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Regulation of the *htpX* Gene of *Xylella fastidiosa* and Its Expression in *E. coli*

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Abstract. *Xylella fastidiosa* was the first phytopathogen to be completely sequenced, and its genome revealed several interesting features to be used in functional studies. In the present work, the *htpX* gene, which encodes a protein involved in the heat shock response in other bacteria, was analyzed by RT-PCR by using cells derived from different cultural conditions. This gene was induced after a temperature upshift to 37°C after growth in minimal medium, XDM, but showed constitutive expression in rich medium or in XDM plus plant extracts. Sequences upstream to the *htpX* gene, containing a putative regulatory region, were also transferred to *E. coli*, and the thermoregulation was maintained in the new host, since it was constitutively transcribed at 37°C or 45°C in all culture media tested, but not at 28°C in minimal culture medium. The gene was also cloned into the expression vector pET32Xa/LIC, and the expression of the corresponding protein was confirmed by Western blotting.

Xylella fastidiosa [24] is a xylem-limited bacterium responsible for several diseases in tropical and subtropical regions, mainly in America. The bacterium causes symptoms in different families of mono- and dicotyledons, such as plum, peach, elm, sycamore, almond, oleander, grapevine, and others (for a review see [8]). In Brazil the major impact of this pathogen was found in sweet orange and coffee plants [1, 20].

The strain 9a5c, isolated from *Citrus sinensis* in São Paulo, Brazil [3], was the first phytopathogen to be completely sequenced [23]. The 2.7-Mb genome revealed 2,838 genes, but only 46% showed homology with other proteins with known functions. Interestingly, no known pathogenicity-related genes such as *avr* or *hrp* genes have been found in the genome of *X. fastidiosa*, indicating that other unsuspected factors might be involved in the elicitation of the symptoms. Currently it is assumed that the pathogen blocks the xylem vessel of infected plants either by secreting some exopolysaccharide or by mechanical occlusion of the vessels leading to a hydric stress and to the appearance of the symptoms [7, 8, 14]. The genome annotation revealed several important ORFs to the comprehension of the interaction of *X. fastidiosa* with its hosts, and also the comparative genomic studies suggested some potential genes involved in pathogenicity [2, 4, 6, 17]. Among these genes, we assumed that some genes involved in adaptation and in response to environmental conditions could be important to offer proper conditions for full bacterial development. The category of heat shock genes is induced after temperature upshift, and the response is characterized by synthesis of several proteins. The major and more studied heat shock genes include *GroEL*, *DnaJK*, *HlsVU*, which have also been found in several bacterial groups as well in *X. fastidiosa*. The proteins encoded by these genes are important in the refolding of denatured proteins or in the degradation of abnormal proteins in the cell. HtpX is a protease that belongs to the heat shock protein (hsp) family, and it is induced in *E. coli* when variations in environmental conditions occur [10, 16, 18, 22].

The present paper describes the induction of *X. fastidiosa htpX* under different growth conditions by transcription analysis (RT-PCR). It was found that the gene was regulated by heat shock and by using complex and minimal medium enriched with plant extracts. The gene was also cloned into an expression vector, and the expression of the corresponding pro-

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tein was confirmed by Western blotting by using His-tag antiserum.

Materials and Methods

Strains and growth conditions. *X. fastidiosa* 9a5c was routinely grown in PW medium [5] at 28°C. RNA was extracted from cells subjected to different treatments after growth in PW medium for 3 days and further transfer to the minimal medium XDM [12] or XDM enriched with plant extracts for four days. Plant extracts were prepared from different plant hosts (*Citrus sinensis*, *Citrus reticulata* cv Ponkan, and *Coffea arabica*) as described [13] and added to the XDM medium in a final concentration of 1%. *E. coli* was grown in LB medium [21] at 37°C, and treatments were similar to those used for *X. fastidiosa* with M9 as minimal medium [21].

