The *htrA* Gene Plays an Important role During *Yersinia Pseudotuberculosis* Growth at High Temperature

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Original article

Keywords: HtrA protein, Heat-shock protein, Chaperone, Yersinia pseudotuberculosis.

Posted Date: August 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-50599/v1

Abstract

*htr*A is a gene coding for the stress inducible HtrA protein, identified as a temperature stress response protein in several Gram positive and Gram negative bacteria. Growth rates at several temperatures (30ºC, 37ºC and 42ºC) were compared for *Yersinia pseudotuberculosis* YPIII wild strain and the isogenic mutant 1YPIII (*htr*A::Km), which was obtained by insertion of a kanamycin resistance cassette into the *htr*A gene.

Y. pseudotuberculosis 1YPIII growth rates did not differ from the *Y. pseudotuberculosis* wild strain growth rates when cultivated at 30°C, which is consistent with a non-essential role for the HtrA protein at this temperature. However, 1YPIII mutant strain growth rate decreased by 18.73% at 37°C, and by 60.14% at 42°C, as compared to the *Y. pseudotuberculosis* YPIII wild strain growth rate. HtrA complementation in the strain 1YPIII/pAHTRA46 suppressed the differences in growth rates. Immunoblot analysis confirmed the absence of the HtrA protein in the 1YPIII mutant strain at any of the growth temperatures under analysis. *In silico* predictions were obtained for the three-dimensional structure of amino acid sequence belonging to HtrA from *Y. pseudotuberculosis* YPIII, *Yersinia pestis* CO92, using the protein data bank structure 1KY9:B from *Escherichia coli*, as template. The model's quality was found to be acceptable. Southern blot analysis shows a single *htr*A gene signal. These data indicate that the unique *htr*A gene in *Y. pseudotuberculosis* YPIII is required for the adaptive response of this species to high temperatures and although it is not a pathogenicity factor, it can be targeted by antibiotics.

Introduction

HtrA (**h**igh **t**emperature **r**equirement A, also called DegP) is a periplasmic heat-shock protein, with ATP-independent serine protease (Resto et al. 2000); (Phillips and Roop, 2001) and chaperone activities. The chaperone function is dominant at low temperatures (Spiess et al. 1999), whereas the protease activity becomes considerable between 32 °C and 42 °C (Skorko-Glonek et al. 1995); (Spiess et al. 1999). This protein can discriminate damaged proteins to be degraded, from partially unfolded proteins amenable to successful refolding and is a key factor in protein quality control in Gram negative bacteria (Pallen and Wren, 1997); (Spiess et al. 1999); (Young and Kim, 2005). Several proteins of the HtrA/DegP family have been identified in *Yersinia enterocolitica* (Li et al. 1996), *Haemophilus influenza* (Craig et al. 2002), *Bartonella henselae* (Resto et al. 2000), *Legionella pneumophila* (Pedersen et al. 2001), *Brucella abortus* (Phillips and Roop, 2001), *Salmonella typhimurium* (Bäumler et al. 1994) and *Helicobacter pylori* (Zawilak-Pawlik et al. 2019; Zarzecka et al. 2019).

The monomer HtrA/DegP from *E. coli* (PDBid 1KY9:B, the S210A mutant; Uniprot P0C0V0, DEGP_ECOLI, full CDS and used for the numbering given here) presents a signal peptide lost at secretion (amino acids 1 to 20) and three functional regions: i) a "Do" protease domain (residues 1-259), ii) core domain containing the catalytic triad of amino acids H105, D135 S210 required for enzymatic function (Clausen et al. 2002), in the carboxy terminus, the Arg-Gly-Asp (RGD) motif, including a conserved sequence signature GNSGG (residues 208–212) as catalytic domain, and iii) two PDZ domains at the carboxy terminal region. PDZ1 comprises residues 260–358 and PDZ2 resides at residues 359–448. These last

PDZ domains are involved in protein-protein interactions and appear to determine target recognition (Table 1).

Table 1
regions and positions of HtrA protein

		1KY9_S210A_B	HtrA_Yptb3
Signal Peptide			**1–27**
Protease Domain Do		1-259	**28–290**
catalytic triad	**H**	105	**136**
	D	135	**166**
	S	210	**241**
GNSGG		208–212	**239–243**
PDZ1		260–358	**291–389**
PDZ2		359–448	**390–481**
LA Loop		44–79	**73–108**
L1 loop		204–214	**235–245**
L2 loop		228–239	**259–270**
L3 loop		187–199	**218–230**
Q linker		55–79	**84–100**
Carboxylate loop		370–374	**402–406**

In *Y. enterocolítica*, the *gsr*A gene (**g**lobal **s**tress **r**equirement **A**) has been identified and sequenced. When the amino acid sequence of GsrA was compared to the HtrA of *Escherichia coli* and *S. typhimurium*, the signatures of the three characteristic HrtA domains were found. GsrA presents a potential signal peptide (residues 1 to 27), a catalytic domain with the sequence Gly-Asn-Ser-Gly-Gly (residues 239–243) (Table 1) and near the carboxy terminus, the RGD motif (Yamamoto et al. 1996).

The mutant *gsr*A^{-} of *Y. enterocolítica* O:8 showed increased sensitivity to oxidative stress, temperature stress and had a reduced virulence in murine yersiniosis bioassays (Li et al. 1996).

A *Yersinia pestis htr*A orthologous was cloned and sequenced its amino acid sequence showed high identity to *Y. enterocolítica* GsrA (95%), *E. coli* HtrA (86%) and *S. typhimurium* HtrA (87%). *Y. pestis htr*A^{-} mutant, in contrast to the wild strain, failed to grow at 39 °C, but showed only a small increase in sensitivity to oxidative stress, and its virulence was only partially attenuated in the animal model. In addition, the protein expression pattern of the mutant differed from that of the wild strain, when grown at

28 °C, conditions similar to those in the flea (Williams et al. 2000). In this study, the molecular genetic approach is used to gain insight on the possible role of the *htr*A orthologous gene product, and to explore the behaviour of an isogenic mutant of *Y. pseudotuberculosis* YP III strain.

Materials And Methods

Bacterial strain and plasmids

The bacterial strains and plasmids used in this work are listed in Table 2.

Table 2
Bacterial strains and plasmids

Strains/plasmids	Relevant characteristics	Source or Reference
Yersinia pseudotuberculosis YPIII	Serotype 3	Wt strain collection
Y. pseudotuberculosis 1 YPIII	Km^r, with *htr*A¯ gene	This work
E. coli S17-1 λpir	(F–) RP4-2-Tc::Mu *aphA*::Tn*7 recA* λ*pir* lysogen	(Simon et al., 1983)
E. coli S17.*1HK4*	*E. coli* SY327 (λpir) with plásmid pDHK4	This work
pCR 2.1 TOPO vector	Cloning vector high number copies Km^R, Ap^R	Invitrogen®
pBSL46	Cloning vector low number copies Km^R, Ap^R	(Schneider et al., 2004)
pACYC 184	Cloning vector low number copies Cm^R, Tc^R	New England Biolabs®
pDS132	Cloning vector suicide in enterobacters *ori* R6K, *mob*RP4, *sac*B, Cm^R	(Kruger *et al.*, 1994)
pCHTRA7	Derivative of pCR 2.1 TOPO, Km^R, Ap^R, with the *htr*A gene	This work
pAHTRA46	Plasmid derivative of pACYC184 Cm^R, Tc^s, with *htr*A gene	This work
pAHTK99	Plamid derivative of pAHTR46 Cm^R, Tc^s, Km^R, with *htr*A¯gene	This work
pDHK4	Derivative of pDS132, *ori* R6K, *mob*RP4, *sac*B, Cm^r, with *htr*A¯ gene	This work

Media and growth conditions

Y. pseudotuberculosis YPIII wild strain was grown at 30 °C in CIN broth (agar DIBICO). *E. coli* strain was grown at 37 °C in Luria-Bertani broth with antibiotic, kanamycin 100 µg mL^{-1} (Km), tetracycline 20 µg

mL^{-1} (Tc), chloramphenicol 35 or 18 µg mL^{-1} (Cm).

