

The ferric aerobactin receptor *IutA*, a protein isolated on agarose column, is not essential for uropathogenic *Escherichia coli* infection¹

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Although many proteins have been described involved in *Escherichia coli* colonization and infection, only few reports have shown lectins as important components in these processes. Because the mechanisms underlying *E. coli* colonization process involving lectins are not fully understood, we sought to identify the presence of other non-described lectins in *E. coli*. Here, we isolated a 75-kDa protein from *E. coli* on Sepharose column and identified it as ferric aerobactin receptor (IutA). Since IutA is controversially associated with virulence of some *E. coli* strains, mainly in uropathogenic *E. coli* (UPEC), we evaluated the presence of *iutA* gene in UPEC isolated from patients with urinary infection. This gene was present in only 38% of the isolates, suggesting a weak association with virulence. Because there is a redundancy in the siderophore-mediated uptake systems, we suggest that IutA can be advantageous but not essential for UPEC.

Descriptors: Uropathogenic *Escherichia coli*; Siderophores; Virulence.

¹ Supported by FAPESP (grant 06/04482-6)

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O receptor de aerobactina *IutA*, uma proteína isolada em coluna de agarose, não é essencial para a infecção por *Escherichia coli* uropatogênica

Apenas alguns relatos na literatura demonstram que lectinas são importantes nos processos de colonização e infecção por *Escherichia coli*. A falta de compreensão clara dos mecanismos envolvendo lectinas, no processo de colonização por *E. coli*, motivou a realização deste estudo para se identificar a presença de outras lectinas não descritas em *E. coli*. Neste trabalho, isolou-se uma proteína de 75kDa de *E. coli* em coluna de Sepharose, correspondente ao receptor de aerobactina férrica (*IutA*). A associação de *IutA* com virulência de cepas de *E. coli* é controversa, principalmente em *E. coli* uropatogênica (UPEC), o que levou a se avaliar a presença do gene *iutA* em UPECs isoladas de pacientes com infecção urinária. O gene estava presente em 38% dos isolados, sugerindo fraca associação com virulência. Devido à existência de redundância nos sistemas de captura de ferro, sugere-se, aqui, que *IutA* possa ser vantajosa, mas não essencial para UPEC.

Descritores: *Escherichia coli* Uropatogênica; Sideróforos; Virulência.

El receptor de aerobactina *IutA*, una proteína aislada en columna de agarosa, no es esencial para la infección por *Escherichia coli* uropatógena

La falta de una clara comprensión de los mecanismos de participación de las lectinas en el proceso de colonización por *Escherichia coli*, nos motivó a identificar la presencia de otras lectinas que no han sido descritas en *E. coli*. En este estudio, se aisló una proteína de 75kDa de *E. coli* en una columna de Sepharosa, correspondiente al receptor de aerobactina (*IutA*). La asociación de *IutA* con cepas virulentas de *E. coli* es controvertido, especialmente en *E. coli* uropatógena (UPEC), lo que nos llevó a evaluar la presencia del gen *iutA* en UPECs aisladas de pacientes con infección urinaria. El gen estaba presente en 38% de los aislamientos, lo que sugiere una débil asociación con la virulencia. Debido a la existencia de redundancia en los sistemas de captura de hierro, se sugiere que *IutA* puede ser una ventaja, sin embargo no es esencial para la UPEC.

Descriptores: *Escherichia coli* Uropatogénica; Sideróforos; Virulencia.

Introduction

The intestinal microbiota of mammals includes several species of bacteria⁽¹⁾, which generally establish a relationship of mutualism with the host. The first step in the process of colonization of a host is the attachment of the colonizing agent on the epithelium of the colonized organism⁽²⁾. In Gram-negative bacteria, a wide variety of molecules or structures can function as adhesins, which promote better adhesion to the epithelial cell receptor⁽³⁾.

