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RESEARCH ARTICLE

In silico analysis of proteins potentially involved in fimbrial biogenesis in *Helicobacter pylori*

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ABSTRACT

Background. Colonization and chronic infection with *Helicobacter pylori* is the major contributing factor to the development of gastric cancer. A large repertoire of adhesins has been described that contribute to the adaptation of bacteria to a specific gastric niche. As in other pathogenic bacteria, *H. pylori* biofilm formation is central to survival in unfavorable environments. Type IV pili or fimbriae are responsible for the adhesion of many pathogenic bacteria (e.g., *Escherichia coli, Pseudomonas aeruginosa* and *Vibrio cholerae*) to various surfaces. The aim of this study was to identify and analyze genes that might encode proteins involved in the biogenesis of fimbriae on *H. pylori* and characterize their expression during biofilm formation.

Methods. PSI BLAST, bioinformatics and molecular tools were used as well as the NCBI database search for sequences related to protein biogenesis of fimbriae. Multiple alignments were performed using the HMMer and T-COFFEE programs. The secondary structure prediction was performed with ANTHEPROT and the tertiary structures were predicted with the I-Tasser.

Results. We identified two counterparts—jhp0257 and HP0272—from PilN protein of *Campylobacter rectus* and *Xilella fastidiosa*, which is part of the machinery of assembly type IV fimbriae. Similarly, proteins jhp0887 and HP0953 showed homology from signal peptide to PilA level of *P. aeruginosa*, and the HP0953 protein was overexpressed during the formation of the biofilm.

Conclusions. *H. pylori* possesses homologous proteins to fimbrial protein families, specifically PilN and PilA, which join type IV fimbriae in other bacteria. The latter has a higher expression level during the initial stage of the formation of biofilm.

Key words: *Helicobacter pylori*, type IV fimbriae, pilin, biofilm.

INTRODUCTION

Helicobacter pylori is a Gram-negative[1] pleomorphic bacteria that chronically colonizes the gastric epithelium and infects half of the population.[2] Its prevalence is higher in developing countries where the infection is associated with the social, cultural and economic level of the population.[3]

H. pylori is considered a prominent member of the human microbial flora and has accompanied *Homo sapiens* throughout history. This microorganism is acquired during childhood and is transmitted within the family environment.[4] Most infected individuals are asymptomatic. However, 10-20% may develop atrophic gastritis, peptic ulcer, gastric adenocarcinoma, or mucosa-associated lymphoid tissue (MALT) and ~3% may develop gastric cancer. *H. pylori* was classified as a type I[5] carcinogen. It is estimated that 5.5% of all cancer cases worldwide and >60% of gastric cancer cases are caused by this microorganism.[4]

H. pylori is also associated with extradigestive infections such as vascular function disorders (primary mi-

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graine and primary Raynaud's phenomenon), ischemic heart disease and cardiac syndromes.[6] The disease generally does not develop during childhood, but develops in adulthood after decades of infection.[7]

The process of colonization with *H. pylori* consists of four steps: (i) transmission to a new host, (ii) adhesion of the bacteria to a specific niche within the host, (iii) prevention, subversion or exploitation of the host defense mechanisms, and (iv) acquisition of nutrients resulting in successful multiplication of bacteria.[8]

H. pylori may be present in the water distribution system, on the surface from underground wells, and in rivers and sewer systems, which allows us to consider the possibility that it exists as biofilm on surfaces exposed to water. This provides another route of infection to humans as no animal reservoirs are known.[9] Other possibilities of infection with *H. pylori* are via the oral-fecal or iatrogenic route, during collection of biopsies through the endoscope or associated with the use of contaminated forceps.[10]

Biofilm formation is critical not only for the survival of the microorganism in unfavorable environments, but also for the successful infection of numerous pathogenic bacteria.[11] It has been documented that fimbriae are important for the formation of biofilms for *Salmonella enteritidis, P. aeruginosa, Staphylococcus epidermidis* and *E. coli*. These protein structures recognize a range of molecular targets, allowing the bacterium to interact with various surfaces and to bind to specific host tissues.[12] Type IV fimbriae are flexible and multifunctional filamentous appendages with virulence properties such as adhesion, contractile movement, aggregation, horizontal gene transfer, and multicellular development and formation of biofilms.[13,14]

The pilus fiber consists mainly of thousands of polymerized pilin subunits on the bacterial surface (PilE in *Neisseria meningitidis* and PilA in *Pseudomonas aeruginosa*).[14] Type IV fimbriae are classified into two types: IVa and IVb. Proteins associated with the biogenesis of type IVb fimbriae are more diverse than those associated with type IVa fimbriae. Some of the proteins that share both types of fimbriae are PilA, PilF ATPase, PilQ outer membrane secretin and pilT ATPase. The genes *pilM, pilN, pilO, pilP* and *pilQ* are found consecutively in the chromosome, which appears to be specifically associated with the biogenesis of type IVa fimbriae.[15]

In recent years, bioinformatics tools have allowed for more efficient analysis of a large number of proteins of yet unknown function in many organisms including *H. pylori*. The objective of this study was to identify in the *H. pylori* genome the genes that encodes for hypothetical proteins, homologous to proteins associated with the biogenesis of fimbriae, and to analyze their expression during biofilm formation using bioinformatic tools.