Y. pseudotuberculosis genomic DNA extraction and general cloning techniques were carried out as described by (Sambrook and Russell, 2001).

The homologous primers were derived from *Y. pestis* strain CO92, covering 457 bp (*bar*A gen) upstream and 412 bp (*dgt* gene) downstream of the *htr*A gene (CP009792.1:4438735–4440180), (Parkhill et al. 2001). To amplify 2315 bp (2.3 kb) fragment containing *htr*A gene by polymerase chain reaction (PCR) with primers (forward 3531) 5'AAGAAGGATCCGGCACGGGATCTCTTAACCA-3' and (reverse 3532) 5'GTAAGGATCCAATTGAAACACGGTTATT-3' in which were designed a *Bam* HI site. The PCR conditions were: 95 °C for 1 min; 15 cycles of 30 s at 95 °C, 1.5 min at 41.8 °C, 2 min at 72 °C; and them 10 min at 72 °C.

The 2.3 kb DNA fragment was cloned into the pCR 2.1 TOPO vector (Invitrogen®) to generate the pCHTRA7 plasmid. This plasmid was sequenced with M13 primers (at Instituto de Biotecnología, UNAM).

Construction of the mutant in *htr*A gene on *Y. pseudotuberculosis* YPIII

Plasmid pCHTRA7 was digested with *Bam* HI to remove a 2.3 kb, and subcloned into the similarly digested pACYC184 plasmid (New England Biolabs). The resulting plasmid pAHTRA46 was transformed into *E. coli* S17-1 cells. The transformants were selected for Cm resistance. Clones were screened by restriction. The Km resistance cassette (1.2 kb) from pBSL46 plasmid (Alexeyev et al. 1995) was inserted into the 2.3 kb fragment in pAHTRA46 using *Eco* RI restriction site to generate a 3.5 kb fragment in pAHTK99 plasmid, screening of transformants with Cm and Km.

The 3.5 kb fragment was amplified by PCR using pAHTK99 DNA as template with 3531 and 3532 primers, was subcloned in *Sma* I site of pDS132 (Schneider et al. 2004), yield the plasmid pDHK4. This plasmid was transformed into *E. coli* S17-1 cells, the transformants were selected with 35 µg mL^{-1} Cm and 50 µg mL^{-1} Km. The pDHK4 plasmid was introduced by conjugation, *Y. pseudotuberculosis* wild strain, (receptor) and *E. coli* S17-1 (donor). The culture was collected and serial dilutions were made in sterile NaCl 0.9% in CIN agar with Km.

The transconjugants were selected with 100 µg mL^{-1} Km and incubated at 30 °C overnight. On the other hand in agar plates with 5% sucrose and 100 µg mL^{-1} Km which were incubated during 48 h at 30 °C. The clones Km resistant and Cm sensitive named 1YPIII were chosen for colony blot hybridization.

The mutation was corroborated by three Southern blot hibridization, as described by Sambrook (Sambrook and Russell, 2001), using as probes the kanamycin cassette, the *htr*A gene from pAHTRA46 plasmid and pDS132 plasmid. The DNA was digested with *Eco*RI for the first and third assay (data not shown), and on the second assay with *Ava*I and *Cla*I.

Characterization of the mutant *Y. pseudotuberculosis* 1YPIII

Using genomic DNA of *Y. pseudotuberculosis* and specific primers (Gene ID: 6090393 NCBI data bank) for the *Yersinia* genus. 16S gene primers are: Forward primer: U16S38D: (5'-GCG GCA GCG GGA AGT AGT TTA-3') and Reverse primer: D16S47R: (5'-GAT TAA GCG TAT TAA ACT CAA CCC C-3').

The PCR conditions were: denaturalization at 95 °C for 7 min; 30 cycles of 30 s at 95 °C, 1 min at 54 °C, 1 min at 72 °C and 10 min at 72 °C.

Growth curves were performed: *Y. pseudotuberculosis* YPIII, 1YPIII and 1YPIII/pHTRA46 strains were incubated at 30 °C on a rotary shaker at 250 rpm during 18 h of this culture one mL was inoculated in 50 mL of LB broth and incubated at 30, 37 and 42ºC during 12 h. $O.D._{600}$ measurements were realized every two hours. In order to determine total proteins, these cultures were incubated until $O.D._{600}$ 0.6 were packed and resuspended in PBS buffer.

The cellular pellet was sonicated using a 20 kHz (GE 130) Ultrasonic Processor equipped, controlled at 70 W, with a wave guided probe type of 3 mm diameter, for 90 s total, using 8 s pulses, at 4^{o}C. The soluble proteins in the supernatant were determined by Bradford method. The proteins separation and analysis were realized in PAGE-SDS and Western blot (Krüger et al. 1994). Proteins in the gels were electroblotted onto nitrocellulose membrane (Hybond^{TM}-ECL, GE Healthcare). Blots were blocked overnight at 4 °C in 3% nonfat milk in TBST, probed with the HtrA1 polyclonal antibody of *Staphylococcus aureus*, washed and incubated with anti-rabbit immunoglobulin G conjugated with phosphatase alkaline. The blots were developed with BCIP and NBT (Merck). Purple staining intensity was observed.

Homology modeling.

Because there was a high level of identity between *Y. pseudotuberculosis* HtrA and *E coli* DegP, we used the threading tools implemented in SWISS PDB viewer (Kaplan and Littlejohn, 2001) and SWISS MODEL workspace (Arnold et al. 2006) to build a model of the wild type *Y. pseudotuberculosis* HtrA. In addition models were also requested to well know I-TASSER server (Zhang, 2008). The resulting models were evaluated with several tools available at the SWISS-MODEL workspace (Arnold et al. 2006) the ANOLEA energy, Dfire, Pro check, GROMOS energy and all these tools revealed and prediction with average to good quality. Finally, predictions were evaluated for biological appropriateness (a measure of the backbone fold adequacy to host the target amino acid sequence) using the Rd.HMM protocol (Martínez-Castilla and Rodríguez-Sotres, 2010). The model was had acceptable Rd.HMM score and E-values for a prediction, and also having canonical geometrical and chemical features. According to this evaluation, the model for the wild type HrtA protein was rated as a good prediction, likely to represent correctly the three-dimensional structure of the real protein in most of the model, whit the exception of the first 40–45 amino acids at the N-terminus. As expected, the model reconstructed the essential amino acids at the serin protease active site and provided a plausible prediction for the active geometry of these residues.

