Investigation note

# Identification of proteins in *Candidatus* Liberibacter asiaticus to develop an immunoenzymatic detection method

Cynthia Guadalupe Rodríguez-Quibrera[1]
Isidro Humberto Almeyda-León[2§]
Felipe Roberto Flores de la Rosa[1]
José Luis Hernández-Mendoza[3]
María Antonia Cruz-Hernández[3]
Alberto Mendoza-Herrera[3†]

[1]Ixtacuaco Experimental Field-INIFAP. Martínez de la Torre-Tlapacoyan highway km 4.5, col. Rojo Gomez, Tlapacoyan, Veracruz. CP. 93600. Tel. 800 0882222, ext. 87601. (flores.felipe@inifap.gob.mx; rodriguez.cynthia@inifap.gob.mx). [2]General Terán Experimental Field-NIFAP. Montemorelos-China Highway km 31, col. Former Hacienda Las Anacuas, General Terán, Nuevo León, Mexico. CP. 67400. Tel. 55 38718700, ext. 83615. [3]Genomic Biotechnology Center-National Polytechnic Institute. Boulevard del Maestro s/n, esq. Elias Pina, col. Narciso Mendoza, Reynosa City, Tamaulipas, Mexico. CP. 88710. Tel. 55 57296300, ext. 87714. (jhernandezm@ipn.mx; macruzh@ipn.mx).

[§]Corresponding author: almeyda.isidro@inifap.gob.mx.

## Abstract

The objective of this work was to identify outer membrane proteins in the genome of *Candidatus* Liberibacter asiaticus (*C*Las) with potential for the development and optimization of an immunoenzymatic detection method. The study was conducted during 2019, and the Predict Protein web server, as well as the HhPred/HhSearch and Pfam databases, were used. Fifty-two outer membrane proteins were detected in the complete genome of *C*Las, of which 11 had not been previously characterized. Predictive analyses performed on the protein B8Y674 generated eight possible epitopes and four of them, experimentally evaluated in B cells, showed percentages of identity between 80 to 90%. *C*Las was detected by endpoint PCR from DNA extracted from Mexican lime with symptoms of Huanglongbing using primers designed on the sequence of the *Omp* gene encoding the protein B8Y674 and 95% identity was recorded between the generated sequences and sequences of *C*Las previously reported. The results obtained allow us to infer that the protein B8Y674 is a potential candidate to be used in the immunoenzymatic detection of *C*Las.

**Keywords:** diagnosis, huanglongbing, serology.

Reception date: July 2022
Acceptance date: November 2022

Huanglongbing (HLB), or Yellow Dragon Disease, is considered the most devastating citrus disease worldwide (Ding *et al*., 2015). The etiological agent is a gram-negative α-proteobacterium and three species are currently known to infect citrus: *Candidatus* Liberibacter asiaticus (*C*Las), *Candidatus* Liberibacter africanus (*C*Laf) and *Candidatus* Liberibacter americanus (*C*Lam) (Achor *et al*., 2020; Andrade *et al*., 2020). In Mexico, the most severe damage of this disease occurs in Mexican lime (*Citrus aurantifolia*) and Persian lime (*Citrus latifolia*), while in other regions of Asia and Africa and in countries such as Brazil, USA and Cuba, the damages are mainly reported in sweet orange (*Citrus sinensis*) (McCollum *et al*., 2016).

The official confirmation of the bacterium in plants is done by real-time PCR, since it is a very sensitive method and is considered the most reliable tool for its detection. However, this method is expensive; therefore, a fast and reliable test is required to corroborate its presence in the field. Immunoenzymatic methods or immunoassays, such as the enzyme-linked-immuno sorbent assays (Elisa) technique, are based on the antigen-antibody (Ag-Ab) reaction, are economical, rapid, and specific to detect low concentrations of Ag or Ab and can be used for their sensitivity to detect phytopathogens (Fundora *et al*., 2013). The results of its application have allowed the development of integrated disease management strategies, improving the quality and health of crops, as well as their competitiveness and profitability (Zherdev *et al*., 2018).