Primers design. Two different sets of primers were used: the first set, named "TR", spanned a 100-bp upstream region with the following sequences: 2625F: 5′CGCTAAACATGGCTAATAAC 3′ and 2625R: 5′TTATCGCTTGATATTCACTG 3′. The second set of primers, "PR", comprising the entire gene, was employed for cloning and in the RT-PCR reactions, 2625petD 5′ GGTATTGAGGGTCGCATGTT-GACTCGTATTG 3′ and 2625petR 5′AGAGGAGAGTTAGAGCCT-TATTGTCTAGCC 3′.

PCR and cloning. The *X. fastidiosa* DNA was isolated according to Rosato et al. [19]. The PCR reactions were performed in a final volume of 25 μL using approximately 50 ng DNA, buffer 1X; 100 μM dNTP mix; 10 pmol of primers (TR or PR); and 0.5 U *Taq* DNA polymerase. The reactions were carried out in an MJ Thermo-Cycler (USA) by using the following program: 3 min at 94°C and 30 cycles of 1 min at 94°C, 30 s at 52°C, 1 min 30 s at 72°C, and 5 min at 72°C for the final extension. After a simple purification procedure, using the Concert kit (Gibco-BRL, USA), the PCR products were cloned either into pGEM T-easy (Promega, USA) or into pET 32 Xa/LIC expression vector (Novagen, USA) by using *E. coli* DH5α as host. The correct insertion of the fragments was confirmed by sequencing reactions with the kit Big Dye terminator (Applied Biosystems, USA) in the ABI 377 sequencer (Applied Biosystems, USA).

RNA extraction and RT-PCR reactions. The RNA extraction was performed with the hot phenol method, which consists, briefly, of a lysis reaction using lysozyme (0.5 mg/mL) and SDS (1%), followed by hot phenol (pH ~ 5) and chloroform extraction. The sample was then precipitated with ethanol and sodium acetate 3 M at −70°C. The RNA was treated with DNase (10 U) at 37°C for 1 h. The concentration of total RNA was estimated by denaturing agarose gel electrophoresis of the 16S and 23S products. Synthesis of cDNAs was performed with random primers (Gibco-BRL, USA) and 0.5 U of reverse transcriptase (SuperScriptII, Invitrogen, USA), and the final concentration was assessed by PCR amplification with 16S primers. The RT-PCR reactions were performed in a final volume of 25 μL with ~50 ng cDNA buffer 1X, 120 μM dNTP mix, 10 pmol of PR primers, and 2.5 U *Taq* DNA polymerase. The reactions were carried out in an MJ Thermo-Cycler under the cycling conditions of 3 min at 94°C and 30 cycles of 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C.

Protein expression. The *htpX* gene was cloned into the pET 32 Xa/LIC expression vector and transferred first to *E. coli* DH5α and then to the expression hosts BL21(λDE3) and BL21(λDE3)pLysS. The recombinants were grown overnight in LB + ampicillin at 37°C. This culture was then transferred to LB medium until A_{600} of 0.6 when the IPTG inductor was added (1 mM or 2 mM), and cell aliquots were collected after 2, 4, 6, and 8 h. The cells were disrupted following the standard protein extraction procedure, and SDS-PAGE was performed [21].

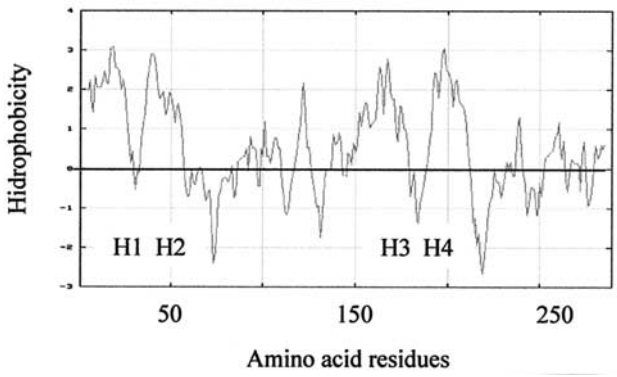

Fig. 1. Hydropathy profile of *X. fastidiosa* HtpX according to Kyte and Doolittle [11] parameters. Hydrophobic regions (H1–H4) are shown in the figure.

