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Characterization of the universal stress protein F from atypical enteropathogenic *Escherichia coli* and its prevalence in *Enterobacteriaceae*

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Abstract: Atypical enteropathogenic *Escherichia coli* (aEPEC) are heterogeneous strains in terms of serotypes, adherence patterns and the presence of novel virulence factors. This heterogeneity is intriguing, promoting studies trying to characterize these novel proteins and to better comprehend this pathotype group. In a previous study analyzing low-molecular mass proteomes of four representative aEPEC strains of three different adhesion phenotypes, we classified proteins according to their annotated function, with most of them being involved in metabolism and transport; while some of them were classified as hypothetical proteins. The majority of the hypothetical proteins were homologue products of genes identified in the genome of enterohemorrhagic *E. coli*. One of the hypothetical proteins was annotated as Z2335, with orthologue in EPEC, and by bioinformatics analysis, this protein was revealed to be the universal stress protein F (UspF). Thus, herein we successfully obtained a recombinant UspF protein from aEPEC, which is a α/β, ATP-binding protein involved in stress response, with comparable protein production among the four studied strains, but showing noteworthy differences when cultivated in different stress conditions, also present in other enterobacterial species, such as *Shigella sonnei* and *Citrobacter freundii*. Furthermore, our results confirm that the Usp protein superfamily encompasses a conserved group of proteins involved in stress resistance in aEPEC and other *Enterobacteriaceae*.

Keywords: atypical EPEC; hypothetical proteins; UspF protein; cloning; expression; α/β protein; stress

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Abbreviations: aEPEC, Atypical enteropathogenic *Escherichia coli*; CAPS, N-cyclohexyl-3-aminopropanesulfonic acid; CD, circular dichroism; CFU, colony-forming unit; EAEC, enteroaggregative *E. coli*; EAF, EPEC adherence factor plasmid; ETEC, enterotoxigenic *E. coli*; LEE, Locus of Enterocyte Effacement; STEC, Shiga toxin-producing *E. coli*; UspF, universal stress protein F

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Introduction

Enteropathogenic *Escherichia coli* (EPEC) remain one the most important enteric pathogens infecting children and they are considered one of the main causes of persistent diarrhea worldwide.[1] The hallmark of EPEC pathogenesis is the ability to cause attaching and effacing (A/E) lesions, which results from intimate bacterial adhesion to the intestinal epithelium, the effacement of local microvilli, with subsequent accumulation of polymerized actin and other host cytoskeleton proteins at the site of bacterial attachment, forming pedestal-like structures.[2] A/E lesion-related genes are located in a pathogenicity island named the Locus of Enterocyte Effacement (LEE).[3,4]

EPEC strains have been categorized into two subgroups, termed typical and atypical EPEC (aEPEC) based on the presence and the absence of EPEC adherence factor plasmid (EAF).[5] The aEPEC subgroup has been considered an emerging bacterial pathogen, associated with both sporadic cases and outbreaks of diarrhea.[6] Indeed, aEPEC isolates have been associated with diarrhea in several worldwide countries, including Brazil.[7–13] The emergence of this pathogen has been intriguing and has triggered new studies in epidemiology and pathogenicity, which have been performed to characterize their virulence profiles and better comprehend this pathogroup.[6,14–28]

One of the most interesting features of aEPEC isolates is their variability in the adherence patterns upon contact with epithelial cells *in vitro*. A previous study from our group analyzed and compared the low-molecular mass proteomes of four representative aEPEC strains by 2D gel electrophoresis and LC–MS/MS, study that comprised three different adhesion phenotypes (localized-like, aggregative and diffuse) and one non-adherent isolate. We identified a total of 59 proteins, according to their annotated function, some of them were conserved in the four studied strains (Ec292/84, 9100/83, BA320, BA4013), with most of them being involved in metabolism, stress protection and transport; and some of them were still classified as hypothetical proteins. We also found that the majority of the hypothetical and filamentous proteins identified in these isolates were previously identified in the genome of enterohemorrhagic *E. coli*.[29]

One of the hypothetical proteins (Z2335, orthologue in EPEC) was revealed by bioinformatics analysis to be the multispecies universal stress protein F (UspF), also identified as the YnaF protein found in soluble *E. coli* K12 extracts, which has conserved an ATP-binding site.[30] The orthologue protein UspF in *Salmonella spp* is designated YnaF.[31] Universal stress proteins (Usp) are widely spread proteins in nature. Usp proteins belong to the PF00582 superfamily (COG0589).[32] The universal stress protein (Usp) superfamily represents a group of small cytoplasmic proteins whose expression is affected by a wide variety of internal or external stresses. As example, UspA is involved in protection of DNA from UV damage. In *S. enterica* serovar Typhimurium UspA is important for resistance to metabolic and oxidative stress and other types of stresses, like starvation, and the protein UspA contribute to pathogenicity of *S*. Typhimurium.[33]