E. coli is the most abundant of all microorganisms that colonize the gut of many animals, and rarely causes

disease except in immunocompromised hosts or when the gastrointestinal barriers are broken. The role of adhesins and lectins in the symbiosis of *E. coli* with the host is not well known, in contrast to pathogenic *E. coli*, which has some fimbriae associated with virulence. After the pioneer work⁽⁴⁾ that obtained the first direct evidence that *E. coli* contains lectin, some authors demonstrated that proteins with lectin property, such as type 1 and P fimbriae, were determinant virulence factors important to the process of colonization and infection, especially of the bladder epithelium. The type 1 fimbria is one of the

most studied virulence factor of *E. coli*, present in 80% of UPEC strains⁽⁵⁾. Besides type 1 and P fimbriae, F17 fimbria was described as important to the pathogenesis of enterotoxigenic *E. coli* in ruminants⁽⁶⁾.

Since lectins have shown to be such important components in the colonization and infection processes by bacteria and because some mechanisms underlying *E. coli* virulence are not fully understood, we sought to identify other non-described lectins as molecular markers.

Molecular markers from pathogens have been useful to hasten diagnosis, prevention and treatment for many infectious diseases. In urinary tract infection (UTI), studies using molecular methods have shown to be essential to understand the epidemiology, because the background rate of UTI is so high, and many different organisms can be the ethiological agent of these infections. Furthermore, the bacteria that most commonly cause UTI are *E. coli* strains⁽⁷⁾, which comprise an extremely heterogeneous group⁽⁸⁾. UTI is a public health problem that requires the development of an effective treatment for prevention⁽⁹⁾. So, an understanding of the principles that guide such studies about molecular markers is essential for nurses in all areas of clinical practice, since these professionals are responsible for the planning, assessment and implementation of such strategies, leading to more effective assistance for patients and community.

In this work, we identified an agarose-binding protein of 75-kDa in *E. coli* as a candidate for a new protein with lectin-activity, with a putative role in *E. coli* virulence. This protein was identified as the precursor of the ferric aerobactin receptor (*IutA*), a siderophore receptor encoded by a gene from the plasmid pColV-30⁽¹⁰⁾, which is associated with virulence of some *E. coli* strains⁽¹¹⁾. We also evaluated the presence of *iutA* gene in UPEC strains to correlate with bacterial infection.

Methods

Isolation of carbohydrate-binding protein from *E. coli*

UPEC isolate (kindly provided by Dr. Ebert Hanna, Invent Biotecnologia Ltda, Ribeirão Preto, Brazil) was cultured in Dulbecco's modified Eagle medium (DMEM)⁽¹²⁾ in house at 37°C, shaking at 200 rpm overnight. In order to evaluate the influence of ions in the production of bacterial proteins, the DMEM was supplemented with 22 mM KH₂PO₄, 10 mM CaCl₂, 0.25 mM Fe(NO₃)₃ or 22 mM

KH₂PO₄ plus 0.25 mM Fe(NO₃)₃⁽¹²⁾. Cells were harvested by centrifugation (10,000 × g for 15 minutes at 4°C), resuspended in 25 mL of TBS-azide (Tris-buffered saline solution containing 10 mM CaCl₂, 2% (v/v) Triton X-100 and 0.02% (m/v) sodium azide, 100 mM MOPS, pH 7.4) and sonicated on ice with 5 pulses of 30 seconds each, 60 Hz (Vibra-cell, Sonics & Materials Inc., Danbury, USA). Bacterial lysates were centrifuged at 10,000 × g for 15 minutes at 4°C. The lysate supernatants were submitted to two processes: a. serial incubation with Sepharose CL-4B and carbohydrate-immobilized beads (d-galactose, d-mannose, d-GlcNAc and lactose) for 1 hour at room temperature. After washing the beads with TBS to remove non-absorbed material, the beads were submitted to SDS-PAGE. b. Affinity chromatography on column of Sepharose CL-4B, bed volume of 1 mL, by using ÄKTA Purifier System (GE Healthcare Life Sciences, Uppsala, Sweden). The material adsorbed to the column was eluted with 1 M NaCl or 0.1% acetic acid. Chromatography was monitored by absorbance at 280 nm and the eluted fractions were collected in pools, concentrated and dialyzed against TBS, using Amicon centrifugal filters Ultra-15 (membrane exclusion size of 10-kDa) (Millipore, Billerica, USA), according to manufacturer instructions. Concentrated eluates were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and the gels were stained with Coomassie blue.