Western blot analysis with anti-His tag serum (Santa Cruz Biotechnology, USA) was carried out to check the identity of the expressed protein. Detection was performed with anti-IgG alkaline phosphatase conjugate followed by chemiluminescent detection (CSPD) (Roche, USA).

Results and Discussion

Sequence analysis. The *htpX* gene of *X. fastidiosa* comprises 870 bp, and the analysis of amino acid sequence revealed a high level of identity with the *E. coli* homologue (53%), where this gene has been analyzed in more detail. The expression of *E. coli htpX* is σ^{32}-dependent and induced by temperature upshift [9, 10]. It has been suggested that *htpX* is under control of the Cpx regulatory system, which is responsible for the quality control of the plasma membrane proteins [18]. A detailed analysis by using the BLASTP program and the primary sequence of *X. fastidiosa* HtpX showed a conserved peptidase domain spanning from residue 82 to 289. Analysis of the hydropathy profile of the putative protein encoded by the *X. fastidiosa* gene employing the parameters set by Kyte and Doolittle [11] indicates that it is a protease containing four conserved hydrophobic regions (Fig. 1), H1 to H4, similar to those described in *E. coli* [22]. Also, a zinc metalloprotease-active site, HEXXH, embedded within the domain II, was found, suggesting that *X. fastidiosa* HtpX is a metalloprotease.

Transcription. The transcription of the *htpX* gene was assessed in *X. fastidiosa* grown in PW and XDM media after 24 h of incubation at both temperatures of 28°C and 37°C by using RT-PCR. As this reaction showed variations in the intensity even though RNA concentrations had been estimated previously, another control of cDNA concentration was introduced by performing RT-PCR reactions with 16S primers (Fig. 2a). Transcription of

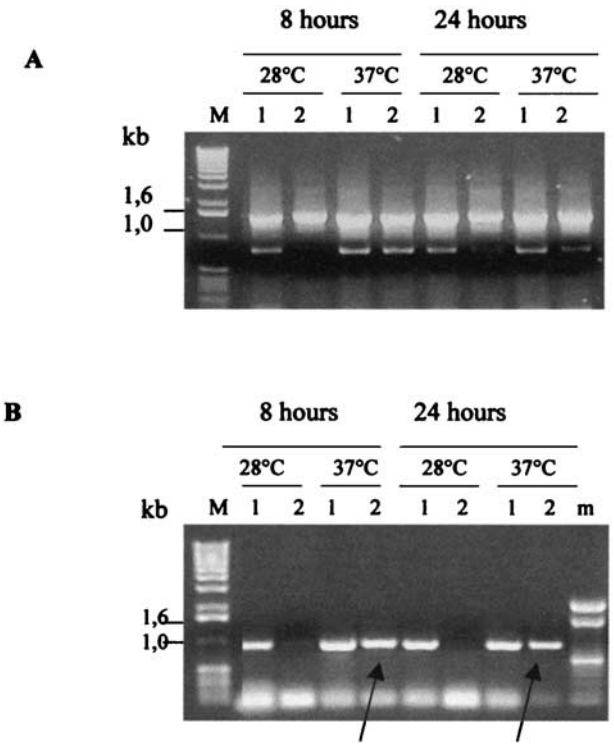

Fig. 2. RT-PCR products of *X. fastidiosa* grown at 28°C and 37°C. The bacterium was grown during 8 and 24 h in rich PW (1) and minimal XDM (2) media. (A) RT-PCR with the 16S primer; (B) RT-PCR with PR primers. M, 1 kb molecular weight (Invitrogen, USA); m, 100 bp molecular weight (Invitrogen, USA). The arrows show the transcription of *htpX* after temperature upshift in XDM.