Also, in *Mycobacterium smegmatis* the Usp Rv1636 was isolated and characterized, this protein binds to cAMP specifically with high affinity and to ATP with lower affinity.[34] In *M. tuberculosis*, proteomic analysis revealed that an increase in protein levels of mycobacterial Usp causes an increase in KatG protein levels, in turn increasing phenotypic susceptibility to isoniazid which is a first line drug for the treatment of active and latent tuberculosis.[35] The UspA protein plays a significant role in protecting *Acinetobacter baumannii* from H_2O_2, low pH, and the respiratory toxin 2,4-dinitrophenol. In a mouse model of pneumonia, UspA is essential for *A. baumannii* pneumonia pathogenesis.[36]

Because in aEPEC no further information about Usp family proteins is available, the aim of the present work was to investigate the function and the prevalence in enterobacterial isolates of the hypothetical protein (Z2335). Thus, *uspF* gene was cloned and the UspF protein was expressed and purified. Herein, we successfully obtained a recombinant UspF protein from aEPEC, which is a α/β, ATP-binding protein, involved in stress responses, with no production differences among the four studied aEPEC strains, but showing significant differences when cultivated in diverse stress conditions. Furthermore, the high prevalence of this protein among the enterobacterial species strength its universal function.

Results

Protein prediction by computational analysis

The *uspF* gene (435 bp) encodes a predicted protein of 168 amino acids, but the signal peptide was not detected by analysis with the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The computational analysis, based on the presence of conserved structural and functional domains,[37] revealed that UspF belongs to the Universal Stress Protein Family. This analiysis showed high similarity values with universal stress proteins of *E. coli*, *Shigella* and *Salmonella* (Table I).

Recombinant multispecies universal stress protein F

The recombinant UspF protein was expressed in *E. coli* BL21 (DE3) pLyS as a cytosolic protein (HT-

Table I. *Identity Analysis of Multispecies UspF With Other Proteins Found in the BLASTp Analysis*

Protein	Gene identification	Multispecies UspF (*Shigella*)
Multispecies UspF (*Shigella*)	gi | 15801753	100%
Putative Filament Protein (*S. flexneri* 2a str. 2457T)	gi | 30062868	100%
Universal Stress Family Protein (*E. coli* MS 69-1)	gi | 301020540	99,4%
Filament Protein (*E. coli* O157:H7 str. Sakai)	gi | 38703971	85,7%
Stress-induced ATP-binding protein (*E. coli* str. K-12)	gi | 89108222	85,1%
Putative Universal Stress Protein (*Salmonella enterica* serovar Tennessee str CDC07-0191)	gi | 238911963	78,6%
Hypothetical Protein (*Salmonella enterica* serovar Paratyphi B str SPB7)	gi | 161613879	78%

UspF) fused at the N-terminal with a His-tag and an additional sequence (20 aa) defining thrombin cleavage site, resulting in protein yields of approximately 100–150 mg/L [Fig. 1(A)]. The protein was purified in a single step after elution of bound protein from loaded resin with imidazole containing buffer at concentrations ranging from 30 to 200 m*M* [Fig. 1(B)]. The purified protein remained completely soluble at high concentrations (20–30 mg/mL) even after prolonged storage at 4°C.

Prediction of secondary structure in different conditions

Further evidence that the recombinant HT-UspF preserved the structure of the native multispecies UspF was obtained by determination of circular dichroism analyses (CD) of the recombinant protein at different pH values. The CD spectrum of HT-UspF (Fig. 2) is characteristic of a α/β protein with minimum at 208 and 222 nm. The CD spectra show that the protein UspF is more stable in neutral and basic pH, but in acid pH a structural loss occurs.

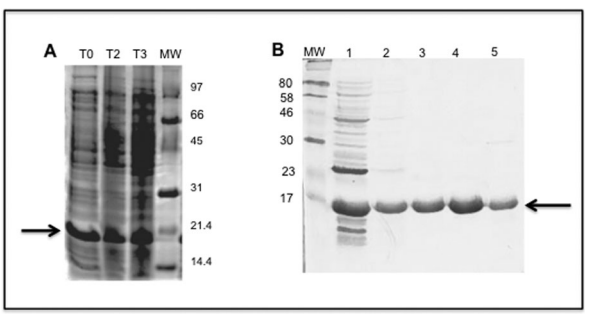

Figure 1. Expression of UspF protein by an IPTG-inducible *E. coli* BL21 (DE3) pLyS strain and purification of soluble fractions containing UspF protein. **A** – T0 total protein extract before induction; T2, total protein extract after induction (3 h); T3, total protein extract after induction (16–18 h). The arrow indicates position of the UspF protein (18.4 kDa). **B** – Purification of soluble fractions or UspF protein by affinity chromatography using a nickel-containing resin. 1–Flow through; 2–30 m*M*, 3–50 m*M*, 4–100 m*M*; 5–200 m*M* of imidazole.

