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Cloning and expression of the porcine attaching and effacing-associated (*paa*) gene of enteropathogenic *Escherichia coli*

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ABSTRACT. Porcine enteropathogenic *Escherichia coli* (PEPEC) produce an outer membrane protein (intimin) called Paa (porcine attaching and effacing-associated), which is involved in the pathogenesis of *E. coli* in piglets with diarrhea. The *paa* gene of a PEPEC strain isolated in Paraná, Brazil, was amplified by polymerase chain reaction, sequenced, and cloned into the pTrcHisTOPO2 vector. The deduced amino acid sequence encoded by the *paa* gene of PEPEC from Paraná, Brazil, showed 99% homology to the sequences from other PEPEC strains. In this study, the overexpression of recombinant Paa (rPaa) using alternative induction strategies was attempted. The auto-induction protocol showed excellent results for rPaa protein production with 0.4% (w/v) lactose. The rPaa protein is insoluble and was purified with Triton

X-100 wash as a total antigen. This method produced a relatively high yield of rPaa. rPaa was recognized by serum from pigs immunized with the PEPEC strain. These results suggest that rPaa could be included in the development of a vaccine against swine colibacillosis.

Key words: Porcine enteropathogenic *Escherichia coli*; *paa* gene; Post-weaning diarrhea; Piglets; Colibacillosis

INTRODUCTION

Non-enterotoxigenic *Escherichia coli* strains have been associated with post-weaning diarrhea and neonatal diarrhea in swine, through adhesion to intestinal epithelial cells in a characteristic attaching and effacing (A/E) pattern (Batisson et al., 2003). Porcine enteropathogenic *E. coli* (PEPEC) produces an outer membrane protein (intimin), which is involved in the intimate attachment of the bacteria to enterocytes and induces typical A/E lesions, as observed in a pig ileal explant model (Zhu et al., 1994, 1995). These A/E lesions contribute to the initial phases of PEPEC pathogenicity (Batisson et al., 2003).

The gene in PEPEC that induces the A/E lesions has been designated *paa* (porcine attaching and effacing-associated) (An et al., 1999). Its sequence revealed an open reading frame of 753 bp encoding a 27.6-kDa protein, which displayed significant similarities with the Paa of enterohemorrhagic *E. coli* (EHEC) O157:H7 strains (Batisson et al., 2003).

The *paa* gene is also associated with other virulence genes of enterotoxigenic *E. coli* (ETEC); its conservation and expression have been shown in the O149 ETEC collection, and all *paa*-positive strains possess ETEC virulence genes. *paa* is mostly found with the enterotoxin gene *estA* and the autotransporter gene *sepA*, and it is carried on high-molecular weight plasmids (Leclerc et al., 2007). In Brazil, the *paa* gene has also been found in association with genes for other adhesins and toxins, and it has been found in 22% of *E. coli* strains isolated from piglets (Vidotto et al., 2013).

A/E lesions have been experimentally reproduced in newborn piglets, and both Paa and Tir proteins of PEPEC have been confirmed to be involved in A/E lesions *in vivo* (Girard et al., 2005). Considering that the Paa protein is important in virulence and that a mutant strain, PEPEC O45 (deltaler), which does not have toxins but expresses the *paa* gene, could be used as an attenuated vaccine candidate against PEPEC O45 (Hu et al., 2009), we cloned and expressed the *paa* gene to produce the recombinant Paa (rPaa) protein in the development of a vaccine against swine colibacillosis.