The model for the HtrA::Km chimera was made using the well know I-TASSER server (Zhang, 2008), and the resulting prediction was also evaluated with the tools provided by SWISS MODEL workspace (Arnold et al. 2006). In contrast to the previous models the I-TASSER predictions for the HtrA::Km chimera where all dramatically different and the Rd.HMM failed to match the target sequence, therefore all predictions were rated as incorrect.

Results

Analysis of the sequence of 2315 bp.

The complete sequence of *htr*A gene was obtained, thoroughly analysed and not showed any mutation (Fig. 1) and aligned to *E. coli* HtrA (1KY9:B) and *Y. pestis* (GI:115349010) with CLUSTALW program, revealing 99% sequence identity (Fig. 2A). The amino acid sequence of HtrA from *Y. pseudotuberculosis* was determined by *in silico* translation of the gene sequence and analysed with the Smart program (http://smart.embl.de/) to identify those sections corresponding to the putative characteristic domains of the HtrA family proteases, *i.e.*: i) signal peptide; ii) catalytic domain; and iii) two PDZ domains (Fig.2B). *In silico* models of HtrA from *Y. pseudotuberculosis* YPIII and *Y. pestis* CO92 were created using Swiss-Pdb-Viewer program taking as template 1KY9:B and threaded onto the crystallographic structure of HtrA from *E. coli*. As expected the threading revealed many coincidences between the different structures of HtrA protein in *E. coli*, *Y. pestis* and *Y. pseudotuberculosis*, but a significant difference in the loop formed by R-44 to G-79 in *E. coli* and the loop formed by P-73 to G-108 in *Y. pestis* and *Y. pseudotuberculosis*. This last loop corresponds to the LA loop (containing Q-linker) in the proteolytic domain (Fig. 2C) The insertion of kanamycin cassette into the *htr*A gene was described in methods, and generated the pAHTK99 plasmid. This plasmid containing the mutated *htr*A CDS (*htr*A::Km) was digested with *Bam* HI (4.2 and 3.5 kb fragments), *Hind* III (7.7 kb fragment) and *Eco* RI (3.9, 2.5 and 1.2 kb fragments) to check its restriction enzyme pattern (Fig.3A), sequenced, analysed and showed the inserted sequence (Fig.3B).

Transforming *Y. pseudotuberculosis* 1YPIII with the pAHTRA46.

The mutant *Y. pseudotuberculosis* 1YPIII was complemented by transformation with the original pAHTRA46 plasmid containing the wild-type copy of the *htr*A gene. These transformed colonies were recovered and tested for the presence of the pAHTRA46 plasmid. The resulting confirmed transformants were designated *Y. pseudotuberculosis* 1YPIII/pAHTRA46.

Characterization of the *Y. pseudotuberculosis* 1YPIII *htr*A^{-} mutant.

In order to prove that the clone of *Y. pseudotuberculosis* 1YPIII did not produce the native HrtA protein, a Western blot analysis was performed using total protein extracts of YPIII wild type and 1YPIII mutant strains using polyclonal antibodies anti HtrA of *Staphylococcus aureus*. In the total proteins extracts from cultures at 30°C, 37°C and 42°C no signal was observed in the protein extracts form the clone *Y. pseudotuberculosis* 1YPIII (Fig. 4A). This was surprising, because no signal was observed at all, neither at the chimeric HtrA::Km expected size, nor not at the wild-type HrtA protein expected size. In contrast, a band with the size expected for HtrA wild type protein was observed for the wild-type strain *Y. pseudotuberculosis* YPIII.

Y.pseudotuberculosis 1YPIII (*htr*A$^-$) mutant strain was grown at different temperatures (30ºC, 37ºC y 42ºC). The mutant growth rates were comparable to those of the wild type strain at 30ºC, but were reduced at 37ºC and severely affected at 42 ºC (Fig. 4B). The data suggest an important role for HtrA protein in the growth of the bacterium at 37 and 42ºC. To confirm the participation of HtrA in the growth at 37 and 42ºC, the growth rates of the complemented strain 1YPIII/pHTRA46 were determined. The complemented mutant showed wild-type like growth rates at 37 and 42°C (Fig. 4B). In agreement, the protein band corresponding to the HtrA wild type protein size was detected in the crude extracts from the complemented mutant to a similar level observed for the wild type strain (data not shown).

Southern blot type hybridizations, using a 2.1 kb fragment as a probe, which contains the *htr*A gen of *Y. pseudotuberculosis*, only one signal was obtained (Fig. 5), also was amplified the *htr*A gene and *htr*A::Km in the YPIII, 1YPIII strains, shown 2.3 kb and 3.5 kb fragments respectively, another amplification using primers 16S specific for *Yersinia* U16S38D and D16S47R, shown 412 bp fragment (Fig. 6).

Discussion

Y. pseudotuberculosis has been subclassified into 21 serotypes, which are considered as causative agents of several human and animal diseases (Skurnik, 1999). *Y. pseudotuberculosis* is the occasional etiologic agent of gastroenteritis, resulting in severe abdominal pain, fever and headache (Putzker et al. 2001).

Y. pseudotuberculosis HtrA protein shares the proteolytic and the two PDZ domains with its orthologues from *E. coli* (Wessler et al. 2017), *Legionella fallonii*, and *Thermotoga maritime* (Hansen and Hilgenfeld, 2013). A regulatory role for the PDZ domains has been demonstrated for HtrA and DegS (Krojer et al. 2010), but currently, there is no evidence for a similar regulation of the *Y. pseudotuberculosis* HtrA protein. According to *in silico* modeling, the differences in the amino acid sequence between the *Y. pseudotuberculosis* HtrA protein and its orthologues from *Y. pestis*, *Chlamydia trachomatis* and *E. coli* (Gloeckl et al. 2012) cluster at the LA loop within the proteolytic domain. The alteration in the coding region introduced in the *htr*A::Km chimeric gene interrupts the gene at the beginning of the proteolytic domain and encodes an 836 amino acid long polypeptide which is coincident only in the first 70 codons and has less than 25% similarity (at the amino acid level thereafter). Contrary to our expectations, the signal of this large chimeric HtrA::Km polypeptide, or any degradation fragment could not be detected

with Abs anti HtrA of *S. aureus*. As expected, the band corresponding to the size of the HrtA wild-type protein was not present either. Either the chimeric transcript encoding HtrA::Km chimera is not transcribed, it is not translated, it is rapidly degraded at the mRNA or protein level, or all the antigenic determinants are lost due to the mutation. Instead, the HtrA protein is present in the *Y. pseudotuberculosis* wild type grown at 30, 37 and 42 °C, and under this conditions, it has been reported to have proteolytic activity (Lopes et al. 2009).

In contrast to the mutation by deletion of *H. pylori* (Zawila-Pawlik *et al*. 2019), to the mutation by deletion of PDZ domain in *Synechocystis* reported by Huesgen *et al.* (Huesgen et al. 2011) and to the site-directed mutagenesis in *C. trachomatis* reported by Gloeckl *et al*. (Gloeckl et al. 2012), the insertion of Km resistance cassette into the proteolytic domain coding region of the *htr*A gene of *Y. pseudotuberculosis* YPIII wild type strain, here reported, lead to the loss of the HtrA protein signal in the immunoblot assay.