UspF protein three-dimensional model

The UspF three-dimensional model was generated with the structural coordinates from putative filament protein/universal stress protein of *K. pneumoniae* (PDB code 3fdx), the orthologues that had the highest sequence identity (76% over the complete sequence of the mature protein) [Fig. 3(A,B)]. The quality of the model is expressed by Z score and showed per residue in Figure 3(C). In the model, it was verified that the protein could bind ATP [Fig. 3(B)], similar to UspG protein that belong to the same sub-family.

UspF is involved in stress responses

Therefore, we investigate if the UspF protein was expressed at different serotypes of atypical EPEC (Ec292/84, 9100/83, BA320, BA4013) in different stress conditions. Immunoblotting assay of the heat-extracted proteins of the strains showed that UspF

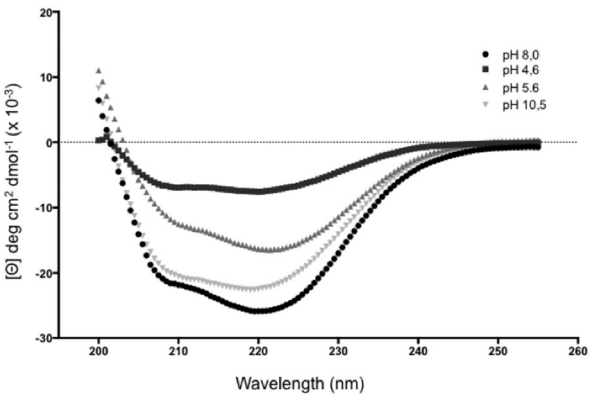

Figure 2. Circular dichroism analyses of the UspF recombinant protein in different pHs. 20 m*M* phosphate buffer at pH 8.0, 5 m*M* sodium acetate pH 4.6, 5 m*M* sodium citrate pH 5.6 and 5 m*M* of CAPS pH 10.5.

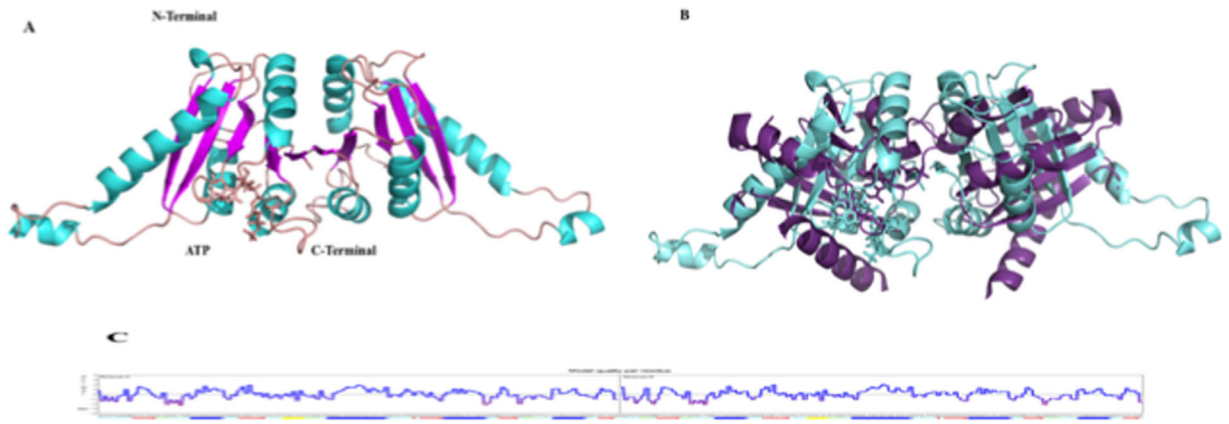

Figure 3. Model of multispecies UspF. **A**. Schematic representation of the multispecies UspF, ATP is represented in stick. **B.** Multispecies UspF superimposed with the PDB putative filament protein/universal stress protein F of *K. pneumoniae* (3fdx), ATP is represented in stick. **C.** Overall quality of the predicted UspF model.

was detected similarly in all strains and in different stress conditions, such as oxidative stress, low pH, high salt concentration and heat (Fig. 4). In addition to this qualitative analysis; we also performed a survival assay, in which colony-forming units (CFU) were counted after stressors exposure. The results showed that differences in grow patterns of the strains in different stress conditions. In presence of 3 M NaCl and H_2O_2, all strains growth was decreased, except in BA4013, where H_2O_2 stimulated it. The growth for BA4013 and 9100/83 strains was also impaired in low pH. On the other hand, change in temperature only decreased the growth of BA4013 (Fig. 5). The presence of UspF indicates the potential role of this protein in resistance and survival of strains in response to adverse conditions.

