctions between typical and atypical strains have been reported. In general, tEPEC strains are more homogeneous in terms of virulence characteristics, while aEPEC strains often carry genes encoding virulence factors of other diarrheagenic E. coli (DEC) pathotypes. Furthermore, phylogenetic studies indicate that aEPEC have a genetic background with characteristics that allow the acquisition, retention and expression of genes encoding virulence factors of other DEC pathotypes. One example is the presence of SPATE-encoding genes in aEPEC, which can confer adaptive advantages and additional pathogenic mechanisms to the A/E lesion.

In a previous study we detected one aEPEC strain (BA589) carrying a DNA fragment corresponding to the Pic encoding gene. In the present study we characterized the biological activities of Pic produced by BA589 both in vitro and in vivo. The virulence features of Pic produced by this particular strain denote the importance of this protein in aEPEC pathogenesis.

**Results**

**Immunodetection of Pic in the culture supernatant of aEPEC BA589**

The secretion of proteins with more than 100 kDa by the BA589 and the control EAEC 042 strains were firstly visualized by SDS-PAGE using culture supernatants (Fig. 1A). Immunoblotting with anti-Pic antibodies indicated that BA589 produces and secretes higher amounts of Pic when compared to EAEC 042 (Fig. 1B). Pic from BA589 supernatant was successfully purified by gel filtration and protein identity was confirmed by mass spectrometry (LC-MS/MS).

**Genetic characterization of BA589**

In order to localize the pic gene into the BA589 genome, BA589 was cured of its plasmids using sub-inhibitory concentrations of acridine orange. Interestingly, PCR analysis showed that the cured strains lost the pic gene (clones 12, 23, 31, Fig. 2A) and consequently, the Pic protein was not detected...
by immunoblotting (Fig. 2B). These data strongly suggest that BA589 pic is encoded in a plasmid, differently from EAEC, UPEC and S. flexneri strains, which is located in the chromosome. To further demonstrate that pic is a plasmid-encoded gene, agarose gel electrophoresis was performed after plasmid extraction. Eight bands corresponding to possible plasmids with different sizes, ranging from 1.2 to >100 kb, were detected (Fig. 3A). Southern blot analysis showed that pic in BA589 is present in a plasmid of high (>100 kb) molecular weight (Fig. 3B).

BA589 has been previously characterized as aEPEC by the eae+/bfpA-/EAF-/perABC-/stx1-/stx2- genotype,26,27 and harboring the pic and espI gene sequences.18 Further analyses by PCR for other autotransporters showed that BA589 also harbors sepA. To identify more virulence genes associated to EAEC, other 22 genes were screened by PCR and among them only astA, irp2, and pilS were detected.

**Sequencing and deletion of pic<sub>BA589</sub>**

Nine pairs of primers with overlapping regions were designed to cover the entire pic sequence in BA589 (Table 1). The corresponding amplicons were sequenced and the contigs assembled as a unique sequence corresponding to 4,119 nucleotides. The complete sequence of pic showed an identity of 99% with the same gene in EAEC 042 and S. flexneri 2a (GenBank accession numbers: FN554766.1 and AF200692.2, respectively), and was deposited in GenBank (accession no. KT362219).

In order to perform functional analyses of Pic protein in aEPEC, BA589 was mutagenized by the λ-Red system generating the BA589Δpic mutant. The absence of pic and lack of Pic production were confirmed by nucleotide sequencing and immunoblotting, respectively. Also, the growth curve of both wild type and pic mutant were similar.

**Pic<sub>BA589</sub> cleaves complement proteins, degrades mucin and mediates hemagglutination**

Recently, we have shown that Pic from EAEC 042 reduces complement activation by all 3 pathways by cleaving key complement proteins.13 To investigate whether Pic is an important virulence factor for the resistance of aEPEC to complement-mediated killing, the viability of BA589 in the presence of human serum was first evaluated. BA589 was able to resist serum bactericidal activity. Cleavage of complement molecules by Pic-secreting E. coli BA589 was then assessed. Bacterial supernatants of BA589 or BA589Δpic were incubated with purified complement molecules, and the degradation products were detected with specific antibodies. Cleavage profiles for C2 were quite similar when supernatants of BA589 and BA589Δpic were used, excepting a more intense cleavage product of approximately 70 kDa in the presence of BA589 supernatant. Cleavage fragments of C3 and C4 were clearly detected only in the presence of BA589 culture supernatants (Fig. 4). However, none of them could cleave C1q (data not shown).