There are reports of the presence of proteins that may be immunogenic and are located in the outer membrane of pathogens (Bastianel *et al*., 2005; Lu *et al*., 2013). They are also identified as determinant antigenic or discrete sites that are recognized by B or T lymphocytes through their own specific receptors (Carrizo *et al*., 2009). These proteins can be used for the generation of antibodies in warm-blooded animals, therefore, the objective of this research was to identify proteins located in the outer membrane of *Candidatus* Liberibacter asiaticus, with antigenic capacity that serve as a basis for the development of an efficient and low-cost immunoenzymatic method for the detection of this bacterium in citrus.

For the bioinformatic analysis of outer membrane proteins, the complete genome of *C*Las strain GX-1 (Genbank Access PRJNA158395) was used, which was reported by Lin *et al*. (2013), in the NCBI (http://www.ncbi.nlm.nih.gov/), later it was analyzed in the UniprotKB database (http://www.uniprot.org) and the amino acid sequences of the outer membrane proteins encoded in the *C*Las genome were obtained.

The prediction of outer membrane protein function was performed using the Predict Protein web server (www.predictprotein.org/) and the Blast2go program (Run Blast, Mapping, Annotation, Inter ProScan) (Conesa *et al*., 2005). A Muscle alignment of the selected proteins based on codons was performed and a dendrogram was generated to determine their similarity by Neighbor Joining, using the MEGA X software. For epitope prediction, the protein B8Y674 was selected because it is repetitive in the *C*Las genome and the immune epitope database and analysis resource (Iedb) (http://tools.immuneepitope.org/tols/bcell/iedb_input) was used, by means of the Kolaskar and Tongaonkar antigenicity scale (Ktas) method.

The detection of *C*Las was performed by endpoint PCR from genomic DNA extracted from Mexican lime (*Citrus aurantifolia*) leaves with symptoms of HLB. DNA extraction was performed by the CTAB method optimized by Rodríguez *et al*. (2010) and in the PCR reactions, the primers designed on the sequence of the *Omp* gene encoding the protein B8Y674 were used (Rodríguez *et al*., 2018). The amplified fragments (two for each pair of primer used) were purified, cloned, and sequenced for online analysis through the NCBI database (http://www.ncbi.nlm.nih.gov/) in the Blast section.

Fifty-three outer membrane proteins were identified (data not shown) and based on their homology, motifs, and domains, 12 of these proteins were selected (Table 1), of which only C6XHX5 had previously been characterized as a protein of tolerance to organic solvent. With HhPred/HhSearch (Söding *et al*., 2005), functional predictions were obtained with probabilities ranging from 62.5 to 99.2% and with acceptable E-values ($3.3E^{-05}$, $1.4E^{-06}$, $2.5E^{-13}$, $2.8E^{-13}$, $3.5E^{-15}$), obtaining alignments with significant results for 10 proteins. In the proteins C6XGP8 and C6XGU8, probabilities of 93.8 and 62.5%, as well as E-values of 0.0027 and 1.1 respectively, were recorded, so they were considered non-significant or unreliable results in their predictions (Table 1).