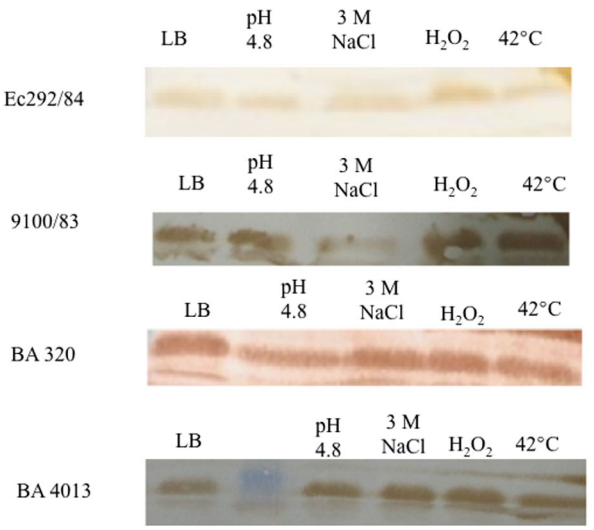

Figure 4. Bacterial lysates from strains Ec292/84, 9100/83, BA320 and BA4013 submitted or not to stress conditions. LB; LB pH 4.8; 3 M NaCl; 0.045% of H_2O_2; LB at 42°C (10 μg) were separated by SDS-PAGE (12,5%) and transferred to a nitrocellulose membrane. Nitrocellulose membranes were incubated with anti-UspF rabbit polyclonal serum followed by goat anti-rabbit IgG peroxidase-conjugate. Immunodetection signals were visualized by addition of DAB/H_2O_2.

Presence of the gene as a multispecies universal stress protein F

The *uspF* gene presence was detected by PCR in the majority of analyzed pathotypes, with some exceptions. The gene was 100% present either in pathogenic bacteria such tEPEC and enteroaggregative *E. coli* (EAEC), or in non-pathogenic bacteria, e.g., in *E. coli* isolates that do not carry virulence factors found in diarrheagenic *E. coli*. In Shiga toxin-producing *E. coli* (STEC) and enterotoxigenic *E. coli* (ETEC) isolates, the gene was present at 95.6 and 91%, respectively. Half of the tested aEPEC isolates presented the gene and in other enterobacterial species, the presence of *uspF* gene was less frequent (33.3%), herein detected only in *S. sonnei* and *C. freundii* (Table II).

Discussion

Different proteomic studies on *E. coli* have been used to compare and to identify proteins differentially expressed in tEPEC E2348/69 versus EHEC EDL933 strains[38]; pathogenic versus commensal *E. coli* strains[39]; tEPEC versus aEPEC[25] and different strains of aEPEC.[29] The proteins identified in our previous data[29] consisted of the outer membrane protein OmpX, caseinolytic protease, chain A of the phosphocarrier protein and structure–function of iron superoxide dismutase, described before in *E. coli* without strain specification. Further, a flavoprotein, Trp repressor-binding protein, glucose-specific enzyme IIA component of PTS and the iron-containing superoxide dismutase were designated in *E. coli* K12 strain.

Concerning proteins previously described in pathogenic *E. coli*, we identified in aEPEC different 50S ribosomal proteins, the alkyl hydroperoxide reductase subunit C, autonomous glycyl radical