MATERIAL AND METHODS

Strains, vectors, and chemicals

The *paa*-positive *E. coli* strain PEPEC was isolated from piglets with diarrhea in Paraná State (Vidotto et al., 2009, 2013). *E. coli* TOP 10 and *E. coli* host strain BL21 (DE3) purchased from the Invitrogen Corporation (San Diego, CA, USA) were used to produce a Paa recombinant protein. Vector pTrcHisTOPO2 (Invitrogen, Carlsbad, CA, USA) was used for cloning and expression studies. All chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cloning and sequence analysis of the *paa* gene from PEPEC

The *paa* gene was amplified from *paa*-positive PEPEC genomic DNA by polymerase chain reaction (PCR). The base sequences for the specific oligonucleotide primers used in this study were based on PAA PEPEC O45, forward: 5'-TCTTCTGCTGCTTATGCTGATATC-3', and PAA PEPEC O45, reverse: 5'- TTACCAGCCATATTTTTTGAATGC-3', annealing at nucleotides 37 to 60 and 718 to 738 of the *paa* gene, respectively (Vidotto et al., 2013).

PCR was carried out in a total volume of 25 µL, containing 5 µL template DNA, 20 pmol of each of the primers, 200 µM dNTPs, 1X PCR buffer, and 1.5 U Taq DNA polymerase (Invitrogen Life Technologies, São Paulo, Brazil). The PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, annealing at 55°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 7 min in a thermal cycler (Biocycler). The amplified DNA was visualized on a 1.5% agarose gel stained with SYBR Safe (Invitrogen). A 100-bp ladder (Promega, Madison, WI, USA) was used as a standard for determining the molecular mass of the PCR products.

The PCR products were quantified and used as inserts in the pTrcHisTOPO2 vector (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. Chemically competent *E. coli* host strain TOP10 cells were then transformed with 3 µL of the cloning reaction product. The transformant colonies were selected on plates containing 50 µg/mL ampicillin, and the presence of the *paa* gene was confirmed by PCR. The recombinant plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen Inc.), and the correct position of the *paa* gene was confirmed by sequencing with primers pTrcHis forward and paa reverse, utilizing a BigDye Terminator commercial kit (Applied Biosystems, CA, USA). The sequences obtained were analyzed using BLASTN accessed through the NCBI website (http://www.ncbi.nlm.nih.gov/) to verify the sequence identity. DNA and amino acid sequence analyses were carried out using the CAP3 Contig Assembly Program, Clustal W (1.81) Multiple Sequence Alignments, and Six Frame Translation of Sequence software.

Expression of the *paa* gene in an *E. coli* strain

Conventional isopropyl-1-β-D-thiogalactopyranoside (IPTG) induction

E. coli BL21 (DE3) was transformed with the recombinant plasmid pTrcHis/paa by thermal shock. The BL21/pTrcHis/paa strain was grown to an optical density at 600 nm of 0.7. IPTG (Invitrogen Life Technologies) was then diluted to 1 mM, and aliquots were removed at different times to determine the best time for expression. The cells were collected by centrifugation, and expression was determined in soluble and insoluble fractions by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Auto-induction

To overexpress the rPaa, we examined four *E. coli* BL21 (DE3) strains (RIL, RP, pLysS, and Rosseta) with inducer molecules such as lactose or IPTG. The possibility of using several types of media and lactose as inducers to increase the yields of the recombinant protein was investigated.

rPaa was overexpressed under auto-induction conditions, as described by Deacon et al. (2008), with slight modifications. Briefly, a seed culture of transformed BL21 (DE3)/pTrcHis/paa was grown O.N. (37°C) to saturation in Lysogeny broth medium. Aliquots of 0.1 mL seed culture were inoculated in Superbroth auto-induction medium (SB auto: tryptone, 32 g/L; yeast extract, 20 g/L). The compositions (g/L) of the supplementary solutions added to the base media were as follows: NPSC stock (Na_2HPO_4, 71; KH_2PO_4, 68; Na_2SO_4, 14.2; NH_4Cl, 53.3; pH adjusted to 7.0 with NaOH; used at a final concentration of 50 mL per liter medium); glucose stock (glucose, 25; used at a final concentration of 10-40 mL per liter medium); glycerol stock (glycerol, 250; used at a final concentration of 10-40 mL per liter medium); and lactose stock (lactose, 100; used at final concentration of 10-40 mL per liter medium and grown for 24 h at 37°C with shaking at 250 revolutions per minute). During auto-induction, expression levels were analyzed at 3-h intervals by 15% SDS-PAGE.