In *Salmonella enterica* serovar Typhimurium, there is a protein homologue to *E. coli* HtrA called DegQ, in addition to classic *S. enterica* HtrA. The protease DegQ has identical activity to HtrA *in vitro*, but the null mutation of *deg*Q gene does not attenuate the virulence of this strain, whereas the double null mutant in *htr*A and *deg*Q present virulence attenuation (Farn and Roberts, 2004).

Two proteases of the type HtrA are found in *S. aureus* and their coding genes are called *htr*A_1 and *htr*A_2. In this case only $HtrA_1$ was found to have a function in the protection against thermal stress, but its protease activity is low or absent, and the role of $HtrA_1$ as a protease may be compensated by a different, yet unidentified protease (Rigoulay, 2005).

Mycobacterium leprae has the gene ML0176, which codes a predicted HtrA-like protease, conserved in other species of mycobacteria (Lopes et al. 2009) and in *Mycobacterium tuberculosis*. This gene, known as *pep*D, is directly regulated by the stress-responsive two-component signal transduction system MprAB and indirectly by the so called extracytoplasmic function (ECF) sigma factor, SigE (White et al. 2011).

It has been reported that three serine proteases, HtrA, HhoA (HtrA homologue A) and HhoB (HtrA homologue B), are important for survival of *Synechocystis* sp. PCC6803 (Huesgen et al. 2011).

In *Campylobacter jejuni* the HtrA has protease and chaperone activities and participates in the interaction between *C. jejuni* and mammalian host cells (Bæk et al. 2011).

In Southern blot type hybridizations were performed, using a 2.1 kb fragment as a probe, which contains the *htr*A gen of *Y. pseudotuberculosis*, and only one signal was obtained. This last result suggests a unique gene encoding HtrA function in *Y. pseudotuberculosis* YPIII strain.

In contrast to *Y. pseudotuberculosis* YPIII wild type strain increase of the temperature strongly reduces the growth of the corresponding 1YPIII *htr*A^- mutant. This result is in agreement with the data reported by Yamamoto (Yamamoto et al. 1996), for a mutant of the *gsr*A gene of *Y. enterocolítica*, and with the Williams' results (Williams et al. 2000) for *Y. pestis htr*A^- mutant. In none of these last two reports did the

mutant grow at temperatures higher than 39 °C, is agreement with the data reported by Zarzecka for *H. pylori* (Zarzecka et al. 2019).

In addition, HtrA from *M. leprae* displayed maximum proteolytic activity at temperatures above 40 °C (Lopes et al. 2009), HtrA from *H. pylori* showed temperature-dependent oligomer dissociation (Hoy et al. 2013) and DegP was important during high-temperature bacterial growth (Kim and Robert, 2012). In our case, *hrt*A^{-} mutant showed virtually a total growth arrest after 4 h at 42ºC, and when compared to the wild strain at 12 h, a culture OD difference of 77.82% was observed. As already mentioned, in mutants of the *htr*A gene of bacteria such as *Streptococcus pneumonie* (Musa et al. 2004) and *S. enterica* (Lewis et al. 2009), the growth at 42 °C is attenuated, while the growth at lower temperatures was normal. Therefore, HrtA must be performing a role at 37ºC and 42ºC which is required for normal growth and that cannot be fulfilled by any other chaperones and/or proteases in any of these bacterial species.

In a similar fashion, mutations in the *htr*A gene of *S. pneumonie* (Musa et al. 2004), *Klebsiella pneumonie* (Cortes et al. 2002) and *Listeria monocytogenes* 10403S (Wonderling et al. 2004) did have no effect in the growth rates at 37ºC, but did reduce the growth and/or survival rates at higher temperatures and in other bacteria as mentioned by Wessler (Wessler et al. 2017). In *L. monocytogenes* 10403S the sensitivity to high osmolarity was also increased in the mutant.

In the present work, there seem to be an increase in the expression of the HtrA band with the growth temperature in the *Y. pseudotuberculosis* YPIII wild type strain, (Fig. 3A). This result is in agreement with the increase in the *rpo*E gene with an increasing growth temperature. This gene encodes a transcriptional regulator which is related to the transcription of the *htr*A gene in *E. coli* and is likely to fulfill a similar role in *Y. pseudotuberculosis*. *Yersinia pseudotuberculosis* HtrA protein is important for cell growth at 37ºC, it is essential at 42ºC, and its molecular features suggest an unshared involvement in protein folding homeostasis at high temperatures.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Please contact to the authors for all request.

Competing interest

The authors declare that they have not competing interest.

Funding

This work was supported in part by grants VIEP-BUAP; ID: 00249 to LJMM and ID: 00122 to LSU), and a grant Ref. 48190 to LJMM from CONACYT-MEXICO.

Author contributions

LJMM and EEG designed this study. EEG performed the experiments. EEG, LSU, RRS and LJMM interpreted the results. RRS supported the project by critical evaluation of experimental results, "*in silico*" analysis and providing facilities required. EEG, RRS LSU and LJMM participated substantially in writing the manuscript, critical evaluation and modifications. LJMM supervised the study. All authors read and approved the final manuscript for publication.

Acknowledgements

This work was supported in part by a grant (Ref. 48190 to LJMM) and scholarship (173674 to EEG) from CONACYT-MEXICO. We would like to thank to Dominique Schneider, Ph.D. for the pDS132 plasmid and Candice Rigoulay, Ph.D. for the antibodies anti HtrA, kindly donate. RRS thanks for their support to UNAM supercomputing facilities, project SC151IR47.

References

1. Alexeyev MF, Shokolenko IN, Croughan TP (1995) Improved antibiotic-resistance gene cassettes and omega elements for *Escherichia coli* vectors construction and in vitro deletion/insertion mutagenesis. Gene 160:63–67
2. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22:195–201
3. Bæk KT, Christina SV, Lone B (2011) HtrA chaperone activity contributes to host cell binding in *Campylobacter jejuni*. Gut Pathogens 3:13. DOI:10.1186/1757-4749-3-13
4. Bäumler AJ, Kusters JG, Stojiljkovic I, Heffron F (1994) *Salmonella typhimurium* loci involved in survival within macrophagues. Infect Immun 62:1623–1630
5. Clausen T, Southan T, Ehrmann M (2002) The HtrA family of proteases: Implications for protein composition and cell fate. Mol Cell 10:443–455