htpX was detected in PW medium when both temperatures were used; however, in the XDM medium the transcript was detected only at 37°C (Fig. 2b). These results indicate that *htpX* is controlled by temperature in minimal medium, but in rich medium the gene is under another control. Similarly, when the minimal medium was enriched by plant extracts, the transcription of the *htpX* gene was observed in all treatments (Fig. 3). Therefore, in *X. fastidiosa* the gene is regulated by both temperature upshift and existence of some nutrients in the culture medium. Regulation of heat shock genes by nutritional conditions has been described in *E. coli*. During carbon starvation, the cells induce several proteins, some of which are also induced during heat shock stress [9, 15]. Most of them are under control of a regulatory protein, RpoH, which encodes the sigma factor σ^{32} induced during heat and starvation [9]. Several promoter regions upstream of the *rpoH* gene have been described in *E. coli*, including a catabolite-sensitive promoter [15]. In the present case, *X. fastidiosa* is not under starvation, since after 3 days of incubation the cells are in the exponential phase of growth [17], suggesting another kind of control. Since HtpX is involved with protease activity in *E. coli*, we reasoned that if the same function is performed in *X. fastidiosa* the induction of *htpX* gene in rich medium could help in the rapid degradation/refolding of the abnormal proteins formed. Therefore, the induction of such a gene could be important to maintain an appropriate growth rate. Interestingly, in *X. fastidiosa* a high number of genes are expressed in rich medium compared with minimal medium [17], and the reasons for such induction are still unclear. Most of these expressed genes encode hypothetical/conserved proteins, but other genes related to DNA modification, phage activities, as well as heat shock genes, have also been found.

The transcription of the *X. fastidiosa htpX* gene, containing the upstream regulatory region, was also detected in *E. coli* cultivated in all media at 37°C (Fig. 4) and 45°C (data not shown), similarly to the pattern obtained with *X. fastidiosa*. The temperature of 28°C was also tested, and no transcription was observed in *E. coli* grown either in minimal or complex media (data not shown), possibly owing to the non-expression of the σ^{32} factor at this temperature. It is known that the expression of the σ^{32} factor in *E. coli* occurs after temperature upshift from 30°C to 42°C [15]. It is conceivable that in *X. fastidiosa* the temperature-dependent regulatory region should be within the 100-bp upstream region of the gene, and it was able to respond properly even in another host (*E. coli*).

Protein expression. The production of HtpX in *E. coli* is important for future protein purification in large-scale enabling structural studies. Therefore, preliminary attempts to express this protein from an expression system were performed. The pET 32 Xa/LIC system was used to express HtpX in the *E. coli* hosts, BL21(λDE3) and BL21(λDE3)pLysS. The higher level of expression was obtained by using the BL21(λDE3)pLysS host induced with 2 mM IPTG during 4 h at 37°C. The protein was obtained in the insoluble fraction of the cellular extract, and attempts at solubilization at lower temperatures (up to 20°C) were unsuccessful. The high insolubility of the protein is commonly found in the expression of different proteins in *E. coli*, and it has been attributed to protein aggregation forming the inclusion bodies [25]. Additionally, HtpX shows hydrophobic residues as predicted in the theoretical model (11; see also Fig. 1), and the topology studies of the HtpX from *E. coli* indicate that the NH_2 end of the protein is immersed within the membrane [22]. The presence of the histidine residues, fused to the protein, was confirmed by Western blot analysis by using an anti-His tag serum, which will help future purification procedures (Fig. 5).

