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Helicobacter pylori Biofilm Formation and Gene Expression on Abiotic Surfaces Using a Cyanobacterial Extract

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Abstract: The effects of a cyanobacterial extract (CE) on *Helicobacter pylori* biofilm formation onto hydrophobic and hydrophilic abiotic surfaces and the expression of *lux*S, *fla*A, *omp*18, *lpx*D and *ure*A genes associated to biofilm were studied. NCTC11638 reference strain and HP796, a resistant clinical isolate, were grown in Mueller-Hinton broth supplemented with 5% fetal calf serum (FCS) or 1% CE. The ability to form biofilm, viability, morphological changes and gene expression of adhered *H. pylori* cells were determined. The strains were able to form biofilm on both surfaces with the nutritional supplements analyzed. *H. pylori* conserved a characteristic bacillary morphology and viability with CE. Cells attachment was higher with CE than FCS regardless of strains and surfaces. The most remarkable increase in gene expression was observed with the *omp*18 gene using the CE supplement, indicating the important participation of outer membrane proteins in biofilm establishment. The clinical isolate showed similar and even greater gene expression than the reference strain. The results obtained indicated that the nutrients provided by CE favored biofilm formation with retained pathogenicity that under certain conditions can occur in natural aquatic environments.

Key words: *Helicobacter pylori*, biofilm, cyanobacterial extract, gene expression.

1. Introduction

Helicobacter pylori is a human pathogen that colonizes the gastric mucosa, resulting in an acute inflammatory response and damage to epithelial cells, progressing to a number of disease states, including gastritis, peptic ulceration, and gastric cancer [1].

Recent studies indicate that *H. pylori* can exist both in human gastric mucosa and on abiotic surface forming biofilms, explaining the ability of the organism to survive within and outside the host. *H. pylori* biofilm survival in drinking water is discussed as a possible waterborne route of transmission [2, 3].

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The biofilm mode of growth confers a protective advantage to the bacteria which are physiologically distinct from the free-swimming counterpart of the same species, becoming more resistant to host defense and adverse environmental conditions [3], and up to 1,000 times more resistant to antibiotics [4, 5].

The metabolic activity of biofilms is controlled by the environmental conditions found at the surfaces and the expression of specific genes induced by adhesion. In many bacteria biofilm formation of is a cell density dependent process that relies upon an intercellular communication system known as quorum sensing (QS), which is also important in the dissolution of biofilm communities [6, 7]. QS signal molecules

regulates a variety of physiological functions including the generation of bioluminescence, sporulation and the expression of virulence factors [8].

The *luxS* gene codifies for the autoinducer 2 (AI-2) of QS system that is functional in stimulating the *H. pylori lux* operon present in several gram-positive and gram-negative bacteria [9, 10].

The expression of *luxS* gene is essential for *H. pylori* colonization of the human stomach and represents a significant indicator of biofilm production in which bacteria migrate and adhere forming microcolonies [9, 11].

Apart from the *luxS* gene, other genes are involved in biofilm formation including genes encoding for flagella (*fla*A), type I and type IV pili and surface adhesins [12].

The flagella play important roles in biofilm formation in several gram-negative bacteria, both as surface adhesins and as providers of force-generating motility [13]. The expression of *H. pylori fla*A is dependent on *lux*S and the *fla*A transcription increases with culture density [14].

Bacterial outer membrane proteins (OMPs) are important for ion transport, osmotic stability, bacterial virulence and adherence. *Omp*18 is a peptidoglycan-associated lipoprotein precursor, present in *H. pylori*, which is involved in adhesion to gastric cell [15]. The cell envelope gene (*lpxD*) encoding the UDP-3-0-(3-hydroxymyristoyl) glucosamine N-acyltransferase is up-regulated after adhesion to gastric cell in vitro [16]. Therefore, *omp*18 and *lpx*D genes could be involved in biofilm formation.

H. pylori urease enzyme (*ure*A) is essential for pH regulation. The loss of urease activity acidifies the biofilm, decreasing the stability of the biofilm community [17].