Pic was also able to promote the degradation of hog stomach mucin in an enzyme assay in which mucin was coupled to biotin and adsorbed to microtiter plates. Degradation assays showed that Pic<sub>BA589</sub> at 1 mg per well was able to degrade mucin. Mucin degradation by Pic<sub>BA589</sub> and Pic<sub>042</sub> was similar to that...
produced by proteinase K (positive control). No degradation was observed by the BA589Δpic, PBS or the supernatant of a non-pathogenic strain (*E. coli* HB101) (Fig. 5).

To evaluate the hemagglutination activity of Pic, purified protein was incubated with rabbit erythrocytes. Pic was able to cause strong hemagglutination. The loss of this activity was observed when BA589Δpic supernatant was used or when anti-Pic042 antibody was previously added to the reaction.

**PicBA589 promotes intestinal colonization in animal model**

The ability of aEPEC BA589 to colonize the intestinal mucosa was evaluated using the streptomycin-treated

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**Table 1.** Primer sequences, amplified product sizes, and annealing temperatures used in sequencing and mutation of *pic* gene in BA589 strain.

| Gene | Primer sequence (5′–3′) | Annealing temp (°C) | Size of PCR product (bp) | Primer source or reference |
|------|-------------------------|---------------------|--------------------------|---------------------------|
| pic1 | GGGTATTGTCCGTTCCGAT ACAACGATACCCGCTCTCGG | 60 | 1.176 | 4 |
| pic2 | CCGGTGACACCTGCATGTAATAACGATACCGTCTGCC | 56 | 978 | This study (GenBank accession no F554766.1) |
| pic3 | GGAAGTGACGGGCCATTTG CTTGATTGACACCACACTG | 56 | 1.011 | This study (GenBank accession no F554766.1) |
| pic4 | ACAGGTTTACCTGGGTGTAGCATCAGACCATATACGGG | 56 | 1.000 | This study (GenBank accession no F554766.1) |
| pic1a | TCTGTTGATAACGTGCGG TATCAATTGACCCAGTTAC | 55 | 333 | This study (GenBank accession no F554766.1) |
| pic2b | CGACAAAATACGGCACA TGCACGGGTTGAAGGTAAC | 56 | 552 | This study (GenBank accession no F554766.1) |
| pic3b | GGGGGGAAAGAAGGTAATCT CTCATTGGACCCGGCTGCTG | 56 | 552 | This study (GenBank accession no F554766.1) |
| pic3c | ACCTGTTAAACCGTGTGTCAC GCTGGAAAGACGGTGATAT | 56 | 485 | This study (GenBank accession no F554766.1) |
| pic4c | TATCTGCTTCCCTTACGTA ATACACTCCTCCCCTACGCA | 56 | 440 | This study (GenBank accession no F554766.1) |
| Δpic | TCATTAGCTCTCAGTATTTACTCTTATATCCCT TGAACATATAGAATCCATCATATGGAATGGCTGAGCTCCCTG TATATTTAAGCTATTCGACA TCGGACGCCCTTTTACCCCACATCATATATC CTTTACCT | 50 | – | This study |

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**Figure 4.** Pic present in the supernatant of *E. coli* BA589 cleaves purified complement proteins. Supernatants of BA589 or BA589Δpic (1.0 μg of total secreted proteins) were incubated with the purified complement proteins C2, C3, or C4 (0.5 μg) for 5 hours at 37°C. The cleavage products were detected by Western blotting with specific antibodies against the complement proteins.
mouse model. BA589 was able to colonize the intestinal mucosa of mice on the 2nd day post infection and up to the 15th day. Fecal samples of colonized mice contained fluctuant amounts of bacteria, around $10^7$ to $10^{10}$. The BA589Δpic mutant was unable to colonize the intestinal mucosa of mice (Fig. 6).