MATERIALS AND METHODS

Strains and culture conditions

Reference strains of *H. pylori* 26695 and *H. pylori* J99 were used. The first one came from a patient with gastritis (U.K.) and the second from a patient with a duodenal ulcer (U.S.). They were cultured in Casman agar supplemented with 5% sheep blood and incubated for 48 h at 37°C in an atmosphere of 5% CO_2 and 95% humidity.

In silico Analysis to Search for Genes Associated with the Biogenesis of Fimbriae in *H. pylori*

Protein sequences associated with the biogenesis of fimbriae on Gram-negative bacteria were obtained from the database of the NCBI.[16] These sequences were compared with the proteome of *H. pylori* 26695 strain through the programs PSI BLAST[17]and HMMER[18,19] to identify homologous sequences of various bacteria. The T-COFFEE[20,21] program was used for carrying out multiple alignments of the identified sequences. Also, the ANTHEPROT[22,23] program was used to predict secondary structures and properties of hydrophobicity, hydrophilicity, antigenicity and transmembrane domains. Finally, the three-dimensional structures of the identified proteins were modeled by the ab-initio method using the I-TASSER program.[24,25]

Production of biofilm analyzed by the crystal violet retention method

In a 96-well plate, 200 µl of Brucella broth was placed (BBL, Franklin Lakes, NJ) supplemented with 1% yeast extract (DIBICO, Mexico, DF) plus an inoculum (6×10^8 cells/ml) of *H. pylori* J99 and 26,695 strains in each well. Eight replicates were performed and incubated at 37°C in a CO_2 atmosphere for 1, 3, 6, 9, 24 and 48 h. After the incubation period, the supernatant was removed and the

wells were washed with 1X PBS solution (Sigma-Aldrich, St. Louis, MO). Glutaraldehyde was added (2.5%, Sigma-Aldrich) to fix the cells and incubated for 10 min at room temperature. Glutaraldehyde was then removed, washed with 1X PBS (Sigma-Aldrich) and stained with crystal violet solution (0.5% HYCEL, Mexico) for 10 min. It was finally washed twice with 1X PBS and allowed to air dry; 200 µl of alcohol-acetone 80:20 (HYCEL) was added to recover the crystal violet resorbed by the biofilms. Finally, absorbance of the crystal violet retained by adhered cells was read in an ELISA reader at 540 nm using alcohol-acetone as a target (Labsystems Multiskan Ascent, Haverhill, MA).

Expression of genes associated with fimbriae

From the proteins identified by the in silico analysis, the sequences that codify them were obtained and their oligonucleotides were designed to amplify the genes. The Primer3 program was used.[26] Oligonucleotides were also designed to amplify the *flh*F gene, which encodes for a regulatory protein of the biosynthesis of the flagellum. The *glm*M gene is a constitutive gene that codifies for phosphoglucosamine mutase and the *hp0015* gene homologous to *virB2*, which encodes for a protein that forms the hair-like pili of the type IV secretion system.

Extraction of *H. pylori* RNA

RNA was extracted after 48-h growth of *H. pylori* 26,695 strain. This was resuspended in 50 ml of Brucella broth supplemented with 1% yeast extract (pH 6) at a concentration equivalent to McFarland tube 2 (6 x 10^8 CFU/ml). The cultures were incubated at 37°C in an atmosphere of 5% CO_2 for 1, 3, 6, 24 and 48 h. The same experiment was performed at pH4 in a culture of AGS cells (ATCC CRL-1739) with 80 to 100% confluence in F12 media solution (GIBCO, Invitrogen, Grand Island, NY). The latter assay was incubated for 1-3 h. All assays were performed in triplicate. The bacterial suspension not adhered to the surface was removed from each bottle. The surface of the bottles was scraped with 1 mL of sterile PBS to obtain possible cell cultures infected with the *H. pylori* strain. Samples were centrifuged at 10,000 rpm for 2 min, the supernatant was removed and 100 µl of SDS (Invitrogen, Carlsbad, CA) at 1% and 800 ul of TRIzol (Invitrogen) were added to extract the RNA. All samples were stored at -70°C.

Then, 200 µl of chloroform (Baker, Xalostoc, Mexico) was added, mixed and incubated at room temperature for 3 min and centrifuged at 13,000 rpm for 5 min. To the aqueous phase, 350 µl of isopropanol (AMRESCO, Solon, OH) was added, gently mixed and incubated at -20°C overnight. It was centrifuged at 13,000 rpm for 15 min. The supernatant was removed and the pellet was washed with 1 ml of 70% ethanol (AMRESCO), centrifuged at 13,000 rpm for 10 min and the pellet was allowed to dry at room temperature and resuspended in 30 µl of nuclease-free water. Obtained RNA was quantified and subjected to agarose gel electrophoresis (Promega, Madison, WI) at 1% stained with ethidium bromide (Sigma-Aldrich). It was finally stored at -70°C.[27]

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA samples were pretreated with DNase I (Promega) to remove residual DNA. cDNA synthesis was performed following the protocol of the commercial brand SuperScrip First-Strand Synthesis System for RT-PCR (Invitrogen). We performed a reaction mixture containing 1,500 ng RNA, 1 µl of dNTP (10 mM), 1 µl of *random hexamers* (50 ng/µl), and up to 10 µl nuclease-free water. The reaction was incubated at 65°C for 5 min and then at 4°C for 5 min. Also, another mixture was prepared with 2 µl of Regulator 10X RT, 4 µl $MgCl_2$ 25 mM, 2 µl DTT 0.1 mM and 1 ul RNaseOUT 40 U/µl. Nine ul of the second reaction was added to the first reaction and incubated at 25°C for 2 min. Subsequently, 1 µl of SuperScript II RT was added and incubated at 25°C for 10 min, 42°C for 50 min, 70°C for 15 min and 4°C for 3 min. Finally, 1 µl of RNase H was added and incubated at 37°C for 20 min. The cDNA obtained was stored at -20°C for up to 72 h.