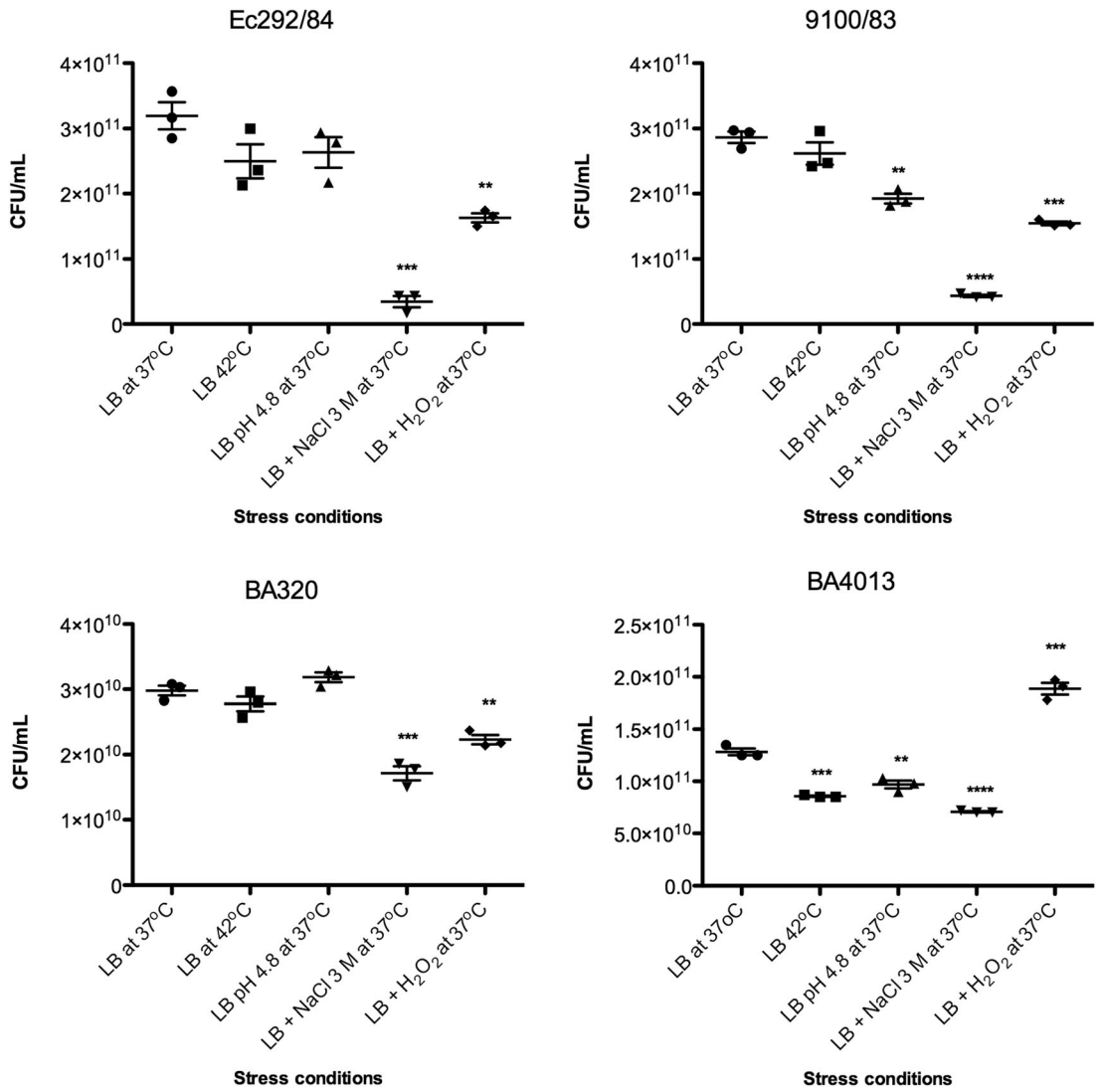

Figure 5. Stress survival assay. After stressors exposure the aEPEC strains: Ec292/84, 9100/83, BA320 and BA4013 were plated on LB agar and then the CFU were counted. The CFU values from triplicates of three independent experiments were analyzed by Graph Prism® 5.01, using unpaired Student's *t*-test. Differences were statistically significant compared the strains incubation in LB at 37°C. **** ($P < 0.0001$); *** ($P = 0.0002$ to 0.0008); ** ($P = 0.002$ to 0.0034) or non-significant.

cofactor, caseinolytic protease, DNA starvation/stationary phase protection protein, DNA-binding transcriptional dual regulator H-NS, ferritin, galactose-binding transport protein, the hypothetical proteins Z0175, Z2335 and Z3776, KHG/KDPG aldolase, universal stress protein A and D, stringent starvation protein A, putative transport protein, a protein of the fucose operon, peptidase E; also a putative filament protein defined in enterohemorrhagic *E. coli* O157:H7 EDL933 and in Sakai strains. Further, hypothetical

Table II. *Presence of* UspF *(%) Gene in Bacterial Isolates Detected by PCR*

Pathotypes	No. of bacterial isolates	*uspF* gene presence	Gene presence (%)	Total
aEPEC	72	43	59.7	43/72
tEPEC	37	37	100	37/37
STEC	46	44	95.6	44/46
ETEC	11	10	90.9	10/11
EAEC	10	10	100	10/10
NVF *E. coli*	6	6	100	6/6
Enterobacterial species	6	2	33.3	2/6

aEPEC = atypical enteropathogenic *E. coli*; tEPEC = typical enteropathogenic *E. coli*; STEC = Shiga-toxin producing *E. coli*; ETEC = enterotoxigenic *E. coli*; EAEC = enteroaggregative *E. coli*; NVF *E. coli* = non-DEC virulence factors *E. coli*.