Epidemiological data suggest that contaminated water is a potential reservoir for this microorganism [18], even specific *H. pylori* DNA fragments have been detected in river water [19, 20].

The presence of cyanobacteria is common in natural aquatic environments where they can generate blooms generally associated with a diverse community of heterothophic cultivable bacteria, some of them considered putative pathogens [21-23]. Aditionally a cyanobacterial extract (CE) obtained from the cyanobacterium *Nostoc* sp. have nutrients, including amino acid, soluble and crude proteins, carbohydrates, and different minerals, useful in the culture of fastidious bacteria [24].

The replacement of fetal calf serum (FCS) by CE in liquid culture media of *H. pylori* increased growth and cell viability with delays in the appearance of coccoid forms considered viable but nonculturable cells (VBNC) [25, 26]. *H. pylori* adhesion and biofilm formation is strongly affected by the presence of certain nutrients [27]. In this work the effects of CE on *H. pylori* biofilm formation and the expression of *lux*S, *fla*A, *omp*18, *lpx*D and *ure*A genes associated to biofilm is presented.

2. Material and Methods

2.1 Strains and Media

H. pylori NCTC11638 (reference strain) a kind gift for Dr. Manuel López-Brea, Microbiology Service of Hospital Universitario de la Princesa, Madrid, Spain and HP796, characterized as clarithromicyn (CLA) and metronidazole (MTZ) resistant strain and *cag*A + and *vac*A s1m1, were used for this study. The bacterial strains were routinely grown on Mueller-Hinton agar (MHA), supplemented with 7% horse blood (MHA-HB), and incubated in a microaerobic atmosphere for 48 h at 37 °C. The identity of *H. pylori* was confirmed by the following criteria: microaerophilic growth requirement, morphology, Gram's stain, oxidase, catalase and urease reactions. Strains were stored in trypticase soy broth supplemented with 20% glycerol at -80 ºC until use.

2.2 Cyanobacterial Extract

The CE was obtained as previously described by

Silva et al. [24].

2.3 Biofilm Assays

The ability of *H. pylori* to form biofilm on abiotic surfaces was determined by total bacterial count and viable cell count methods using 2 cm^2 coverslips of glass and polypropylene (PP) placed on 90 mm Petri dishes added with 12 mL of 0.3% glucose Mueller-Hinton Broth (MHB) supplemented with 5% FCS (MHB-FCS) or 1% CE (MHB-CE). The biofilm formation was initiated by inoculating *H. pylori* cells at a final concentration of 1×10^6 colony forming units (CFUs)/mL in each plate. Cultures were incubated under microaerophilic conditions for 196 h at 37 ºC without shaking. In order to analyze biofilm evolution, coverslips were sampled at different times, rinsed three times with phosphate-buffered saline (PBS) to remove planktonic cells and biofilm debris and stained with 0.1% crystal violet (CV). Also rinsed coverslips were vortexed for 3 min in PBS to allow cell detachment from biofilm. Total bacterial count was performed using the Breed Counting Method by spreading 0.01 mL of resuspended biofilm cells over 1 cm^2 microscope slide, the smear was dried and stained with 0.1% CV for 30 min and the cells counted with an optical microscope. For viable cell counts, undiluted and 1:10 dilution resuspended biofilm cells were plated onto MHA-HB by duplicate. CFUs were counted after incubation for three days at 37 ºC.

2.4 Fluorescence Microscopy

To assess the membrane integrity of sessile bacteria, the coverslips were washed with 0.9% saline and stained with fluorescent dyes of the Live/Dead BacLight kit (Molecular Probes, Invitrogen Corporation) and incubated for 20 min in dark. Coverlips were observed with a Zeiss Axioplan 2 fluorescent microscope. Images were acquired by a camera using Axiovision 3.0 software.

2.5 Scanning Electron Microscopy

Biofilms were analyzed by scanning electron microscopy (SEM) using a Zeiss LEO 1450VP microscope. Biofilms formed on abiotic surfaces were washed with 0.9% saline solution, dried and dehydrated using a graded ethanol series (70%, 95%, and 100% three times for 10 min each). Samples were mounted on aluminium stubs and coated with a gold layer and processed in a standard sputter. Observations were made at 20 KV.