6. Cortes G, de Astorza B, Benedi VJ, Alberti S (2002) Role of the *htr*A gene in *Klebsiella pneumoniae* virulence. Infect Immun 70:4772–4776
7. Craig JE, Nobbs A, High NJ (2002) The extracytoplasmic sigma factor, σE, is required for intracellular survival of nontypeable *Haemophilus influenza* in J774 macrophagues. Infect Immun 70(2):708
8. Farn J, Roberts M (2004) Effect of inactivation of the HtrA like serine protease DegQ on the virulence of *Salmonella enterica* serovar Typhimurium in mice. Infect Immun 72:7357–7359
9. Gloeckl S, Joel DA, Tyndall SH, Stansfield PT, Wilhelmina MH (2012) The active site residue V266 of Chlamydial HtrA is critical for substrate binding during both in vitro and in vivo conditions. J Mol Microbiol Biotechnol 22:10–16
10. Hansen G, Hilgenfeld R (2013) Architecture and regulation of HtrA-family proteins involved in protein quality control and stress response. Cell Mol Life Sci 70:761–775
11. Hoy B, Hans B, Silja W (2013) The stability and activity of recombinant *Helicobacter pylori* HtrA under stress conditions. Journal of Basic Microbiology 53:402–409
12. http://smart.embl.de/ Accessed 23 Feb 2015
13. Huesgen PF, Helder M, Xuan TL, Perthold M, Holger S, Iwona A, Christiane F (2011) Recombinant Deg/HtrA proteases from *Synechocystis* sp. PCC 6803 differ in substrate specificity, biochemical characteristics and mechanism. Biochem J 435:733–742
14. Kaplan W, Littlejohn TG (2001) Swiss-PDB Viewer (Deep View). Brief Bioinform 2:195–197
15. Kim S, Robert TS (2012) Cage assembly of DegP protease is not required for substrate-dependent regulation of proteolytic activity or high-temperature cell survival. PNAS 109(19):7263–7268
16. Krojer T, Sawa J, Huber R, Clausen T (2010) HtrA proteases have a conserved activation mechanism that can be triggered by distinct molecular cues. Nature Structural Molecular Biology 17:844–852
17. Krüger E, Völker U, Hecker M (1994) Stress induction of *clp*C in *Bacillus subtilis* and its involvement in stress tolerance. J Bacteriol 176:3360–3367
18. Lewis C, Skovierova H, Rowley G, Rezuchova B, Homerova D, Stevenson A, Spencer J, Farn J, Kormanec J, Roberts M (2009) *Salmonella enterica* Serovar Typhimurium HtrA: regulation of expression and role of the chaperone and protease activities during infection. Microbiology 155:873–881. doi:10.1099/mic.0.023754-0
19. Li SR, Dorrell N, Everest PH, Dougan G, Wren BW (1996) Construction and characterization of a *Yersinia enterocolítica* O:8 high-temperature requirement (*htr*A) isogenic mutant. Infect Immun 64:2088–2094
20. Lopes R-GM, Blini ME, Tempone AJ, Jablonski AJ, Klitzke CF, da Silveira EKX, Vieira PFC, Vidal PMC (2009) Cloning, expression and characterisation of an HtrA-like serine protease produced in vivo by *Mycobacterium leprae*. Mem. Inst. Oswaldo Cruz, Rio de Janeiro. 104(8): 1132–1138
21. Martínez-Castilla LP, Rodríguez-Sotres R (2010) A score of the ability of a three-dimensional protein model to retrieve its own sequence as a quantitative measure of its quality and appropriateness. PLoS One 5(9):e12483. DOI:10.1371/journal.pone.0012483

22. Musa YI, Kerr AR, McCluskey J, Mitchell TJ (2004) Role of HtrA in the virulence and competence of *Streptococcus pneumonia*. Infect Immun 72 (6): 3584–3591. DOI: 10. 1128 /IAI.72.6.3584-3591
23. Pallen MJ, Wren BW (1997) The HtrA family of serine proteases. Mol Microbiol 26:209–221
24. Parkhill J, Wren BW, Thomson NR, Titball RW, Holden MT, Prentice MB, Sebaihia M, James KD, Churcher C, Mungall KL, Baker S, Basham D, Bentley SD, Brooks K et al.„Cerdeño-Tarraga AM Chillingworth T, Croin A, Davis RM, Davis P, Dougan G, Feltwell T, Hamlin N, Holroyd S, Jagels K, Karlyshev AV, Leather S, Moule S, Oyston PC, Quail M, Rutherford K, Simmonds M, Skelton J, Stevens K, Whitehead S, Barrel BG, (2001) Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature. 413: 523–527
25. Pedersen LL, Radulic M, Doric M, Abu KY (2001) HtrA homologue of *Legionella pneumophila*: an indispensable element for intracellular infection of mammalian but not protozoan cells. Infect Immun 69(4):2569
26. Phillips RW, Roop RM (2001) *Brucella abortus* HtrA functions as an authentic stress response protease but is not required for wild-type virulence in BALB/c mice. Infect Immun 69:5911–5913
27. Putzker M, Sauer H, Sobe D (2001) Plague and other human infections caused by *Yersinia* species. Clin Lab 47:453–466
28. Resto S, Sweger D, Widen RH, Valkov N, Anderson B (2000) Transcriptional activation of the *htr*A (high-temperature requirement A) gene from *Bartonella henselae*. Infect Immun 68:5970–5978
29. Rigoulay C (2005) Comparative analysis of the roles of HtrA-like surface proteases in two virulent *Staphylococcus aureus* strains. Infect Immun 73:563–572
30. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
31. Schneider D, Philippe N, Alcaraz JP, Coursange E, Geiselmann J (2004) Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. Plasmid 51(3):246–255
32. Simon R, Priefer U, Puhler A (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Biotechnology 1:784–791
33. Skorko-Glonek J, Krzewski K, Lipinska B, Bertoil E, Tanfani F (1995) Comparison of the structure of wild-type HtrA heat shock protease and mutant HtrA proteins. A Fourier transform infrared spectroscopic study. J Biol Chem 270:11140–11146
34. Skurnik M (1999) Molecular genetics of *Yersinia* lipopolysaccharide. In Genetics of bacterial polysaccharides. Edited by J. B. Goldberg. Boca Raton, FL: CRC Press. 23–51
35. Spiess C, Beil A, Ehrmann M (1999) A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell 97:229–347
36. Wessler S, Schneider G, Backert S (2017) Bacterial serine protease HtrA as a promising new target for antimicrobial theraphy? Cell Communication Signaling 15:4. DOI:10.1186/s12964-017-0162-5
37. White MJ, John PS, Daniel J, Bretl H, He RM, Penoske SST, Thomas CZ (2011) The HtrA-like serine protease PepD interacts with and modulates the *Mycobacterium tuberculosis* 35-kDa antigen outer

envelope protein. PLoS ONE. DOI: 10. 1371/journal.pone.0018175

38. Williams K, Oyston PCF, Dorrell N, Li SR, Titball RW, Wren BW (2000) Investigation into the role of the serine protease HtrA in *Yersinia pestis* patogénesis. FEMS Microbiol Letters 186:281–286
39. Wonderling LD, Wilkinson BJ, Bayles DO (2004) The *htr*A (*deg*P) gene of *Listeria monocytogenes* 10403S is essential for optimal growth under stress conditions. Appl Environ Microbiol 70:1935–1943
40. Yamamoto T, Hanawa T, Ogata S, Kamiya S (1996) Identification and characterization of the *Yersinia enterocolitica gsr*A gene, which protectively responds, to intracellular stress induced by macrophage phagocytosis and to extracellular environmental stress. Infect Immun 64:2980–2987
41. Young K, Kim K (2005) Structure and function of HtrA family proteins, the key players in protein quality control. J Biochem Mol Biol 38:266–274
42. Zhang Y (2008) I-TASSER server for protein 3D structure prediction. BMC Bioinformatics 9:40. DOI:10.1186/1471-2105-9-40
43. Zarzecka U, Modrak-Wójcik A, Figaj D, Apanowicz M, Lesner A, Bzowska A, Lipinska B, Zawilak-Pawlik A, Backert S, Skorko-Glonek J (2019) Properties of the HtrA protease from bacterium *Helicobacter pylori* whose activity is indispensable for growth under stress conditions. Front Microbiol 10:961. doi:10.3389/fmicb.2019.00961
44. Zawilak-Pawlik A, Zarzecka U, Żyła-Uklejewicz D, Lach J, Strapagiel D, Tegtmeyer N, Böhm M, Backert S, Skorko-Glonek J (2019) Establishment of serine protease *htrA* mutants in *Helicobacter pylori* is associated with *secA* mutations. Scientific Reports 9:11794. doi.org/10.1038/s41598-019-48030-6