The reaction mixture for the PCR contained 1.5 µl of cDNA 120 ng/µl, 1 µl of each oligonucleotide 10 pmol/µl, 5 µl of Go Taq Green Master Mix (Promega) (*Taq* DNA polymerase, 0.4 mM dATP, 0.4 mM dGTP, 0.4 mM dCTP, 0.4 mM dTTP, 3 mM de $MgCl_2$) and 1.5 µl of nuclease-free water. The amplification program for genes *glm*M and *flh*F was an initial denaturing cycle at 94°C for 5 min and 24 cycles at 94°C for 1 min, 65°C for 1 min for alignment and for genes *hp0015* and *hp00953* was a denaturation cycle at 94°C for 5 min and 26 cycles at 94°C for 1 min and 68.4°C. The extension for both

was done at 65°C for 1 min and a final extension cycle of 65°C for 7 min. Then, 5 µl of the PCR product was subjected to electrophoresis on a 1% agarose gel stained with ethidium bromide (100 V, 50 mA for 40 min). The gel was observed under ultraviolet light.

Statistical analysis

Data were expressed as mean and standard deviation. For the statistical analysis we used Student t test; a p value <0.05 was considered statistically significant.

RESULTS

In silico Analysis to Search for Genes Associated with the Biogenesis of Fimbriae in *H. pylori*

Protein sequences that comprise the various types of fimbriae were compared such as type P fimbriae, type I and curly type with the proteome of the *Helicobacter* genus without significant results. Moreover, using the NCBI database, proteins possibily associated with the biogenesis of fimbriae in the *Helicobacter* genus were sought, and two hypothetical proteins were identified from *H. winghamensis* and *H. acinonychis*. The protein sequence with accession number ZP_0453914 of *H. winghamensis* was compared with the proteome of different *Helicobacter* species, using the HMMER program,[19] but no significant results were obtained. The protein sequence with accession number CAJ99352 of *H. acinonychis* was compared in the same way. We found two proteins with an expected value of 0.05 for jhp0257 and HP0272, one belonging to *H. pylori* J99 and the other to *H. pylori* 26695, respectively.

The sequence of the jhp0257 protein was analyzed with PSI BLAST[17] to search for its remote homologues. It was found that the protein was homologous to PilN, a protein that assembles the type IV fimbria in different bacteria. Similar results were obtained with the protein HP0272.

The closest homologous proteins were for the PilN protein of *Campylobacter rectus* with accession number ZP_03611073 and the fimbrial assembly protein of *Xylella fastidiosa* with accession number ZP_00652831. These sequences and those of the *H. pylori* were aligned using the T-COFFEE program.[21] The alignment shows highly variable areas and only a small area preserved that is marked in red (Figure 1a).

PilN proteins from *C. rectus* and *X. fastidiosa* were obtained during the first iteration of the analysis; therefore, they were used in the comparison of predictions of biological properties of proteins jhp0257 and HP0272, which were obtained with the ANTHEPROT program.[23] The graphs show a similar pattern in the hydrophobicity, hydrophilicity, antigenicity properties, and transmembrane domains (Figure 2a).

Furthermore, a prediction of the secondary structure of the four proteins was carried out with the ANTHEPROT program. Great similarity is seen between the graphs, indicating that jhp0257 and HP0272 proteins have very similar secondary structures of PilN proteins of *X. fastidiosa* and *C. rectus* (Figure 2b).

Using as reference the precursor of type IV fimbrial protein of *P. aeruginosa* (NP_253215 according to the NCBI database[16]), two conserved domains were revealed: one related to the pilins superfamily with reference PF00114 of the Pfam database and the other concerning the pilins family for the Tfp pilus assembly of different bacteria with COG4969 access. We analyzed protein sequence alignments comprised of both families, and hidden Markov models were created using the program HMMER.[19] These were subsequently compared with the group of proteomes of the different *H. pylori* species, yielding three proteins similar to the pilins superfamily (Pfam) with expected values >0.05.