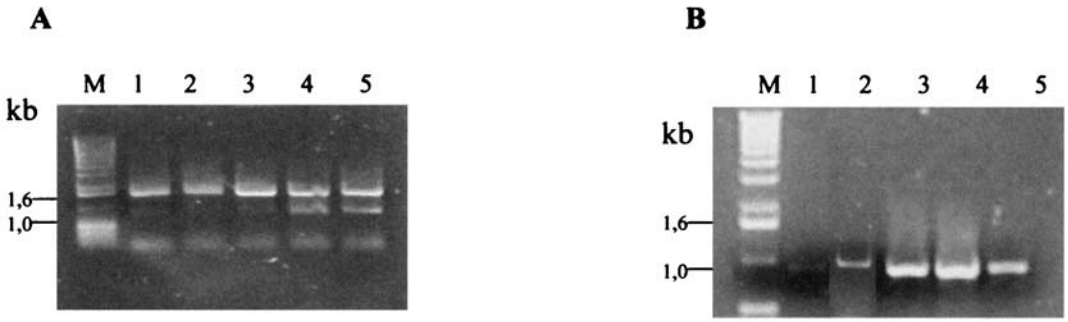

Fig. 3. RT-PCR products of *X. fastidiosa* grown in different media. (A) RT-PCR with the 16S primer. (B) RT-PCR with PR primers. (1) Minimal medium XDM; (2) XDM+ sweet orange extract; (3) XDM+ "Ponkan" extract; (4) XDM+ coffee extract; and (5) PW medium. M, 1 kb molecular weight (Invitrogen, USA).

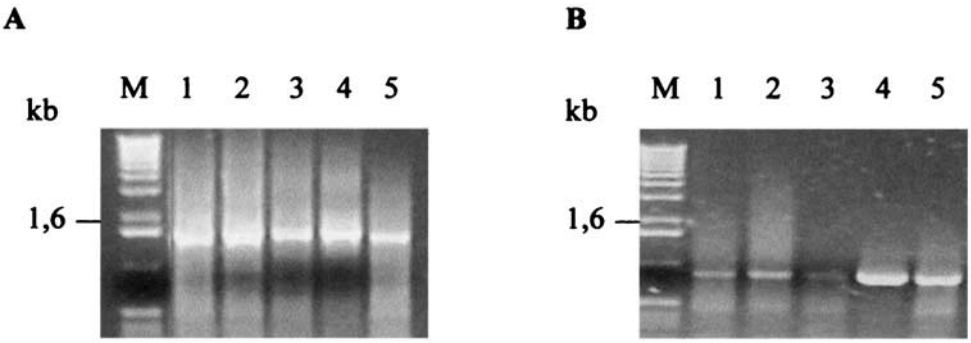

Fig. 4. RT-PCR products of *E. coli* recombinant cells grown in different media. (A) RT-PCR with the 16S primers; (B) RT-PCR with PR primers. (1) Minimal medium M9; (2) M9+ sweet orange extract; (3) M9+"Ponkan" extract; (4) M9+ coffee extract; and (5) LB rich medium. M, 1 kb molecular weight (Invitrogen, USA).

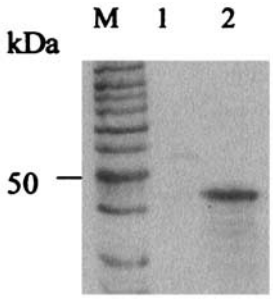

Fig. 5. Western blot of induced BL21(λDE3)pLysS cells containing pET 32 Xa/LIC −HtpX with anti-His tag serum. M, protein ladder Benchmark (Invitrogen, USA). (1) Non-induced control; (2) induced with 2 mM IPTG.