Figures

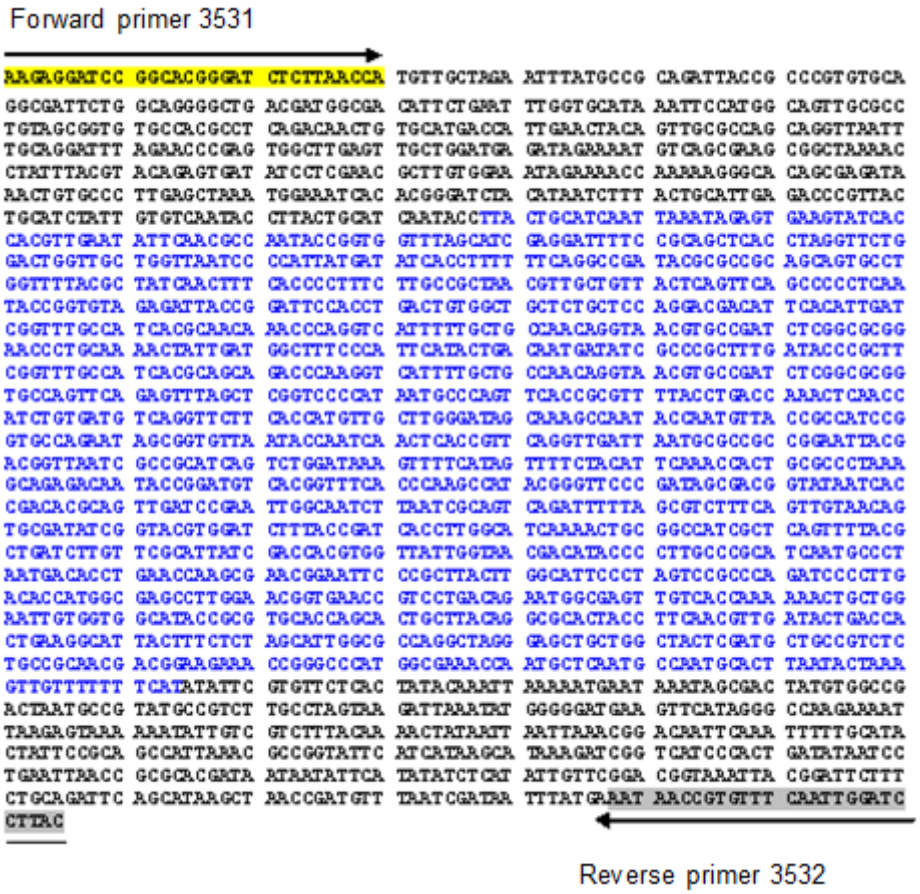

Figure 1

Sequence of a 2315 bp fragment of DNA including the htrA gene (blue) of Yersinia pseudotuberculosis.

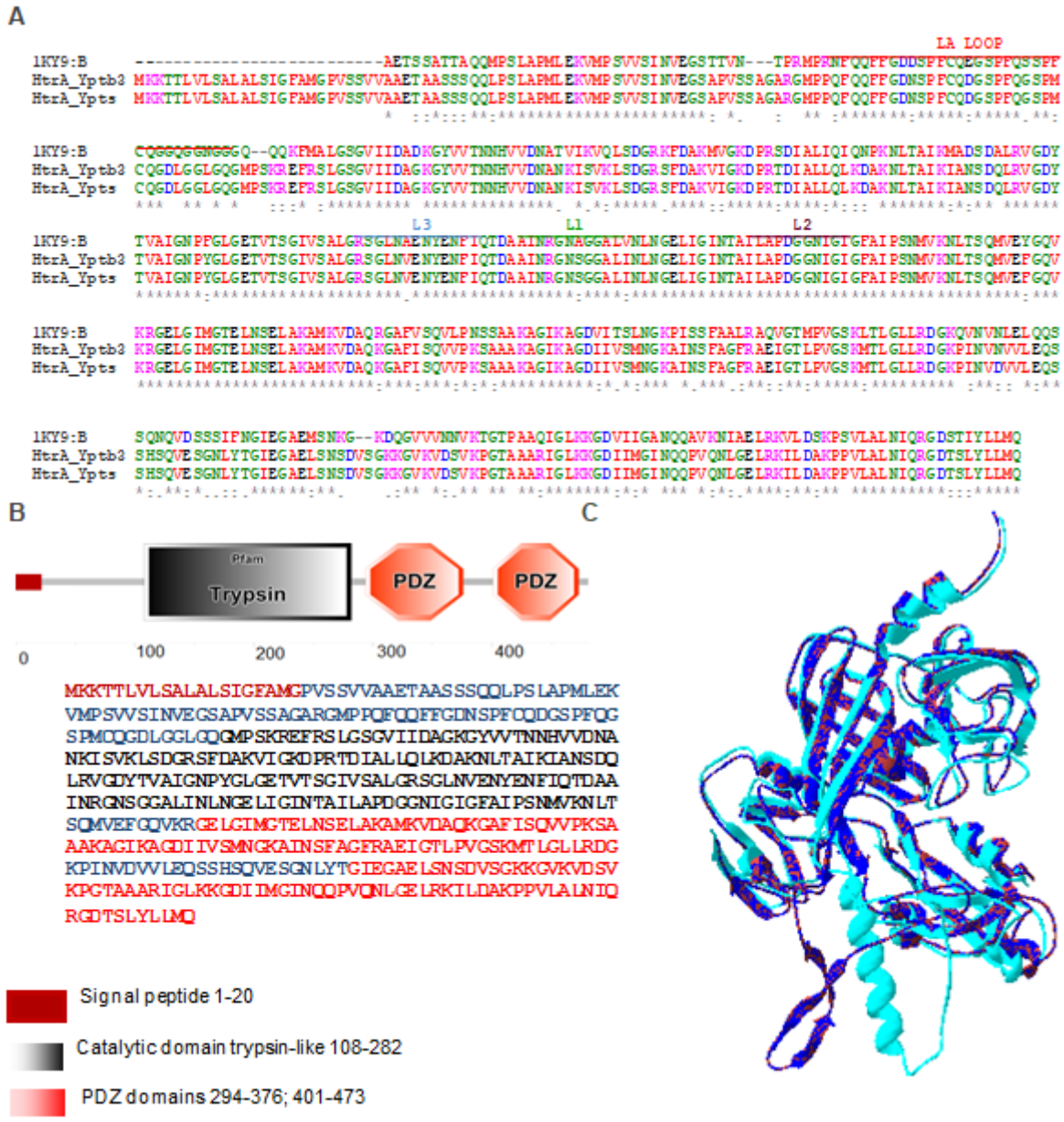

Figure 2

A.- Amino acid alignment (CLUSTALO http://www.ebi.ac.uk/Tools/msa/clustalo/) of HtrA protein from E. coli 1KY9 S210A:B (1KY9:B), Yersinia pseudotuberculosis (HtrA_YptB3), and Yersinia pestis (HtrA_Ypts) strains. B.- Schematic representation of the predicted domain organization for the amino acid sequence of the HtrA protein from Y. pseudotuberculosis (http://smart.embl.de/.). The entire sequence was 481 amino acids long, with a predicted molecular mass of 49.8 kDa and a predicted signal peptide. The mature protein was predicted to be 47.1 kDa without signal peptide, and have a pI 8.76. C.- In silico models of HtrA from Y. pseudotuberculosis (brown) and 1KY9 S210A:B Escherichia coli (light blue). The models were prepared by threading using Swiss-Pdb-Viewer. Rd.HMM scores were 102.7, E-value 1.2x10-24, ratio score to sequence length 0.233, for Y. pestis prediction, and 110.6, with E-value 5.1 x 10-27 and score ratio to sequence length 0.251 for Y. pseudotuberculosis prediction. The HMMer alignment showed

coincidence between the backbone and the threaded residues in all but a small 40 amino acid segment at the amino-terminus. The model is therefore imperfect in this side.