**Table 1. Prediction of functional domains in 12 outer membrane proteins of *C*Las by HhPred/HhSearch**.

| No. | No. of access to UniprotKB | Probability (%) | E-value | ID* Pfam |
|---|---|---|---|---|
| 1 | B8Y674 | 98.9 | $2.5E^{-13}$ | PF01103 |
| 2 | B8Y671 | 98.9 | $2.5E^{-13}$ | PF01103 |
| 3 | B2KNJ1 | 98.9 | $2.8E^{-13}$ | PF01103 |
| 4 | J7H0I4 | 99.2 | $3.5E^{-15}$ | PF01103 |
| 5 | B8Y672 | 98.9 | $2.5E^{-13}$ | PF01103 |
| 6 | B8Y673 | 98.9 | $2.5E^{-13}$ | PF01103 |
| 7 | B8Y675 | 98.9 | $2.5E^{-13}$ | PF01103 |
| 8 | C6XGP8 | 93.8 | 0.0027 | PF01389 |
| 9 | C6XHX5 | 99.9 | $4.1E^{-27}$ | PF04453 |
| 10 | C6XF21 | 97 | $1.4E^{-06}$ | PF04355 |
| 11 | C6XGU8 | 62.5 | 1.1 | PF01389 |
| 12 | C6XFB8 | 96.2 | $3.3E^{-05}$ | PF01389 |

*= Identification number or entry access to the Pfam database.

Annotations of functional domains indicated predictions of surface antigen for proteins B8Y674, B8Y671, B2KNJ1, J7H0I4, B8Y672, B8Y673, B8Y675, C6XGP8, C6XGU8 and C6XFB8, suggesting that they have antigenic capacity, C6XHX5 had prediction of tolerance to organic solvents and C6XF21 maintenance of cell envelope integrity. The multiple alignment of the proteins encoded by the *Omp* gene (B8Y671, B8Y672, B8Y673, B8Y674 and B8Y675) showed an identity among them of 97% (Figure 1).

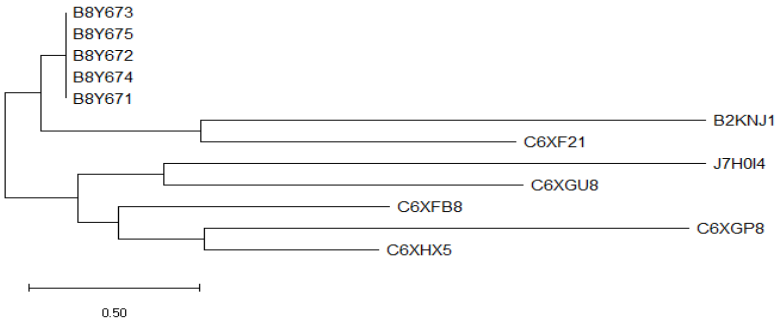


**Figure 1. Dendrogram generated by alignment of sequences of outer membrane proteins of *C*Las.**

The prediction of epitopes for the protein B8Y674 allowed obtaining eight B or T epitopes, with a specificity of 91% (Table 2). The IEDB database determined that the epitopes FSEVNISQLP ANDVSDYVILRVSVKQLSAGSVGIA, RHAVQNYTLSVEDPYFLGSPISAGFD, IPSIYTTLI EHG, and FSSHSISQSIIYNY, have been evaluated experimentally with identities of 90%, 80%, 80%, and 80%, respectively (http://www.iedb.org/). In addition, it revealed that the type of cell involved in most cases was the B cell. Some of the important functions of B cells are the production of antibodies against antigens and eventually transform into memory B cells after being activated by interaction with an antigen (Mauri and Bosma, 2012).

**Table 2. Prediction of epitopes in the protein B8Y674 using the Kolaskar and Tongaonkar antigenicity scale method**.

| No | Position | Epitope | Length |
|---|---|---|---|
| 1 | 11 | DSVIRRE | 7 |
| 2 | 44 | FSEVNISQLPANDVSDYVILRVSVKQLSAGSVGIA | 35 |
| 3 | 104 | RARLAAG | 7 |
| 4 | 113 | RHAVQNYTLSVEDPYFLGSPISAGFD | 26 |
| 5 | 155 | SAAVRMIVPITE | 12 |
| 6 | 173 | KYDLRFLQYGAI | 12 |
| 7 | 190 | IPSIYTTLIEHG | 12 |
| 8 | 203 | FSSHSISQSIIYNY | 14 |

Fragments of 1 121 bp and 904 bp respectively of the *Omp* gene of *C*Las encoding the protein B8Y674 were amplified from DNA extracted from Mexican lime leaves with symptoms of HLB using the primers reported by Rodríguez *et al*. (2018). The four sequences obtained from the amplified fragments presented 98% identity with *C*Las sequences previously reported in GenBank (CP019958.1, CP004005.1, KC473159.1, KC473156.1, JQ928886.1, JQ928883.1, JQ928882.1, JN049494.1).