Purification of rPaa

The culture of induced bacteria was centrifuged, lysed by sonication, and then frozen in liquid nitrogen. This suspension was then centrifuged and the supernatant utilized for protein purification using Ni-nitrilotriacetic acid (NTA) resin columns (Qiagen, Valencia, CA, USA).

The rPaa was also purified as a total antigen; the induced bacteria were collected by centrifugation and incubated in 50 mL buffer (Tris 0.1 M, pH 7.0; ethylenediaminetetraacetic acid 1 mM; lysozyme 1 mg/mL) for 1 h at room temperature. The cells were sonicated on ice with three 5-s pulses at high intensity and then frozen in liquid nitrogen to ensure cell lysis. The rPaa was washed in buffer: Tris 0.1 M, pH 8.0, NaCl 1.5 M, ethylenediaminetetraacetic acid 20 Mm, and Triton X-100 2%. The protein content of the purified rPaa was measured using the Bradford method and analyzed by 12% SDS-PAGE.

SDS-PAGE and western blot

Lysates were suspended in electrophoresis sample buffer (0.025 M Tris-HCl, 2% SDS, 15% glycerol, 2.5% 2-mercaptoethanol, pH 6.8), boiled for 5 min, and analyzed by 10% SDS-PAGE. The gels were either stained with Coomassie blue or used for western blots. For western blots, proteins were transferred onto nitrocellulose membranes (Pharmacia Biotech) (Towbin and Gordon, 1984), and the membranes were blocked in blocking buffer [phosphate-buffered saline (PBS) + 0.1% Tween 20 + 5% nonfat dry milk] for 1 h at room temperature with agitation. The membranes were washed in PBS-T (PBS + 0.1% Tween 20) and incubated for 1 h with serum. The proteins were then incubated with anti-pig antibodies conjugated with peroxidase (Sigma Immunochemicals). The membranes were washed and rPaa was detected by incubation with substrate/chromogen solution. Protein molecular mass markers (Rainbow™ colored, Amersham Life Science) were used as standards.

RESULTS

paa gene isolation from a positive PEPEC genome was performed by PCR. For the PCR, the primers were designed based on conserved sequences of the *paa* gene coding region

from some *E. coli* strains. Sequences from *E. coli* O55, PEPEC O45, and ETEC 70463 were aligned using Clustal W (1.81) Multiple Sequence Alignments (data not shown). The forward primer was designed from the 37-bp position, and the reverse primer up to the 738-bp position. After *paa* open reading frame cloning and sequencing, the sequence was analyzed for similarity to other strains. The analysis of the *paa* gene coding region from the PEPEC strain indicated that the nucleotides showed high identity (99%) with the sequence from the other PEPEC strains.

When the amino acid sequence of Paa protein from the Brazilian PEPEC strain was compared with other strains, we observed 91% similarity with PEPEC 045 (Bruant et al., 2009), 99% similarity with *E. coli* 0103 and 055, and 100% similarity with *E. coli* O157:H7 strain EC4115.

The recombined plasmid pTrcHis/paa showed that the *paa* gene had been inserted at the correct position in the vector. The amount of time that was adequate for the induction of the protein with IPTG was 18 h. The induced Paa protein was recovered from the pellet of the lysed bacteria, as it is insoluble (data not shown).

The auto-induction protocol showed excellent results for rPaa protein production in simple batch cultivations. *E. coli* BL21 (DE3)-RP and BL21 Rossetta exhibited the highest quantity with 0.4% (w/v) lactose, an induction time of 16-18 h, and an induction temperature of 37°C (Figure 1A, line 2).