Furthermore, when comparing the largest subunit (PilA) of the family of pilins, three proteins were found. One corresponded to that found during the analysis with the pilins superfamily (Pfam). As in the prior analysis, these latter proteins are not statistically significant because the expected value was >0.05. The proteins found belong to *H. mustelae*, *H. pylori* P12, *H. pylori* G27, *H. hepaticus* and *H. felis*. The protein of *H. mustelae* has the accession number YP_003517399 of the NCBI database[16] and contains sequences similar to pilins of the pilins superfamily and to the pilin family for the assembly of the Tfp pilus. The sequence of this protein was used to perform a search for remote homologues using the NCBI PSI BLAST tool.[17] This protein also has the preserved domain of the pilin A (PilA) family and is very similar to PilA protein of other bacteria. Also, proteins of different species of *Helicobacter* have been found that are homologous to that of *H. mustelae*, including *H. pylori* proteins similar to the superfamily of pilins. Those mainly considered were

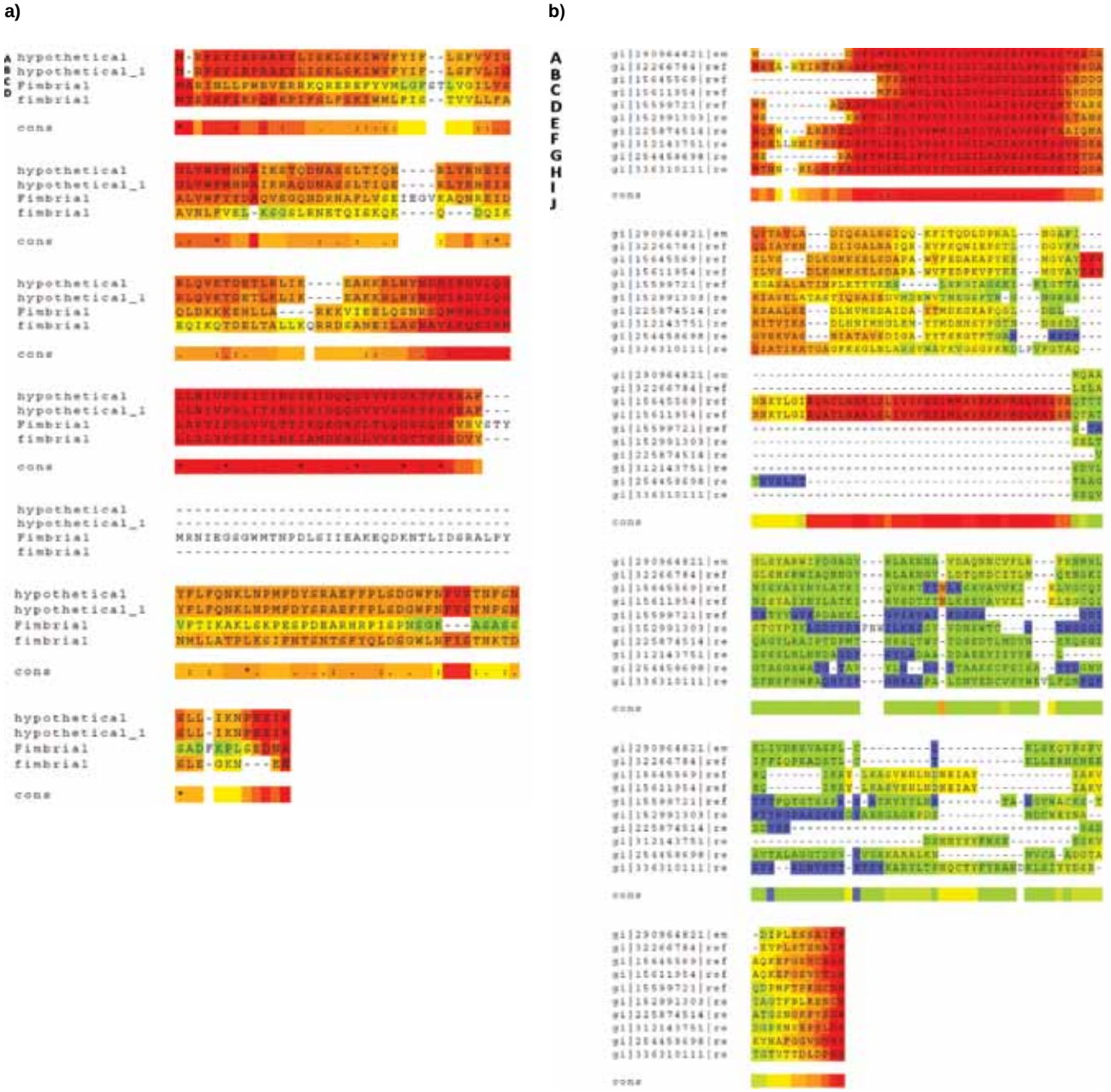

Figure 1. Multiple sequence alignments. **(a)** Alignment of homologous protein sequences to PilN fimbrial protein. (A) HP0272 of *H. pylori* 26695. (B) jhp0257 *of H. pylori* J99. (C) Fimbrial assembly protein of *X. fastidiosa.* (D) Fimbrial assembly protein (PilN) of *C. rectus*. Highly preserved regions are highlighted in red and the most variable in green. **(b)** Alignment of homologous protein sequences to the PilAC fimbrial protein. (A) Hypothetical protein *(H. mustelae*). (B) Hypothetical protein HH1285 (*H. hepaticus* ATCC 51449). (C) Hypothetical protein HP0953 (*H. pylori* 26695). (D) Hypothetical protein jhp0887 (*H. pylori* J99). (E) Type IV PilA fimbrial precursor (*P. aeruginosa* PAO1). (F) Fimbrial protein (*Nitratiruptor* sp.). (G) Prepilin type N-terminal domain cleavage/methylation (*Acidobacterium capsulatum* ATCC 51 196). (H) Assembly protein of the Tfp pilus, principal PilA piline (*Halanaerobium hydorgeniformans*). (I) Prepilin type N-terminal domain cleavage/methylation (*Campylobacter bacterium* GD 1). (J) Fimbrial pilin (*Shewanella* sp. HN-41). The highly preserved regions are shown in red, whereas green represents the most variable regions. The regions at the beginning of the sequences are highly preserved.

the reference strain proteins HP J99 and 26695, which are jhp0887 and HP0953, respectively.