Intestinal fragments were analyzed by scanning electron microscopy (SEM). BA589 was able to induce high levels of mucus production. The same level of mucus production was not observed neither on control tissue obtained from uninfected mice, nor on intestinal fragments from mice infected with BA589Δpic. Bacterial adhesion to the intestinal cells surface was observed on ileum and more intensely on cecal portions of mice infected by the wild type BA589, but not in the mice infected with the pic isogenic mutant (Fig. 7).

**Discussion**

Several studies have shown high frequency of SPATEs, including Pic, in bacterial isolates from blood, stool and urine. Boisen et al. demonstrated that 94.5% of the EAEC strains carried at least one SPATE-encoding gene, among which 63.6% harbored pic. In another study with EAEC, pic was found in 57% of the strains. On the other hand, frequency of pic or even other SPATE genes in EPEC is low.

In our EPEC collection, pic was detected in strain BA589. This strain was further characterized, confirming some aEPEC characteristics such as the localized adherence-like (LAL) pattern of adherence to HEp-2 cells and the capacity to induce the A/E lesion. These attributes were not affected by pic mutation, indicating no polar effects in the A/E lesion encoding-genes (Suppl. Fig. 1). Interestingly, our results showed that differently from EAEC 042, S. flexneri 2a and UPEC, pic from BA589 is present in a high molecular weight plasmid (> 100 kb), indicating that pic may be carried by plasmids and horizontally transferred.

A few studies have shown that SPATE genes are present in EPEC, but production of the corresponding proteins has been demonstrated only for Pet. Our data show that Pic is secreted by BA589 and displays the same biological activities of the prototype EAEC 042. The production of some SPATEs, such Pic, shows maximal expression at 37°C in alkaline pH, conditions employed in our study. Also, Pic production by BA589 was similar to EAEC 042 until 4 hours of incubation when Pic production was higher in BA589 (Suppl. Fig. 2).

PicBA589 promotes cleavage of key molecules of the complement system (C2, C3 and C4) that contributes to immune evasion of this particular aEPEC strain. Thus, by promoting serum resistance, Pic may contribute, to increase the severity of the disorders caused by these E. coli. Infection by aEPEC is restricted to the intestinal mucosa, but some studies have demonstrated the ability of some aEPEC strains to invade epithelial cells in culture, and translocate through the small intestine to mesenteric lymph nodes, spleen and liver in animal models. In this context, survival of these pathogens in the bloodstream is of great importance and requires factors that enable bacteria
to circumvent host’s complement system attack. Thus, the ability of aEPEC to produce Pic and translocate would result in an essential mechanism of immune response evasion.

We have also investigated if PicBA589 would display hemagglutination and mucinolytic activities as previously shown.4 PicBA589, but not BA589Δpic, presented hemagglutination activity against rabbit erythrocytes. Like Pic042, PicBA589 was also able to degrade mucin, but this capacity was inhibited in the presence of BA589Δpic. It is worth to mention that the ability to degrade mucin is not an attribute of all SPATEs. Beside Pic, mucin has been shown to be degraded by Tsh, EatA, Vat and TleA.4,9,10,36-39 It has been proposed that the main role of Pic in pathogenesis could be attributed to its mucinolytic activity.4,9 As described by Navarro-Garcia et al.11 and also observed in this study, Pic is secreted in the initial stage of bacterial colonization, during adhesion to epithelial cells.

Pic and SigA secreted by hybrid EAEC/Shiga toxin-producing E. coli (serotype O104:H4) were essential for colonization in rabbits.7 Pic secreted by EAEC 042 was also involved in intestinal colonization of mice treated with streptomycin.40 By using a similar approach, we were able to demonstrate that BA589 colonized the intestinal mucosa of mice treated with streptomycin for 15 days. On the other hand, mice infected with EAEC 042Δpic presented reduced levels of colonization while BA589Δpic was not able to colonize. It is important to note that EAEC 042 has factors strongly involved in colonization, such as AAF/II fimbria41 that are absent in our strain. The role of Pic in colonization has been controversial in other strains such as Citrobacter rodentium Δpic that showed an increase in colonization.36 However, it is not appropriate to compare these experiments, since animal models, bacterial inoculums, and methods used to count bacteria were different from those used in our and EAEC 04240 studies. In addition, it is necessary to use a higher number of bacterial inoculum (>1 × 10⁷ CFU/ml) in order to promote EPEC colonization in mice,42,43 corroborating data obtained in our laboratory with other strains of aEPEC lacking pic (unpublished data). In our study, although using a lower bacterial inoculum (5 × 10³ CFU/ml), BA589 was able to colonize, suggesting that Pic is a virulence factor involved in colonization by aEPEC, in this animal model.