Identification of proteins

Coomassie blue-stained proteins were excised and trypsinized in the gel prior to analysis by 1D nLC-MS-MS, using a 4000 QTrap (Applied Biosystems) tandem MS system (Fingerprints Proteomics Facility, University of Dundee). The raw QTrap data were analyzed using Mascot (www.matrixscience.com).

Polymerase chain reaction (PCR) of *iutA* gene in UPEC strains

UPEC strains, which were isolated from urine of hospitalized patients, were collected in the Emergency Unit of the University Hospital of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, in the period from July to September 2009. These strains were submitted to PCR to detect *iutA* gene. The plasmid pPOC9-*iutA*, kindly provided by Dr. Michael O'Connell, Dublin City University, Ireland⁽¹³⁾, was used as positive control. The PCR assays was carried out with 20 µL of

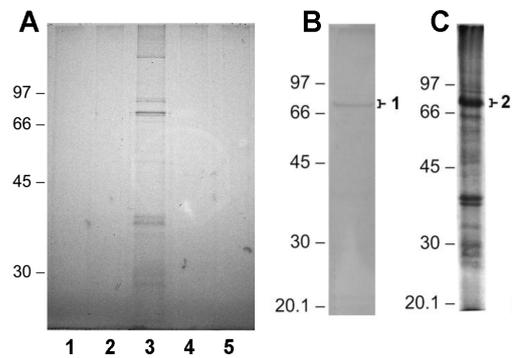
reaction mixture, containing 1 colony of each tested strain, 1,25 μM of the primers 5'-AAATTCATATGATGATAAGCAAAAAG-3' and 5'-TTCAAGCTTTCAGAACAGCACAGTAG-3', 0.2 mM of each deoxynucleoside triphosphate, 10X PCR buffer, 1.5 mM MgCl_2 , 2 U of Taq DNA polymerase (Fermentas, Burlington, Canada), performed in an *Eppendorf Master Cycler Gradient Thermocycler* (Eppendorf Scientific, Inc., Westbury, USA), as follows: 94°C for 5 min, 30 cycles at 94°C for 1 minute, 51°C for 1 minute and 72°C for 2.5 minutes and, at the end of these 30 cycles, 72°C for 5 minutes. Reactions were kept at 4°C until use. PCR products were analyzed on 1% agarose gel after ethidium bromide staining and the amplicons were identified based on the size of the amplified product.

This work was approved by the Ethics Committee of the College of Nursing of Ribeirão Preto, University of São Paulo, protocol 0737/2006.

Results

E. coli presents an agarose-binding 75-KDa protein

To identify carbohydrate-binding proteins that could be specific for colonization we first cultured *E. coli* in minimum medium (DMEM) that mimics the gastrointestinal tract conditions⁽¹²⁾. When the supernatant lysate from *E. coli* was serially incubated with Sepharose CL-4B and carbohydrate-immobilized resins, we did not detect adsorbed proteins to Sepharose (Figure 1A, lane 1) or carbohydrate-immobilized resins (data not shown). Based on the work of Kenny et al.⁽¹²⁾, who obtained an increase in the production of virulence factors of EPEC when the minimum medium was supplemented with salts, we cultured bacteria in DMEM, supplemented with 22 mM KH_2PO_4 , 10 mM CaCl_2 , 0.25 mM $\text{Fe}(\text{NO}_3)_3$ or 22 mM KH_2PO_4 plus 0.25 mM $\text{Fe}(\text{NO}_3)_3$. The induction of Sepharose-binding proteins was observed only in bacteria grown in DMEM supplemented with CaCl_2 (Figure 1A, lane 3). In the preparation, we obtained a major protein of 75-kDa, followed by minor proteins of >97-kDa, ~85-kDa and a duplex of ~40-kDa. Because agarose is a support to immobilize carbohydrate, we first incubated the lysate with Sepharose CL-4B, followed by others resins. Because the 75-kDa protein bound Sepharose CL-4B, we were not able to detect proteins in other resins.