It is important to note that the sequences used to date for detection by ELISA have been isolated from sweet citrus (Lu *et al*., 2013) or psyllids carrying the bacterium (Yuan *et al*., 2016), unlike the sequences of our study that were isolated from Mexican lime, finding five nucleotide variations,

corroborating what was mentioned by Tomimura *et al*. (2009), who reported that the gene that codes for the outer membrane protein has nucleotide variations that allow differentiating between them. In 2010, Chen *et al*. pointed out that, at the OMP locus, the variation is limited to a few SNPs, validating the results of this research.

These data highlight the potential of the findings obtained in this study with the identification of surface antigens, from which specific antibodies that avoid cross-reactions with bacteria other than *C*Las can be obtained, in an immunoenzymatic method for the early detection of the causative agent of citrus HLB disease.

## Conclusions

Based on the prediction of functional domains, proteins B8Y674, B8Y671, B2KNJ1, J7H0I4, B8Y672, B8Y673, B8Y675, and C6XFB8 were identified as proteins with antigenic capacity. The identification of four epitopes, as well as the detection of *C*Las by endpoint PCR using the primers designed on the sequence of the *Omp* gene encoding the protein B8Y674, allows inferring that this is a potential candidate to produce antibodies and implementation of an immunoenzymatic method for the detection of *C*Las.

## Cited literature

Achor, D. S.; Welker, S.; Ben-Mahmoud, S.; Wang, C.; Folimonova. S. Y.; Dutt, M.; Gowda, S. and Levy, A. 2020. Dynamics of *Candidatus* Liberibacter asiaticus movement and sieve pore plugging in citrus sink cells. Plant Physiol. 182(2):882-891. Doi: 10.1104/pp.19.01391.

Andrade, M. O.; Pang, Z.; Achor, D. S.; Wang, H.; Yao, T.; Singer, B. H. and Wang, N. 2020. The flagella of "*Candidatus* Liberibacter asiaticus" and its movement in planta. Molecular plant pathology. 21(1):109-123. Doi: 10.1111/mpp.12884.

Bastianel, C.; Garnier-Semancik, M.; Renaudin, J.; Bové, J. M. and Eveillard, S. 2005. Diversity of "*Candidatus* Liberibacter asiaticus," based on the *omp* gene sequence. Appl. Environ. Microbiol. 71(11):6473-6478. Doi: 10.1128/AEM.71.11.6473-6478.2005.

Carrizo, A.; Brihuega, B.; Etchechoury, I.; Arese, A.; Romero, S.; Gioffré, A., Romano, M. F. and Caimi, K. 2009. Identification of immunoreactive antigens of *Leptospira interrogans*. Rev. Argentina de Microbiol. 41(3):129-133.

Chen, J.; Deng, X.; Sun, X.; Jones, D.; Irey, M. and Civerolo, E. 2010. Guangdong and Florida populations of '*Candidatus* Liberibacter asiaticus' distinguished by a genomic locus with short tandem repeats. Phytopathology. 100(6):567-572. Doi: 10.1094/PHYTO-100-6-0567.

Conesa, A.; Gotz, S.; García-Gómez. J. M.; Terol, J.; Talón, M. and Robles, M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics. 21(18):3674-3676.

Ding, F.; Duan, Y.; Paul, C.; Brlansky, R. H. and Hartung, J. S. 2015. Localization and Distribution of "*Candidatus* Liberibacter asiaticus" in citrus and periwinkle by direct tissue blot immuno assay with an anti ompa polyclonal antibody. Plos One. 10(5):e0123939-10. http://doi.org/10.1371/journal.pone.0123939.