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Literature Cited

1. Beretta MJG, Harakava R, Chagas CM (1996) First report of *Xylella fastidiosa* in coffee. Plant Dis 80:821
2. Bhattacharyya A, Stilwagen S, Reznik G, et al. (2002) Draft sequencing and comparative genomics of *Xylella fastidiosa* strains reveal novel biological insights. Genome Res 12:1556–1563
3. Chang CJ, Garnier M, Zreik L, Rossetti V, Bove JM (1993) Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. Curr Microbiol 27:137–142
4. Costa de Oliveira RC, Yanai G, Muto NH, Leite DB, Souza AA, Coletta-Filho HD, Machado MA, Nunes LR (2002) Competitive hybridization on spotted microarrays as a tool to conduct comparative genomic analysis of *Xylella fastidiosa* strains. FEMS Microbiol Lett 216:15–21
5. Davis MJ, French WJ, Schaad NW (1981) Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr Microbiol 6:309–314
6. Dow JM, Daniels MJ (2000) *Xylella* genomics and bacterial pathogenicity to plants. Yeast 17:263–271
7. Hopkins DL (1989) *Xylella fastidiosa*—xylem-limited bacterial pathogen of plants. Annu Rev Phytopathol 27:271–290
8. Hopkins DL, Purcell AH (2002) *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. Plant Dis 86:1056–1066
9. Jenkins DE, Auger EA, Matin A (1991) Role of RpoH, a heat shock regulator protein, in *Escherichia coli* carbon starvation protein synthesis and survival. J Bacteriol 173:1992–1996
10. Kornitzer D, Teff D, Altuvia S, Oppenheim AB (1991) Isolation, characterization, and sequence of an *Escherichia coli* heat-shock gene, *htpX*. J Bacteriol 173:2944–2953

11. Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132
12. Lemos EGM, Alves LMC, Campanharo JC (2003) Genomics-based design of defined growth media for the plant pathogen *Xylella fastidiosa*. FEMS Microbiol Lett 219:39–45
13. Mehta A, Rosato YB (2001) Differentially expressed proteins in the interaction of *Xanthomonas axonopodis* pv. *citri* with leaf extract of the host plant. Proteomics 1:1111–1118
14. Mircetich SM, Lowe SK, Moller WJ, Nyland G (1976) Etiology of almond leaf scorch disease and transmission of causal agent. Phytopathology 66:17–24
15. Nagai H, Yano R, Erickson JW, Yura T (1990) Transcriptional regulation of the heat shock regulatory gene *rpoH* in *Escherichia coli*: involvement of a novel catabolite-sensitive promoter. J Bacteriol 172:2710–2715
16. Neidhardt FC, Vanbogelen, RA, Lau ET (1983) Molecular cloning and expression of a gene that controls the high-temperature regulon of *Escherichia coli*. J Bacteriol 153:597–603
17. Nunes LR, Rosato YB, Muto NH et al (2003) Microarray analysis of *Xylella fastidiosa* provides evidence of coordinated transcription control of laterally transferred elements. Genome Res 13:570–578
18. Raivio TL, Silhavy TJ (2001) Periplasmic stress and ECF sigma factors. Annu Rev Microbiol 55:591–624
19. Rosato YB, Neto JR, Miranda VS, Carlos EF, Manfio GP (1998) Diversity of a *Xylella fastidiosa* population isolated from *Citrus sinensis* affected by citrus variegated chlorosis in Brazil. Syst Appl Microbiol 21:593–598
20. Rossetti V, Garnier MB, Beretta JM, Teixeira MJG, Quaggio ARR, Negri JA (1990) Presénce de bactéries dans lê xyléme d'orangers atteints de chorose variegée, une nouvelle maladie dês agrumes au Brésil. Comp Rend Acad Sci Ser 3:345–349
21. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
22. Shimohata N, Chiba S, Saikawa N, Ito K, Akyiama Y (2002) The Cpx stress response system of *Escherichia coli* senses plasma membrane proteins and controls HtpX, a membrane protease with a cytosolic active site. Genes Cells 7:653–662
23. Simpson AJG, Reinach FC, Arruda P et al. (2000) The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406:151–157
24. Wells JM, Raju BC, Hung HY, Weisburg WG, Mandelcopaul L, Brenner DJ (1987) *Xylella fastidiosa* gen-nov, sp-nov, Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas*-Spp. Int J Syst Bacteriol 37:136–143
25. Yasukawa T, Kaneiishii C, Maekawa T, Fujimoto J, Yamamoto T, Ishii S (1995) Increase of solubility of foreign proteins in *Escherichia coli* by coproduction of the bacterial thioredoxin. J Biol Chem 270:25328–25331