Using the T-COFFEE server,[21] a multiple alignment of several randomly chosen PilA proteins belong to different bacteria was performed. It was noted that there is a small preservation zone marked in red at the beginning of the sequences with the remainder being variable. Similarly, a multiple alignment was performed between

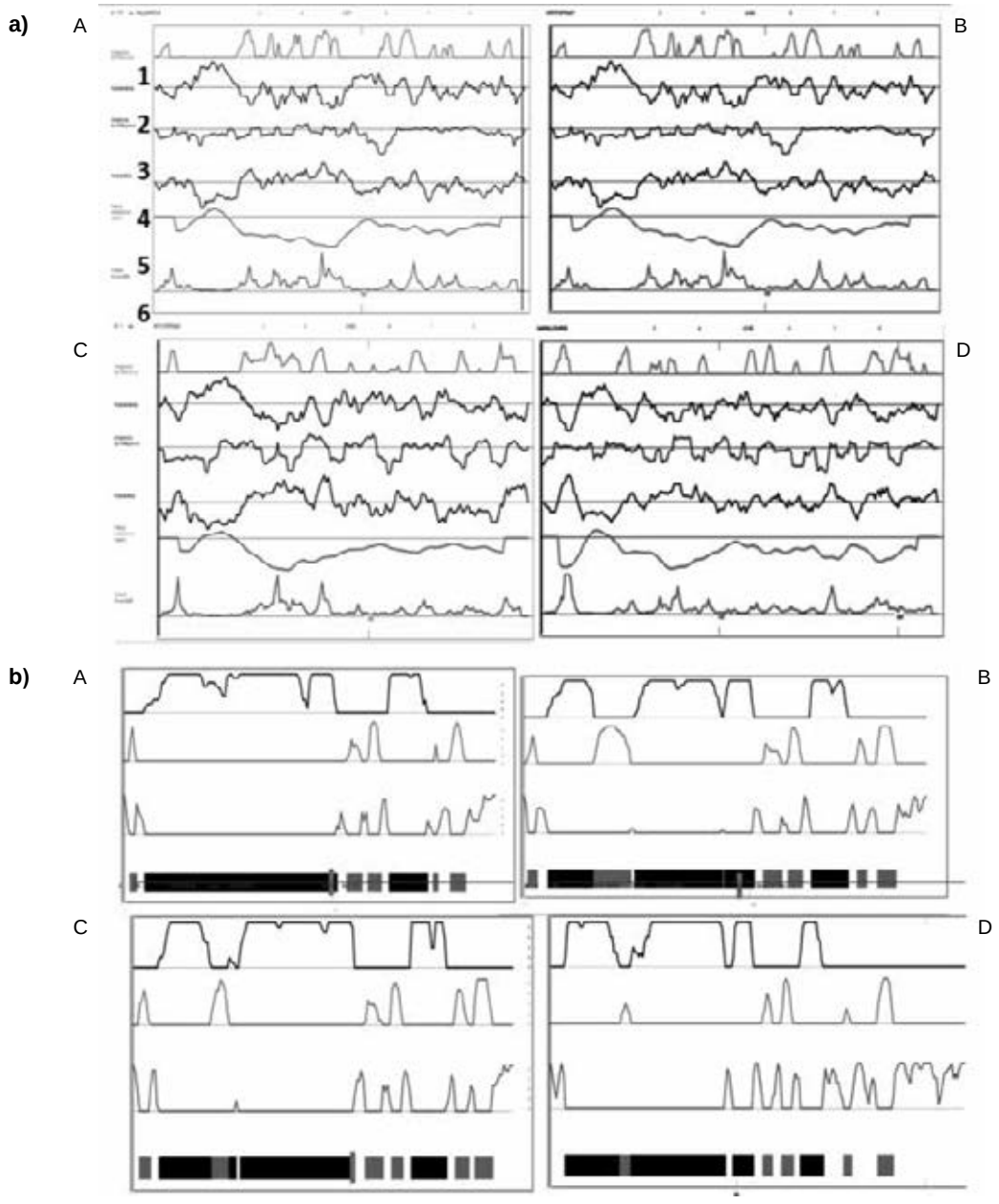

Figure 2. Prediction of biological properties and secondary structure of proteins. **(a)** Predicted biological properties of proteins. (A) jhp0257 of *H. pylori* J99. (B) HP0272 of *H. pylori* 26695. (C) Fimbrial assembly protein (PilN) of *C. rectus*. (D) Fimbrial assembly protein of *X. fastidiosa*. 1) Parker antigenicity. 2) Hydrophobicity. 3) Welling antigenicity. 4) Hydrophilicity. 5) Transmembranal regions. 6) Accessibility to solvent. **(b)** Prediction of the secondary structure of proteins. (A) jhp0257 of *H. pylori* J99. (B) HP0272 of *H. pylori* 26695. (C) Fimbrial assembly protein (PilN) de *C. rectus*. (D) Fimbrial assembly of *X. fastidiosa* protein. 1) a-helix. 2) Folded b-sheets. 3) Coil.

the same proteins of different bacteria and the proteins obtained from different *Helicobacter* species, including *H. mustelae* and *H. pylori*, which showed a similar behavior to the above (Figure 1b).