Fundora, H. H.; Puig, P. Y.; Chiroles, R. S.; Rodríguez, B. A. M.; Gallardo, D. J. y Milián, S. Y. 2013. Métodos inmunológicos utilizados en la identificación rápida de bacterias y protozoarios en aguas. Rev. Cubana de Hig. Epidemiol. 51(1):84-96.

Lin, H.; Han, C. S.; Liu, B.; Lou, B.; Bai, X.; Deng, C.; Civerolo, E. L. and Gupta, G. 2013. Complete genome sequence of a chinese strain of "*Candidatus* Liberibacter asiaticus". Genome Announcements. 1(2):e00184-13. Doi: 10.1128/genomeA.00184-13.

Lu, L.; Cheng, B.; Yao, J.; Peng, A.; Du, D.; Fan, G.; Hu, X.; Zhang, L. and Chen, G. 2013. A new diagnostic system for detection of '*Candidatus* Liberibacter asiaticus' infection in citrus. Plant Dis. 97(10):1295-1300. http://dx.doi.org/10.1094/ PDIS-11-12-1086-RE.

Mauri, C. and Bosma, A. 2012. Immune regulatory function of b cells. Annual Review of Immunol. 30:221-242. Doi: 10.1146/annurev-immunol-020711-074934.

McCollum, G.; Hilf, M.; Irey, M.; Luo, W. and Gottwald, T. 2016. Susceptibility of sixteen citrus genotypes to '*Candidatus* Liberibacter asiaticus'. Plant Dis. 100(6):1080-1086. http://dx.doi.org/10.1094/PDIS-08-15-0940-RE.

Rodríguez, Q. C. G.; Alanís, M. E. I.; Velázquez, M. J. J. y Almeyda, L. I. H. 2010. Optimización de la técnica de extracción del DNA de plantas de cítricos para el diagnóstico del HLB. En 1er. simposio nacional sobre investigación para el manejo del psílido asiático de los cítricos y el huanglongbing en México. Monterrey, Nuevo León. 22-29 pp.

Rodríguez, Q. C. G.; Almeyda, L. I. H.; Alvarez, O. M. G.; Hernández, G. C. y Mendoza, H. A. 2018. Detección de *Candidatus* liberibacter asiaticus mediante PCR-punto final, utilizando iniciadores diseñados a partir de los genes *omp* y *clibasia*_02425. *In*: memoria del XXX1 Simposio de avances en Investigación Agrícola, Pecuaria, Forestal, Acuícola, Pesquería, Desarrollo Rural, Transferencia de Tecnología, Biotecnología, Ambiente, Recursos Naturales y Cambio Climático. Veracruz. 1644-1655 pp.

Söding, J.; Biegert, A. and Lupas, A. N. 2005. The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res. 33(2):244-248. Doi: 10.1093/nar/ gki408.

Tomimura, K.; Miyata, S.; Furuya, N.; Kubota, K.; Okuda, M.; Subandiyah, S.; Hung, T.; Su, H. J. and Iwanami, T. 2009. Evaluation of genetic diversity among '*Candidatus* Liberibacter asiaticus' isolates collected in southeast Asia. Phytopathology. 99(9):1062-1069. Doi: 10.1094/PHYTO-99-9-1062.

Yuan, Q.; Jordan, R.; Brlansky, R. H.; Minenkova, O. and Hartung, J. 2016. Development of single chain variable fragment (scFv) antibodies against surface proteins of '*Ca*. Liberibacter asiaticus'. J. Microbiol. Methods. 122:1-7. Doi: 10.1016/j.mimet.2015.12.015.

Zherdev, A. V.; Vinogradova, S. V.; Byzova, N. A.; Porotikova, E. V.; Kamionskaya, A. M. and Dzantiev, B. B. 2018. Methods for the diagnosis of grapevine viral infections: a review. Agriculture. 8(12):1-19. Doi: 10.3390/agriculture8120195.