proteins c1034, c2185 and c4636, dihydropteridine reductase, ribose-5-phosphate isomerase A previously described in uropathogenic *E. coli* CFT073 strain were identified. The inorganic pyrophosphatase described in tEPEC O127:H6 E2348/69 strain, the molybdenum cofactor biosynthesis protein described in atypical EAEC 101-1 strain and the hypothetical protein O2ColV76 described in a bird pathogenic *E. coli* strain A2363.

In this previous work from our group, we described the first low-molecular mass comparative proteomic study of extracted proteins from four representative aEPEC isolates. After fimbrial extraction, we sought that analyzing low-molecular proteins one can found some fimbrial adhesin involved in the *in vitro* interaction between bacteria and cell lines, but we observed proteins usually involved in cell structure, protection, metabolism, transport, as well as in gene regulation.[29] One of the identified proteins was annotated with the hypothetical name of Z2335 orthologue in EPEC, and by bioinformatics analyses we observed that this protein belongs to the UspF. The annotation has changed recently and now the protein (NP_287771.1) in PubMed is denominated the multispecies universal stress protein F (*Shigella*). The alignment of the multispecies UspF (*Shigella*) with UspF of *E. coli* K12 showed a high identity (99.3%). No signal peptide was observed by using signal 4.1 P server, although a 24 residues in the N-terminal region of the Z2335 sequence does not seems to be part of the codifying sequence as observed by the ORF finder program.

Herein, for the first time a hypothetical protein from aEPEC was successfully cloned, expressed, purified and characterized. The newly characterized UspF preserved the structure of the native multispecies UspF as a α/β and it is an ATP binding protein. The production of this protein was detected using a specific rabbit serum in the four studied strains in diverse stress conditions, which indicates its potential role in resistance and survival of the strains in response to adverse conditions.

Our result emphasizes previous data showing that the Usp protein superfamily encompasses a conserved group of proteins involved in stress resistance, these proteins promote cell survival during prolonged exposure to stress and may activate a general mechanism for stress endurance. UspA in *Salmonella* was showed causing resistance to oxidative stress.[33] Cells of *E. coli* BL21 harboring a SbUSP gene from *Salicornia brachiata*, an extreme halophyte, showed about 1.5- to 1.8-fold increased stress tolerance (1.8 and 1.4-fold for NaCl and KCl, respectively, and 1.5-fold for osmotic stress) compared to control *E. coli* BL21-DE cells and cells expressing GST only.[40] *Salmonella* needs to enter in host organism, and therefore, the bacteria is exposed to a hostile environment with low pH, lack of oxygen and need to survive to the immune response of the host, so the tolerance to diversity of stress in *Salmonella* is probably mediated by Usps proteins (UspA, UspE, UspF).[41]

Indeed, the stress assays showed that when the strains were exposed to different conditions, such as oxidative, temperature, osmolarity and low pH, significant changes in growth were observed, mainly increase with osmolarity and oxidative. It's worth to mention that when the aEPEC strains were cultivated 24 h in those stressors conditions, the growth was completely abolished (data not shown). These data suggest that this UspF is important for maintenance of aEPEC in adverse conditions. Indeed, Usps are among the most highly induced genes when bacteria are subjected to several stress conditions, such as heat shock, nutrient starvation or the presence of oxidants or other stress agents.[42] Therefore, expression of this protein is plausible and important for bacterial stress resistance.[32,43–47]

Here, we showed a high prevalence of UspF either in commensal or in the different *E. coli* pathotypes, which reinforces that the UspF presence might be important for *E. coli*. By circular dichroism it was verified that the protein is more stable in basic pH, which is in agreement with the intestine pH, where aEPEC strains colonize humans. In conclusion, a hypothetical protein from aEPEC was characterized showing that it is UspF which preserved the structure of the native multispecies UspF as a α/β and ATP binding protein which is involved in bacterial stress.