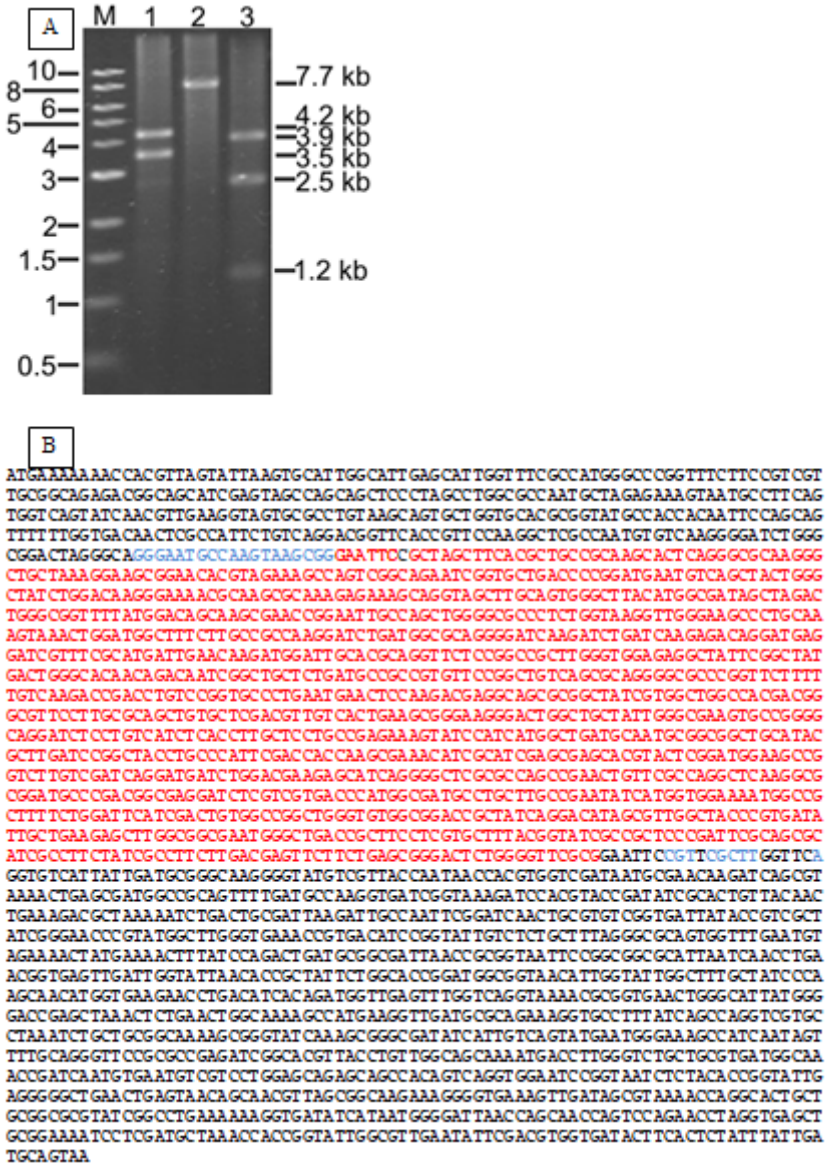

Figure 3

Restriction profile of pAHTK99 with Bam HI, Hind III and Eco RI. A.- Restriction profile of pAHTK99 plasmid. M; 1 kb mw marker. line 1; Bam HI restriction, two fragments are released 4.2 kb vector and 3.5 kb htrA::km construction. line 2; Hind III restriction, 7.7 kb linearized plasmid. line 3; Eco RI restriction, three fragments are released 3.9 kb and 2.5 kb correspond at pAHTRA46 plasmid and 1.2 kb fragment correspond at kanamycin cassette. B. Analysis of sequence of htrA::Km mutant in Yersinia pseudotuberculosis strain Kanamycin cassette insertión (red) into the Eco RI site of htrA gen (black) of Y. pseudotuberculosis YPIII.