Using the I-TASSER program, a three-dimensional modeling of the proteins obtained was done. It was observed that PilN proteins and the hypothetical HP proteins (jhp0257 and HP0272) are very similar, mainly with an α-helix (Figure 3). Meanwhile, the three-dimensional structure of type IV pilin of *P. aeruginosa* PilA has a carboxy terminal (C-terminal) of 122 residues that is folded in different ways. The amino terminal (N-terminal) is an α-helix surrounded by a set of four folded antiparallel β-sheets and ending in α-helices despite being different from the structures of the hypothetical proteins of *H. pylori* J99 and 26695 with those of the PilA model. The jhp0887 model shows an α-helix in the N-terminal where the signal peptide is located (Figure 3, blue). Furthermore, the tertiary structure of the HP0953 protein has an α-helix surrounded by folded β sheets, similar to PilA as well as other α-helices.

Biofilm production

The strain 26696 increased the production of biofilm 10 times from an optical density of 0.351 to 3 h at 540 nm increased to 3.673 OD at 48 h of incubation (Figure 4).

Expression of genes associated with fimbriae

Biofilm formation was monitored in a liquid media at pH 6 after incubating for 1, 3, 6, 24 and 48 h, and it was observed that *hp0953* gene expression, which encodes for the protein HP0953, homologous to PilA, is significantly increased at 3 h of incubation (data not shown). Further experiments were only monitored at this time.

Expression of genes *flh*F, *glm*M, virB2 and *hp0953* was analyzed during the process of biofilm formation (at 0, 1 and 3 h) at pH 4, pH 6 and in the presence of gastric cancer cells (AGS). It was found that at pH 4 at 1 h incubation, the genes *flh*F, *hp0015 and hp0953* flhF are expressed in greater quantity and their expression decreases at 3 h of incubation (Figure 5). Moreover, at pH 6, expression of the *flh*F gene begins to increase after 1 h of incubation and the expression of *hp0953* increases significantly (p <0.05) at 3 h, compared with the other genes. In the presence of cells, expression of *flh*F and *hp0015* increases at 1 h. However, expression of *flh*F and *hp0953* increases at 3 h at higher levels than the other genes.

Figure 6 shows the expression of genes *flh*F, *hp0015 and hp0953* with respect to the constitutive gene *glm*M (p <0.05). It is observed that at time 0, the expression of genes *hp0015* and *hp0953* were below the expression of the GLMM gene. In contrast, at 3 h of incubation it was observed that expression of the *hp0953* gene is significantly higher with respect to the *glm*M gene.

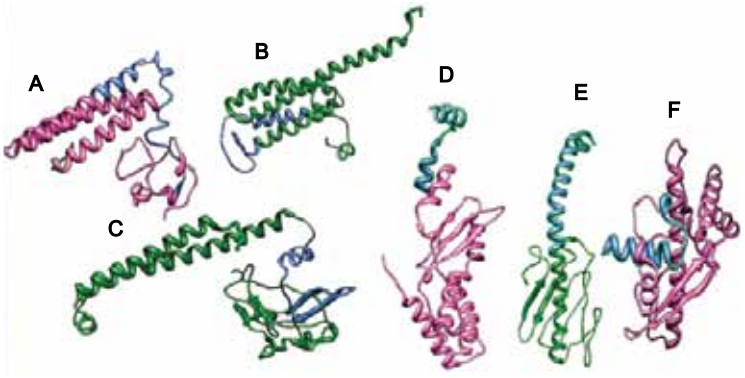

Figure 3. Prediction of three-dimensional protein structure. **(A)** Hypothetical protein jhp0257 of *H. pylori* J99. **(B)** Fimbrial protein assembly PilN of *C. rectus*. **(C)** Fimbrial protein assembly of *X. fastidiosa*. The most preserved zones in multiple alignment sequences are highlighted in blue. The three proteins have a region of α-helices that are arranged in a similar manner. **(D)** Hypothetical protein jhp0887 of *H. pylori* J99. **(E)** Fimbrial precursor type PilA of *P. aeruginosa*. **(F)** Hypothetical protein HP0953 of *H. pylori* 26695. Blue highlights the most preserved region in the multiple alignments showing that the preserved region has an α-helical shape in both proteins.

DISCUSSION

Identification of homologous proteins is increasingly important in modern biology. Traditional biological studies have focused on model systems, and these studies provide significant resources to investigate other species.[28] Identification of homologues can be performed with software by comparing a query sequence with similar sequences found in a database. However, this process is not trivial because two homologous proteins may be separated by a long developmental period. Therefore, its evolutionary relationship may be very difficult to detect. Evolutionarily distant proteins are called remote homologues. A large proportion of previewed proteins (between ~30 and 40%) does not have a particularly assigned function and cannot be associated with any known function in the database. This occurs even in well-studied organisms.[28]

Our study found *H. pylori* proteins that are remote homologues of those that comprise fimbriae in other bacteria. This suggests that there is a large evolutionary distance between them; therefore, it is difficult to predict whether their function is the same. However, analysis of hydrophobicity, hydrophilicity, antigenicity and transmembrane sequence domains indicates that the hypothetical proteins found (jhp0257 and HP0272) and fimbrial assembly proteins of *C. rectus* and *X. fastidiosa* are very similar, so that these proteins may have the same function.