Material and Methods

Bacterial strains and plasmids

The following *E. coli* K12 strains were used: DH10b (Stratagene, USA) and BL21 (DE3) pLyS (Novagen, USA). The plasmid vector pET28a (Novagen, USA) and the pGEM-T Easy Vector System kit (Promega, USA) were used in order to construct the pGEM_uspF and pET28a_uspF plasmids, respectively. Bacterial isolates used in this study consisted of four atypical EPEC strains presenting different adhesion patterns, i.e., Ec292/84, 9100/83, BA320 and BA4013.[29] Also, a collection of different bacterial pathogroups were analyzed for the presence of the *uspF* gene, i.e., typical EPEC (tEPEC), atypical EPEC (aEPEC), ETEC and STEC,[17,48–51] as well as other *Enterobacteriaceae* isolates, including *Morganella morganii*, *Klebsiella pneumoniae*, *Shigella boydii*, *Proteus mirabilis*, *Salmonella* spp., and *Citrobacter freundii*. Further, groups of *E. coli* isolates that do not carry virulence factors found in diarrheagenic *E. coli* and belonging to our bacterial collection were also analyzed.

Computational analysis

The nucleotide sequence and corresponding amino acid sequence of UspF protein [*E. coli* O157:H7 strain EDL933] and gene (Gene ID: 961019) were retrieved from gene bank (Accession No. NP_287771.1). Search of orthologs sequences were carried out using BLASTP, available at the National Center of Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST) and ClustalW (www.ebi.ac.uk/clustalw/). Prediction of signal peptide and transmembrane sequences were determined with SignalP and DAS programs, respectively (http://www.cbs.dtu.dk/services/SignalP/) and (http://www.sbc.su.se/~miklos/DAS/). Protein parameters of UspF were calculated applying programs available at the Expasy Bioinformatics Portal (http://www.expasy.org/).

Cloning of the gene that encodes the multispecies uspF gene

The nucleotide sequence (gi|16445223:2115267-2115773) (without the first 72 base pairs, as indicated by ORF finder program) was amplified by PCR (forward primer 5' GGA TCC ATG AAC AGA ACG ATT CTT GTC C 3' and reverse primer 5' AAG CTT TCA GCG CAC AAC CAG CAC 3') using Platinum Taq High Fidelity (Invitrogen) and standard amplification conditions: an initial step at 95°C for 5 min, 95°C for 1 min; followed by 30 cycles at 55°C for 1 min, at 68°C for 2 min; and followed by a final extension at 68°C for 10 min. The forward primer included a *Bam*HI site, and the reverse primer a *Hind*III site (underlined). The resulting amplified fragment, with a total length of 435 nucleotides, was first cloned into the vector pGEM T-Easy Vector (Promega).

After transformation into *E. coli* DH10b cells and screening of recombinant colonies, a recombinant plasmid, named pGEM_uspF, was selected, amplified, and cleaved with *Bam*HI and *Hind*III enzymes (Invitrogen) to release the 435 bp fragment, which was purified in agarose gels and subsequently cloned into the expression vector pET28a (Novagen), previously treated with *Bam*HI/*Hind*III. Transformation efficiencies of approximately 10^7 CFU/log DNA were routinely achieved with chemically competent *E. coli* DH10b cells. One recombinant colony, selected out of 10 chosen colonies, was subjected to restriction analysis and nucleotide sequencing. The recombinant plasmid, named pET_UspF, was further purified and transformed into the *E. coli* BL21 (DE3) pLyS strain (Novagen). One recombinant clone was chosen at random among the recombinant colonies and selected for further analysis for protein expression and purification. The recombinant UspF protein was expressed as a His6-tagged cytoplasmic protein genetically fused at the N-terminal end (HT-UspF).

Expression and purification of HT-UspF recombinant protein

Cultures of the recombinant *E. coli* BL21 (DE3) pLyS strain-carrying pET_UspF were grown aerobically in Erlenmeyer flasks containing LB medium with 50 μg/mL kanamycin until mid-log phase (OD600 0.4–0.6) before adding the inducer (0.1 m*M* IPTG). The cultures were induced aerobically (200 rpm) either, for 4 h at 37°C. Cells were collected by centrifugation and stored at −20°C for approximately 16 h before cell extracts preparation.

Cell pellets from 1 L of bacterial culture were resuspended in 10 mL in 20 m*M* Tris–HCl, pH 8.0, containing 500 m*M* of NaCl, 0.5 m*M* of PMSF and 20 m*M* of imidazole and incubated with lysozyme (final concentration of 100 μg/mL) for 30 min on an ice bath. Cells were maintained on ice and disrupted by sonication after 4 pulses of 20 s in a cell disruptor (Bandelin) with 30% amplitude, followed by centrifugation at 12,000*g* for 30 min, in order to obtain the soluble and non-soluble cellular fractions. The HT-UspF protein was purified from soluble protein extracts after addition of a nickel-charged Sepharose (ProBond, Invitrogen) slurry (1 mL of resin for 15 mg of total protein) previously washed with two volumes of water and one volume of 20 m*M* Tris–HCl or NaCl, pH 8.0, containing 500 m*M* of NaCl, 0.5 m*M* of PMSF and 20 m*M* of imidazole. The charged resin was transferred to a plastic column and washed with 10 volumes with 20 m*M* Tris–HCl, pH 8.0, containing 500 m*M* of NaCl, 0.5 m*M* of PMSF and 20 m*M* of imidazole followed by washing with three volumes of 20 m*M* Tris–HCl or NaCl, pH 8.0, containing 500 m*M* of NaCl, 0.5 m*M* of PMSF and 30 m*M* of imidazole.