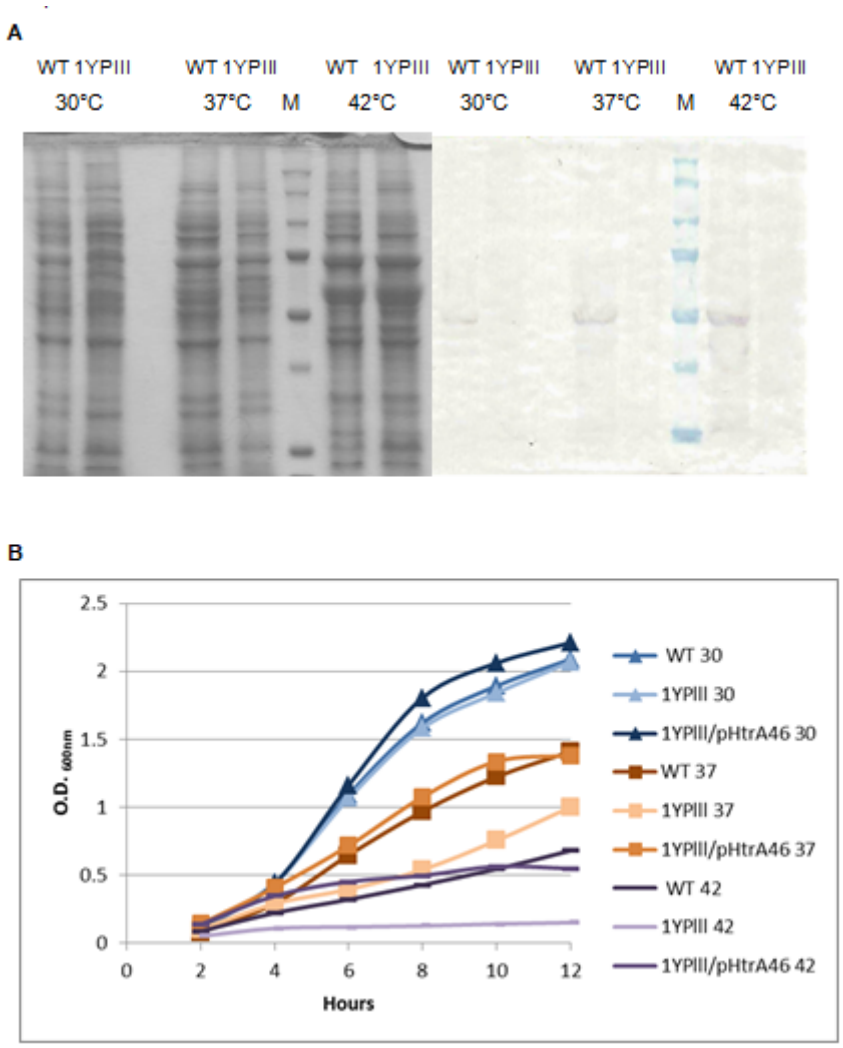

Figure 4

A.- PAGE-SDS (Coomasie) and Western blot assay using antibodies anti HtrA of Staphylococcus aureus. Crude cellular extract protein obtained of Yersinia pesudotuberculosis YPIII wild type and 1YPIII (htrA-) mutant strains, incubated to 30°C (wt and htrA-); to 37°C (wt and htrA-); line M, mw marker (prestained SDS-PAGE standards, broad range, BIO-RAD); and to 42°C (wt and htrA-). Any 1YPIII (htrA-) strain produce the HtrA protein. B.- Growing curves of Y. pseudotuberculosis YPIII wild type strain, 1YPIII (htrA-) mutant strain and 1YPIII/pHTRA46 complemented strain to 30, 37 and 42ºC. The O.D.600 nm of each culture was measured every two hours and plotted. The results represent the values of three independent experiments.

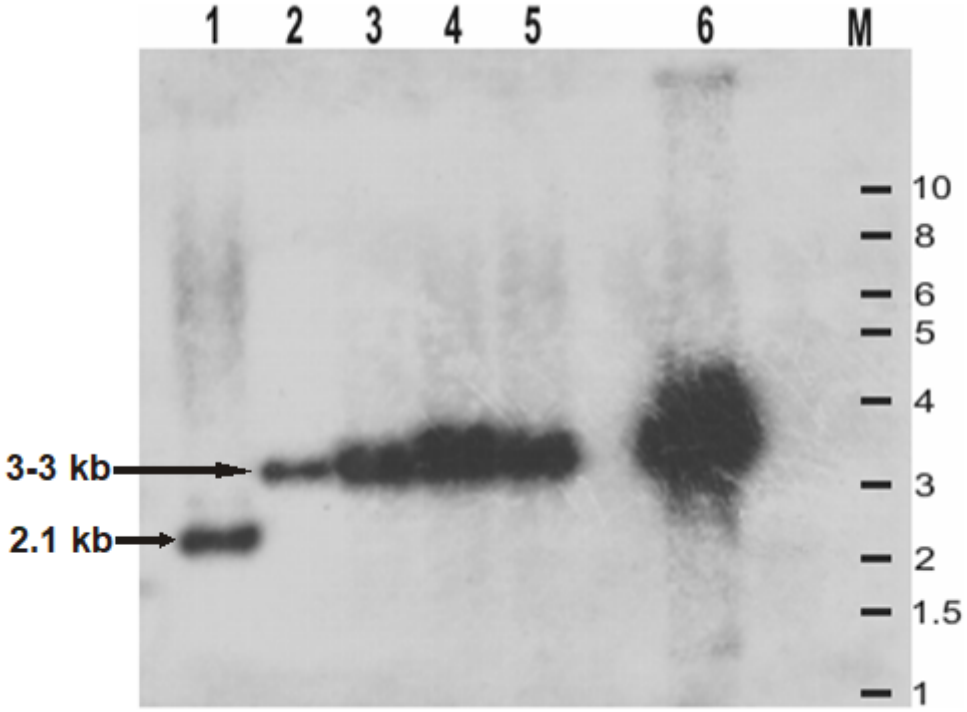

Figure 5

Film of the Southern blot hybridization using DNA from Yersinia pseudotuberculosis strains restricted with the enzymes Ava I and Cla I, using 2.1 kb fragment obtained pAHTRA46 restricted with the enzymes Ava I and Cla I as probe. Lane 1, genomic DNA of Y. pseudotuberculosis YPIII WT. Lane 2, clon 1N DNA. Lane 3, clone 13N DNA. Lane 4, clone 1YPIII DNA. Lane 5, clone 4YPIII DNA. Lane 6, plasmid pDHK4 under Ava I and Cla I restriction. Lane M, molecular weight marker 1 kb Biolabs®.

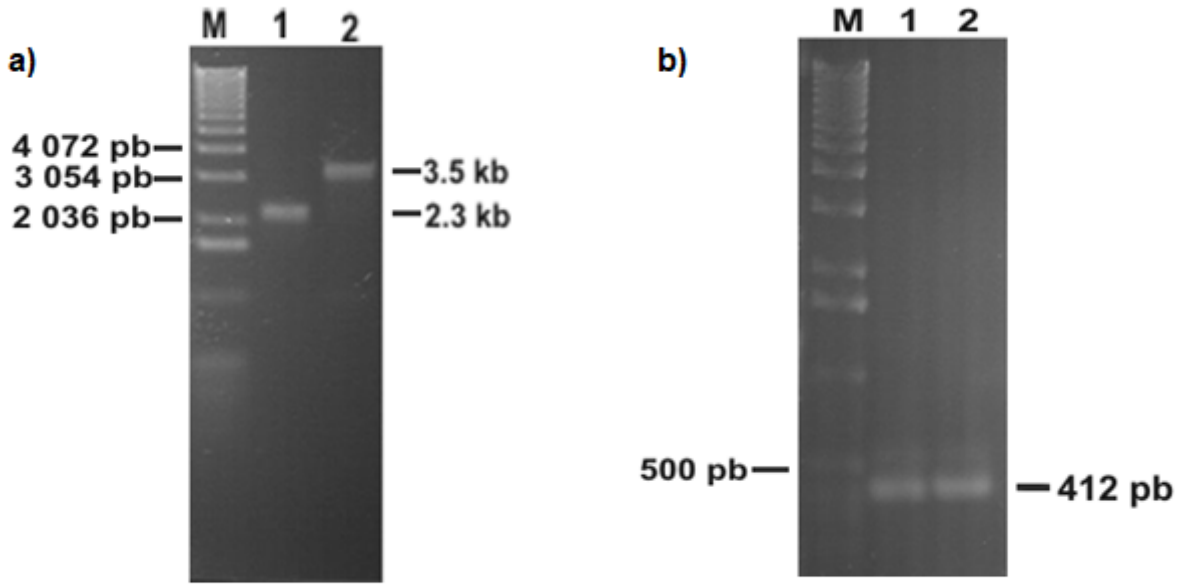

Figure 6

Amplification by PCR of htrA::Km gene and 16S of Yersinia pseudotuberculosis. Amplification of gen htrA::Km of Y. pseudotuberculosis YPIII. a. Line M, mw marker. Line 1 amplification of 2.3 kb fragment with primers 3531 y 3532, using DNA of wild type Y. pseudotuberculosis YPIII as template. Line 2 amplification of 3.5 kb fragment (htrA::Km) with primers 3531 y 3532, using DNA of 1YPIII Y. pseudotuberculosis clone. b. Line M, mw marker. Amplification using genomic DNA of Y. pseudotuberculosis YPIII wt (line 1) and 1YPIII strain (line 2), with primers 16S Yersinia specific U16S38D and D16S47R, resulting an amplification of 412 pb both.

Supplementary Files

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