Studies by Castric and Deal with PilA of different strains of *P. aeruginosa* showed that there is only one preserved area at the beginning of the sequence and the rest is variable.[29] The same results were obtained for the protein HP0953 of *H. pylori*. The preserved region belongs to the subunit of protein processing. Meanwhile, Keizer *et al*. performed alignment of proteins of two strains of *P. aeruginosa* and found only 40% identity between them, which indicates the great variability among such proteins.[30] At the C-terminal domain, the junction to the cellular receptors is found, which also interact with inert surfaces such as stainless steel. However, this domain is semipreserved.[31] Alignment obtained in this study showed similarity to the C-terminal end of the sequences analyzed. Tertiary structures obtained demonstrate differences because in modeling the process does not take into account that the processing may experience some proteins. However, the form presented by the signal peptide indicates that it can be transported to the same region in the cell and, therefore, provide the same function. Moreover, the proteins found belong to the same group of fimbrial proteins and both assembled type IV fimbriae.

When performing the biofilm assay, we observed that a longer incubation time increases the amount of bacteria attached to the surface of the slide. This agrees with the stages of biofilm formation. During the first stage the bacterium interacts with the surface and there must be a Brownian movement, sedimentation or flagella movement. Subsequently, bacteria adhere irreversibly by producing an extracellular polymeric substance, and due to stimulation of receptor proteins of the bacterial wall, development of cell-to-cell bridges takes place. This stabilizes the structure formed during the maturation process. Finally, surface colonization takes place where bacteria grow and form microcolonies.[32]

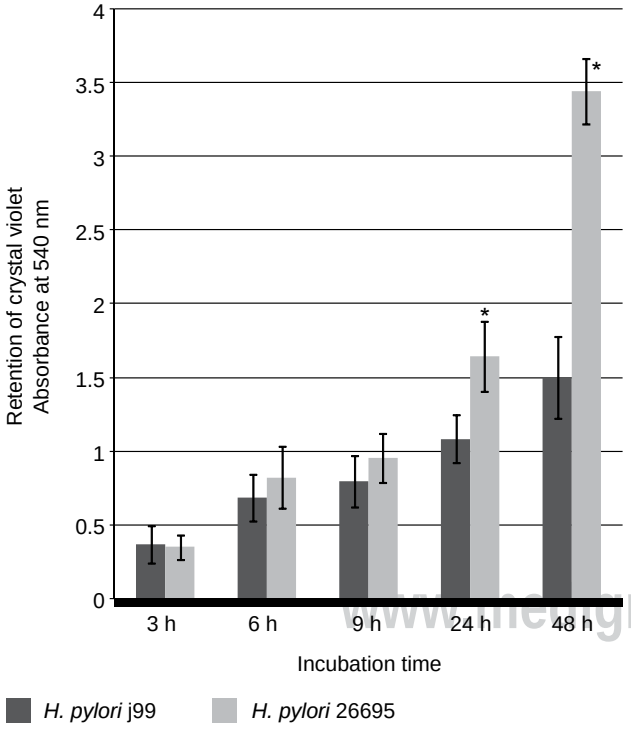

Figure 4. *H. pylori* strains 26,695 and J99 produce a biofilm after 3 h of incubation. Significantly, the 26,695 strain produces a greater quantity of biofilms than the J99 strain at 24 and 48 h. *p <0.05.