(A) Lysate obtained from *E. coli*, which was grown in minimum medium (1) without additional supplementation with salts or supplemented with (2) KH_2PO_4 , (3) CaCl_2 , (4) $\text{Fe}(\text{NO}_3)_3$, (5) KH_2PO_4 and $\text{Fe}(\text{NO}_3)_3$, was incubated with Sepharose CL-4B. After washing, the beads containing adsorbed proteins were submitted to SDS-PAGE. Lysate from *E. coli* grown in minimum medium supplemented with CaCl_2 was also submitted to affinity chromatography on Sepharose. Adsorbed material was eluted with (B) 1 M NaCl and (C) 0.1% acetic acid, dialyzed and submitted to SDS-PAGE. Gels were stained with Coomassie Blue. Migration positions of molecular mass markers are shown on the left in kDa.

Figure 1 - Protein of 75-kDa from UPEC binds to Sepharose CL-4B in a manner dependent on Ca^{2+}

Protein of 75-kDa correspond to *iutA* from *E. coli*

The 75-kDa proteins from *E. coli* obtained by affinity chromatography on Sepharose column, eluted with 1 M NaCl and 0.1% acetic acid, were submitted to electrophoresis (Figure 1B and C, respectively) and to identification by MS. The tryptic peptides of the 75-kDa protein showed high identity with protein involved in iron uptake by bacteria, *IutA*.

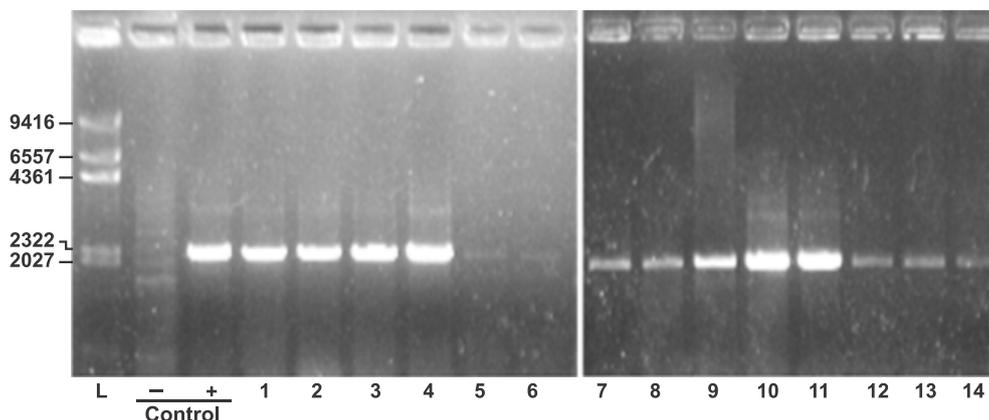
Detection of *iutA* gene in UPEC strains

Since *IutA* is expressed in conditions that mimic the site of *E. coli* colonization and has been associated with virulence⁽¹¹⁾, we sought to determine the presence of the *iutA* gene in *E. coli* strains and correlated to bacterial infection. To detect the *iutA* gene in UPEC strains, initially, we performed an amplification reaction in an *iutA*-subcloned plasmid (pPOC9-*iutA*). As shown in Figure 2, lane +, amplified product had a size between 2,322 and 2,027 bp, consistent with the expected size of 2,202 bp for *iutA* gene. The pPOC9-*iutA* was used as positive control (Figure 2, lane +) in all reactions.

The gene was detected in 14 out of 37 UPEC isolates (data not shown). To confirm the amplifications, positive isolates were submitted to another amplification reaction (Figure 2). Though the samples of lanes 5 and 6 had a weak amplification, we validated that 14 UPEC isolates

contained *iutA* gene, accounting for 38% positivity among UPECs. There was no association between the positivity of IutA in UPEC and severity of infections (data

not shown), making us question whether IutA is really a virulence factor of *E. coli*.