The bound HT-UspF was eluted with buffers containing increasing imidazole concentrations (50 m*M*, 100 m*M*, 200 m*M*, 500 m*M*). Eluted HT-UspF fractions were dialyzed with 20 m*M* Tris–HCl pH 8.0 and 50 m*M* NaCl. Samples were concentrated with Ultrafree MWCO 10,000 centrifugal filters (Amicon Millipore) to a final concentration of 15 mg/mL. The eluted protein fractions were analyzed by 12% SDS–PAGE gels.[52]

Prediction of secondary structure in different conditions

All experiments were carried out using a JASCO J-810 spectropolarimeter equipped with a Peltier-type temperature controller and a thermostatic cell holder, interfaced with a thermostatic bath. CD spectra were recorded using 0.1 cm path length quartz cells at a protein concentration of 0.25 mg/mL (10 μM). The protein stability was determined at four different pHs (25 m*M* sodium acetate at pH 4.6; 5 m*M* sodium citrate at pH 5.6; 20 m*M* sodium phosphate at pH

8.0, and 5 m*M* N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) at pH 10.5.

Polyclonal antibody

Polyclonal serum was obtained from a New Zealand white female rabbit (60 days old) which was immunized intramuscularly three times at 2-week intervals with a dose of 200 μg of UspF recombinant protein adsorbed to 2.5 mg alum (Al^{3+}) as adjuvant. Serum was obtained 45 days after immunization. Immune serum reactivity was tested by indirect ELISA. Serum samples were obtained in order to be used as negative control in specific antibody evaluation, just before immunization by auricular-venom method. The experiments were conducted in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College of Animal Experimentation, and they were approved by the Ethical Committee for Animal Research of Butantan Institute (571/09).

Stress assays and heat extracted proteins analyses

The aEPEC strains: Ec292/84, 9100/83, BA320 and BA4013, were grown 16–18 h on LB media. Thus, the bacterial cultures concentrations were adjusted spectrophotometrically (600 nm) to 4 x 10^9 CFU. The cultures were pelleted by centrifugation at 3,000 *g* for 10 min, and then the pellets were resuspended in media (LB, LB pH 4.8, LB with 0.045% H_2O_2, LB with 3 M NaCl),[33,44] and submitted to 30 min of stress conditions at 37°C. Also, one tube containing only LB was placed at 42°C for the same time. After that bacterial cultures were diluted and plated onto Luria Bertani (LB) agar plates. The number of bacteria was determined by counting the CFU.[53] The CFU values from triplicates of three independent experiments were analyzed by Graph Prism® 5.01, using unpaired Student's *t*-test. The differences were considered statistically significant when $P \leq 0.05$.

For protein heat extraction, after the bacteria was submitted to the above mentioned stress conditions, the samples were incubated at 60°C for 30 min, and then pelleted by centrifugation at 3,000*g* for 10 min. The supernatant was transferred to a new tube, SDS-sample buffer was added, and samples were boiled at 100°C for 10 min. The samples were separated in 12.5% SDS-PAGE gels and immunoblotting was performed using the anti-UspF polyclonal antibody.

Presence of the uspF *gene in different bacterial pathotypes*

The presence of *uspF* gene was investigated by PCR reaction in a collection of different bacterial pathotypes. The PCR reaction was performed using the primers sequence (forward 5' GGA TCC ATG AAC AGA ACG ATT CTT GTC C 3' and reverse 5' AAG CTT TCA GCG CAC AAC CAG CAC 3') using Taq recombinant enzyme (Invitrogen) and amplified using standard conditions: an initial step at 95°C for 5 min, 95°C for 1 min; followed by 30 cycles at 55°C for 1 min, 72°C for 2 min; and a final extension at 72°C for 10 min. The gene amplification in different bacterial isolates was analyzed in agarose gels staining with Gel Red (Biotium).

Modeling

The structural model of the UspF protein was constructed using the Yasara software. The pdb used to construct the model was the 3fdx (Putative filament protein/universal stress protein F of *Klebsiella pneumoniae*). For model validation, the Yasara used the WHAT CHECK Program.[54]

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