Figure 7. Mucus production and bacterial adherence to intestine after mouse infection with wild type and mutant strains. Scanning electron micrographs of intestinal fragments (duodenum, jejunum, ileum and caecum) obtained from mice infected with the wild type strain BA589 and the mutant BA589Δpic for 15 d. In general, BA589 induced higher levels of mucus production (stars). Bacterial adhesion (arrows) to the intestinal surface was more intense on caecal portions of mice infected by the wild type BA589. Non-infected intestinal fragments were used as respective negative controls. Scale bars, 5 μm.
Analyses of different portions of the intestines collected from mice infected with BA589 and BA589Δpic showed a remarkable colonization of the wild type strain on the cecal fragment. Other intestinal portions such as the duodenum, jejunum and ileum were not colonized. It is worth noting the large amount of mucus present in sections obtained from mice infected with BA589. As previously described, Pic is able to induce intestinal mucus hypersecretion.11 These authors showed that Pic secreted by EAEC, UPEC and S. flexneri 2a stimulated an increase in the number of mucus-contained goblet cells.

Unfortunately, our numerous attempts of transformation using the minimal clone pPic,4 in order to obtain a complemented mutant, were fruitless. BA589 has at least 8 plasmids of different molecular weights (Fig. 3) that certainly belong to different incompatibility groups giving a quite restrictive status to our strain.

Taken together our data point to an essential role of Pic in intestinal colonization by aEPEC. Genetic and functional characterization of Pic BA589 indicates that this protein is highly conserved, promotes mucin degradation, causes hemagglutination, cleaves key complement molecules and, most importantly, promotes intestinal colonization in the mouse model. All these features may contribute to invasion and immune evasion of aEPEC in systemic infections. To our knowledge, this is the first description of Pic production in aEPEC, and an evidence that pic can be horizontally transferred by plasmids.

**Materials and methods**

**Bacterial strains and growth conditions**

The BA589 strain (serotype O5:H2) was isolated during an epidemiological study of the etiology of acute diarrhea in Brazil.27 EAEC strain 042 (serotype O44:H18)14 and HB101 E. coli B/K12 hybrid45 was respectively used as positive and negative controls for Pic production. Bacterial strains were aerobically grown in Luria-Bertani (LB) broth at 37°C for 18 h, unless otherwise stated. Apramycin (100 µg/ml) or chloramphenicol (20 µg/ml) were used when necessary. All strains were kept in LB broth supplemented with 15% glycerol at −80°C.

**Detection of virulence genes**

The presence of sepA and sigA encoding autotransporters and 22 genes encoding EAEC virulence related proteins (aafA, aafC, aap, aaiA, aaiG, aggABCD, agg3A, aggC, agg4A, agg4C, aggR, ap58, asiA, irp2, rfbU, pilS, pilV, shf and virK) was assessed by PCR. Primer sequences, sizes of amplified products, and annealing temperatures were previously described.18,28,31,46-49 Amplification was performed in a total volume of 50 µl containing 40 pmol of each primer; dATP, dTTP, dCTP, and dGTP (0.1 mM each); 1.5 U Taq DNA polymerase (Invitrogen); 5.0 µl 10xPCR buffer (Invitrogen); MgCl₂ (2 mM); and 2.0 µl of DNA template, obtained from a colony from culture on LB agar, boiled in 500 µl of water for 10 min.

**DNA sequencing and analysis**

Double-stranded DNA was sequenced in a MegaBACE 1000 (Amersham Pharmacia Biotech) sequencer. Reactions were performed according to the manufacturer’s instructions, using the APBiotech DYEnamic ET Dye Terminator Cycle Sequencing Kit, and primers described in Table 1. Sequence analysis was performed using the SeqManTM 4.5 software (Analysis Expert DNA Star Software, Inc. PC, Madison, WI, USA) and BioEdit Sequence Alignment Editor 7.0.5.3 (Carlsbad).