PCR products were submitted to electrophoresis in 1% agarose to detect the *iutA* gene in different isolates of UPEC. (L) 1 kbp DNA ladder, (-) negative control, (+) positive control and (1-14) UPEC isolates. Gels were stained with ethidium bromide. Migration positions of DNA ladder segments are indicated on the left in bp

Figure 2 - Detection of *iutA* gene in UPEC strains

Discussion

Up to date, only a few carbohydrate-binding proteins have been identified in *E. coli*. Moreover, their role in protein-carbohydrate interaction mediated bacterial colonization is scarcely known⁽⁶⁾. Therefore, we attempted to identify new carbohydrate-binding proteins that could be involved in *E. coli* colonization. Here, we report the isolation and identification of a protein of 75-kDa as the ferric aerobactin receptor (IutA), which appears to have carbohydrate-binding property and to be important for colonization.

Interestingly, IutA was expressed only in *E. coli* grown in DMEM supplemented with calcium that mimics conditions found in the gut environment⁽¹²⁾. In addition, this protein was not produced by bacteria cultured in LB broth, even when supplemented with CaCl_2 (data not shown), suggesting that additional environmental signals, such as nutritional stress, may be responsible for producing this protein.

The adsorption of 75-kDa protein to Sepharose was reproducible, even with the affinity chromatography on Sepharose column, which made us suggest that the protein is a ligand of galactose, since Sepharose is a polymer consisting of alternating residues of β -D-galactopyranose 3-O-linked and 3,6-anhydro- α -L-galactopyranose 4-O-linked⁽¹⁴⁾. Although no 75-kDa protein has been eluted with 0.1 M D-galactose or lactose from the column (data not shown), we hypothesized that a higher specificity of this protein by the disaccharide repeating units that compose the agarose and a low specificity by monosaccharide or

disaccharide, as occurs with some plant lectins, avoided the elution of the protein. There is no doubt that this is one of the reasons why the classification of lectins according to monosaccharide specificity sometimes masks the strange characteristics these proteins often display for di, tri or tetrasaccharide, with association constants up to 1,000 times higher compared to monosaccharides. In addition, many times, the three-dimensional conformation of the oligosaccharide provides complex structures that cannot be mimicked by simple sugars⁽¹⁵⁾. The identification of the 75-kDa protein from *E. coli* as IutA, which is a siderophore receptor to ferric aerobactin, was an interesting result, as this molecule involved in the capture of iron is involved in the colonization process and essential for bacterial growth in the host^(10,16). Siderophore and siderophore-mediated uptake systems play a crucial role in microbial virulence, since they are essential for bacterial pathogenesis. However, there is a redundancy of these systems, which makes it difficult to associate virulence with a specific siderophore receptor⁽¹⁷⁾. Although IutA is expressed in pathogenic bacteria and frequently associated with virulence⁽¹¹⁾, we did not detect preferentially *iutA* gene among UPEC isolates, because only 38% of the UPEC isolates presented *iutA* gene. These findings corroborate a recent study⁽¹⁸⁾ that showed positivity only in 14% of urine isolates.

Several studies have demonstrated the mode of action of receptor-Fe siderophores, such as aerobactin receptor, but no mention has been made of the dependence on

protein-carbohydrate interaction in this system. Though the glycosylation of proteins in bacteria is limited, other glycoconjugates are abundant⁽¹⁹⁾. These glycoconjugates could somehow interact with protein, like in organisms of higher complexity. Moreover, glycosylated siderophores have been found, such as salmochelin, although it is not clear why this modification occurs⁽²⁰⁾.

Conclusion

The correlation between Sepharose-binding IutA and protein system for iron uptake of *E. coli* offers an interesting study of the participation of lectins in this process. The identification of novel carbohydrate-binding proteins is an open field for many discoveries, as a possible involvement of these proteins in the tissue colonization process. In this study, we demonstrated that, although IutA might be important to the EPEC infection, it did not play an essential role in this process.

Acknowledgements

We thank Dr. Leandro L. Oliveira, Denissami A. S. F. Lima, Izaira Brandão, Ana Paula Masson and Ana Flávia Gembre for technical assistance; Dr. Ebert Hanna from Invent Biotecnologia, Brazil, for providing commensal *E. coli* and Dr. Michael O'Connell from Dublin City University, Ireland, for the generous donation of plasmid pPOC9-*iutA*. We are grateful to Dr. Andrea Kaufmann Zeh for a critical review of the manuscript.

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