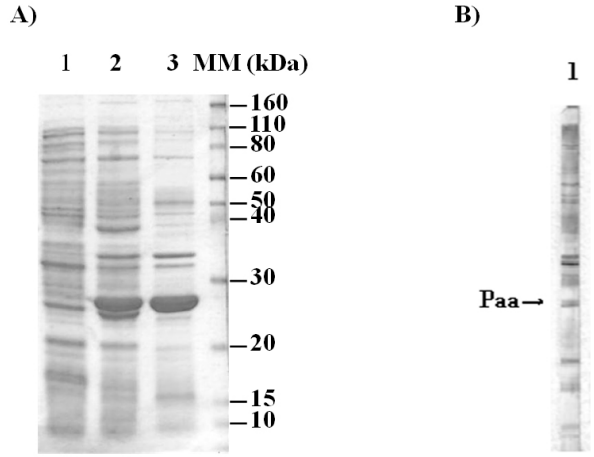

Figure 1. Expression and purification of recombinant Paa (rPaa) protein from clone BL21/pTrcHis/paa. **A.** 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie brilliant blue. *Lane MM* = molecular mass. *Lane 1*, BL21; *lane 2*, BL21/pTrcHis/paa induced with 1 mM isopropyl-1-β-D-thiogalactopyranoside (IPTG); *lane 3* = Paa protein. **B.** Western blotting of rPaa with polyclonal serum produced against the porcine enteropathogenic *Escherichia coli* (PEPEC) strain.

The rPaa was not purified by the Ni-NTA resin columns method. Because the rPaa protein is insoluble, it was purified with Triton X-100 wash as a total antigen. The SDS-PAGE results showed a 28-kDa rPaa, which was absent in the negative control (Figure 1A line 3). Western blotting analysis showed that the antibody reacted with the induced 28 kDa protein (Figure 1B).

DISCUSSION

The association of the *paa* gene of PEPEC with other ETEC virulence genes suggests its importance in the virulence of PEPEC (Leclerc et al., 2007; Vidotto et al., 2013).

In this study, the sequence of the Paa protein from a Brazilian PEPEC showed 99% similarity with the Paa protein previously characterized in other strains from different countries. Although there was 91% similarity with PEPEC 045 (Bruant et al., 2009), the similarity with the *E. coli* O157:H7 strain EC4115 was 100%. Trabulsi et al. (2002) also observed that some atypical EPEC strains are genetically closer to the EHEC strains of the O157:H7 serotype than to typical EPEC strains. Similarly, Leclerc et al. (2007) found that the sequence of the Paa protein is highly conserved among ETEC strains and is very similar to that of porcine EPEC and EHEC, suggesting common ancestry and recent dissemination of the gene coding for this virulence factor.

The serogroup O45 is important among PEPEC strains, and based on its virulence gene content, the O45 PEPEC strain is an atypical EPEC locus of enterocyte effacement-positive *E. coli* lacking the *stx*1 and *stx*2 genes (Bruant et al., 2009). The PEPEC O45 deletion mutant lost its toxigenicity to Vero cells and was found to be safe for mice and pigs. Oral immunization can induce specific immune responses in mice and pigs, and this mutant strain could be used as an attenuated vaccine candidate against PEPEC O45 (Hu et al., 2009).

The cost and composition of culture media are critical for commercial-scale production of recombinant proteins in *E. coli*. The rPaa protein was produced with the use of lactose instead of IPTG for induction. Generally, the protein is expressed by recombinant bacteria following induction with IPTG, which is a good inducer and is commonly used in molecular biology. However, it is costly and potentially toxic. Lactose is a natural substrate of the bacterial enzyme that is controlled by the *lac* operon, which is natively induced. Lactose is a low-cost, harmless, and avirulent compound (Donovan et al., 1996; Deacon et al., 2008).

Additionally, a simple purification process for the recombinant protein is important for commercial-scale production. The rPaa was purified with Triton X-100 wash as total antigen instead of being purified with affinity chromatography in columns loaded with Ni^{2+}. The genetic construct included an N-terminal histidine tag sequence that facilitated recovery, purification, and proper refolding of the vaccine candidate by affinity chromatography; however, this procedure is expensive.

The results suggest that rPaa could be included in the development of a vaccine against swine colibacillosis.

Conflicts of interest

The authors declare no conflict of interest.

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