**Plasmid profile analysis**

Plasmid extraction was performed using the Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA, USA) according to manufacturer’s recommendations, analyzed by agarose gel electrophoresis (0.7%), stained in a Gel Red solution (Uniscience, Woburn, MA, EUA) and visualized under UV light. The apparent molecular weight of each plasmid was determined by comparison with plasmids of known molecular weights of E. coli 39R86150 extracted under the same conditions.

**Southern blot analysis of pic**

Plasmid extraction from BA589 was analyzed by Southern blot assays. Plasmids were resolved by agarose gel (0.7%) electrophoresis and transferred to a positively charged Hybond-N+ nylon membrane (GE Healthcare, USA). The membrane was hybridized with a DNA probe obtained by PCR amplification of pic from EAEC strain 042 (Table 1). Probe labeling,
hybridization and detection were performed using the ECL Nucleic Acid Labeling and Detection System (GE Healthcare) following the manufacturer’s instructions.

**Construction of the pic mutant**

The BA589Δpic strain was constructed using the λ Red system. Specific primers flanked by 50 nucleotides extensions homologous to the regions adjacent to the pic gene were used to amplify the chloramphenicol cassette from plasmid pKD3 (Table 1). The purified PCR product was electroporated into strain BA589 containing the λ Red recombinase plasmid pKOBEC. Transformed bacterial cells were plated and grown on LB agar containing apramycin and kanamycin at 37°C. Deletion of pic was confirmed by PCR and DNA sequencing.

**Culture supernatants and Pic production**

Culture supernatant fractions of E. coli BA589, BA589Δpic, and 042 were obtained from 100 ml of bacterial overnight cultures. After centrifugation at 12,000 × g for 10 min, supernatants were concentrated 100-fold with an Ultrafree centrifugal filter device with a 100-kDa cutoff (Millipore). Pic was purified by gel filtration chromatography using the Superdex gel filtration column - HiLoad Superdex 16/60 (GE Healthcare), resulting in a unique protein of 109 kDa, visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting using polyclonal specific antibodies and mass spectrometry (LC-MS/MS) confirmed Pic identity.

**Proteolytic activity against complement proteins**

BA589 and BA589Δpic culture supernatants (1.0 μg of total secreted proteins) were incubated for 5 h at 37°C with purified complement proteins (0.5 μg of C1q, C2, C3 or C4). The cleavage products were analyzed by Western blot using specific antibodies.

**Mucinolytic activity**

Mucinolytic activity was assessed by incubating Pic with hog gastric mucin (Sigma) followed by a colorimetric detection. Mucin was coupled to biotin, adjusted to different concentrations (0.1, 0.2, 0.4 and 0.8 mg/ml), adsorbed to microtiter plates, and exposed to 1 mg of proteinase K or Pic preparations. The absorbance was measured at 490 nm in a Multiskan Ex ELISA Reader.

**Hemagglutination**

Pic preparations were incubated with rabbit erythrocytes for hemagglutination detection, as previously described. Clumping of erythrocytes was considered as a positive result, and inhibition of hemagglutination was performed incubating Pic protein (1μg/ml) with anti-Pic antisera (diluted 1:50 in PBS) at room temperature for 30 min prior to the addition of the erythrocytes.

**Mouse colonization**

The streptomycin-treated mouse model was used to investigate the intestinal colonization by aEPEC. Twelve week-old female BALB/c mice provided ad libitum with drinking water containing 5 g/l of streptomycin from 48 h prior to the inoculation and for the duration of the experiment were used. Bacterial suspensions of BA589 and BA589Δpic were prepared at a final concentration of 5 × 10³ CFU/ml and 0.2 ml of these suspensions were administrated orogastrically by gavage. Fresh fecal samples were collected, weighted, diluted, and homogenized in sterile PBS. Serial dilutions of these preparations were then plated onto MacConkey agar containing streptomycin (100 μg/ml) for determination of CFU/ml. Bacteria were quantified by plate counts for 15 consecutive days post infection. PCR for detection of pic was also performed to confirm intestinal colonization. Fragments of tissue collected from different portions of intestines of infected mice were analyzed by SEM, as previously described, and examined in a QUANTA 250 SEM (FEI Company, Netherlands) operating at 12.5kV.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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