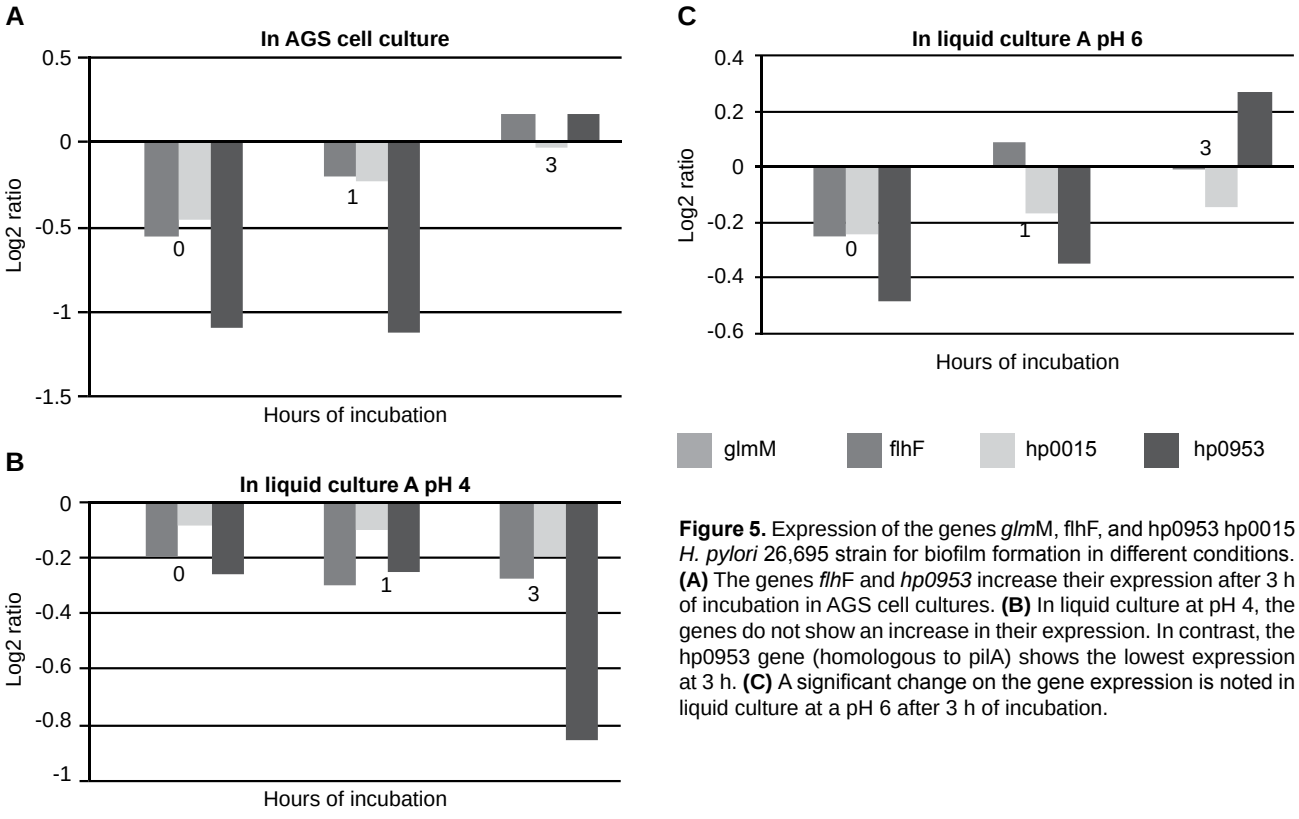

Figure 5. Expression of the genes *glm*M, flhF, and hp0953 hp0015 *H. pylori* 26,695 strain for biofilm formation in different conditions. **(A)** The genes *flh*F and *hp0953* increase their expression after 3 h of incubation in AGS cell cultures. **(B)** In liquid culture at pH 4, the genes do not show an increase in their expression. In contrast, the hp0953 gene (homologous to pilA) shows the lowest expression at 3 h. **(C)** A significant change on the gene expression is noted in liquid culture at a pH 6 after 3 h of incubation.

De Souza et al. found that the *pilA* gene that encodes for the PilA protein in *X. fastidiosa* is overexpressed during the formation of the biofilm.[33] It is known that these adhesins participate in the initial adhesion to surfaces. However, Caserta *et al*. confirmed that the PilA protein of *X. fastidiosa* was expressed considerably during the early stages of biofilm formation, whereas some outer membrane adhesins remain at low levels or its expression did not present any changes.[34] It was observed during this study that the *p0953* gene was expressed in greater proportion during the first 3 h of biofilm formation in liquid culture medium and in the presence of cells, which suggests that its expression may be related with the events of adherence to inert and cellular surfaces.

Therefore, it is concluded that *H. pylori* has proteins homologous to the family of fimbrial proteins, specifically PilN and PilA, which assemble type IV fimbriae in other bacteria. PilA has a higher level of expression during the initial stage of biofilm formation. However, it is necessary to continue with the characterization of these genes to determine their relationship with the production of type IV fimbriae.

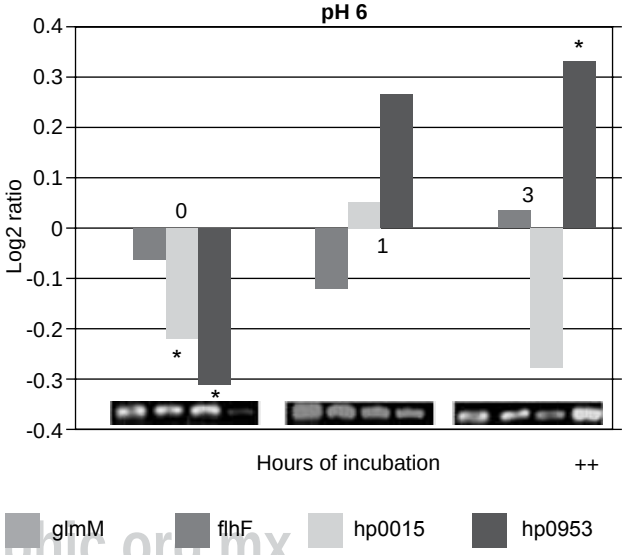

Figure 6. Expression of the genes *glm*M, *flh*F, *hp0015* and *hp0953* of *H. pylori* 26695 strain at 0, 1 and 3 h of formation of the biofilm in liquid media (pH 6). The difference in expression of the genes *hp0015* (homologous to vir B2 of the SSTIV) and *hp0953* (homologous to PilA) is observed relative to the constitutive *glm*M gene at time 0. Expression of the *hp0953* gene increases significantly at 3 h of incubation. **p* <0.05.

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