2.6 Gene Expression

Cells coming from biofilms developed on the abiotic surfaces and planktonic cells were treated with TRIzol reagent (Invitrogen) for total RNA extraction.

The QS *lux*S, virulence *ure*A, *fla*A, adhesion *lpx*D, *omp*18 and housekeeping 16S rRNA genes were analyzed. cDNA was performed with random hexamer and 200 U Moloney murine leukaemia virus reverse transcriptase (Invitrogen). The identification of amplified fragments of 465-, 411-, 111-, 1001- 165- and 390- bp for, *lux*S, *ure*A, *fla*A, *lpx*D, *omp*18 and 16S rRNA genes respectively, was performed with 1.8% agarose gel electrophoresis at a constant voltage of 80 V/cm for 45 min. The gels were stained with GelRed nucleic acid gel stain (Biotium, Inc.), visualized under UV light and photographed. The DNA fragment size was determined by comparison with molecular weight markers with a range of 50 bp to 1,000 bp.

2.7 Statistical Analysis

The statistical analysis was determined by Tuckey-Kramer multiple comparisons tests. Differences were considered statistically significant at $P \leq 0.05$.

Semi-quantification of the bands was performed with an image analyzer (ImageJ WCIF) against the constitutive gene 16S rRNA.

3. Results

3.1 Biofilm Formation

H. pylori strains were able to form biofilm on both

surfaces with the nutritional supplements analyzed (FCS or CE) (Table 1). The attachment of cells increased up to 96 h and in all the cases was higher with CE ($P \leq 0.05$) and with the HP796 strain ($P \leq 0.05$).

The viable counts for the strains attached to the hydrophilic surface (glass) at several times of incubation showed a similar pattern (Fig. 1). The same results were obtained with the hydrophobic surface PP (data not shown). NCTC11638 and HP796 strains biofilm on glass surface showed a major prevalence of bacillary forms at 196 h of incubation when culture was supplemented with CE (Fig. 2). The evolution of HP796 strain biofilms on PP surface at 48, 96 and 196 h, with CE or FCS supplements by optical microscopy is showed in Fig. 3.

The membrane integrity of biofilm cells developed on glass surface with MHB-FCS and MHB-CE media after 96 h of incubation, was assessed by fluorescence microscopy using the Bacto Ligth Live/Dead kit, with green labeled cells (viable) and red cells (nonviable).

The biofilms of HP796 produced with MHB-FCS presented a higher proportion of nonviable coccoid forms than MHB-CE, additional, with MHB-CE the biofilm showed a great amount of spiral, viable, aggregated bacteria (Fig. 4). Viability staining of the biofilms showed a loss of viability after long term incubation.

Table 1 Attachment of *H. pylori* strains to abiotic surfaces in MHB supplemented with FCS or CE. Data are represented as means log10 ± *S.D.* of total cells counts of three independent experiments.

Time (h)	Glass				PP			
	NCTC11638		HP796		NCTC11638		HP796	
	FCS	CE	FCS	CE	FCS	CE	FCS	CE
24	3.9 ± 0.2	4.3 ± 0.2	5.3 ± 0.1	5.9 ± 0.1	3.3 ± 0,2	3.5 ± 0.2	4.6 ± 0.1	4.9 ± 0.1
48	5.3 ± 0.1	5.6 ± 0.1	6.1 ± 0.2	6.7 ± 0.2	4.7 ± 0.1	5.1 ± 0.2	5.7 ± 0.2	6.7 ± 0.2
96	5.5 ± 0.2	5.8 ± 0.2*	6.3 ± 0.2	6.9 ± 0.2*	4.9 ± 0.1	5.3 ± 0.1*	5.9 ± 0.2	6.9 ± 0.2*
196	4.5 ± 0.1	5.1 ± 0.2	5.1 ± 0.2	5.7 ± 0.1	3.9 ± 0.2	4.9 ± 0.2	5.3 ± 0.1	5.5 ± 0.1

*: $P \leq 0.05$.

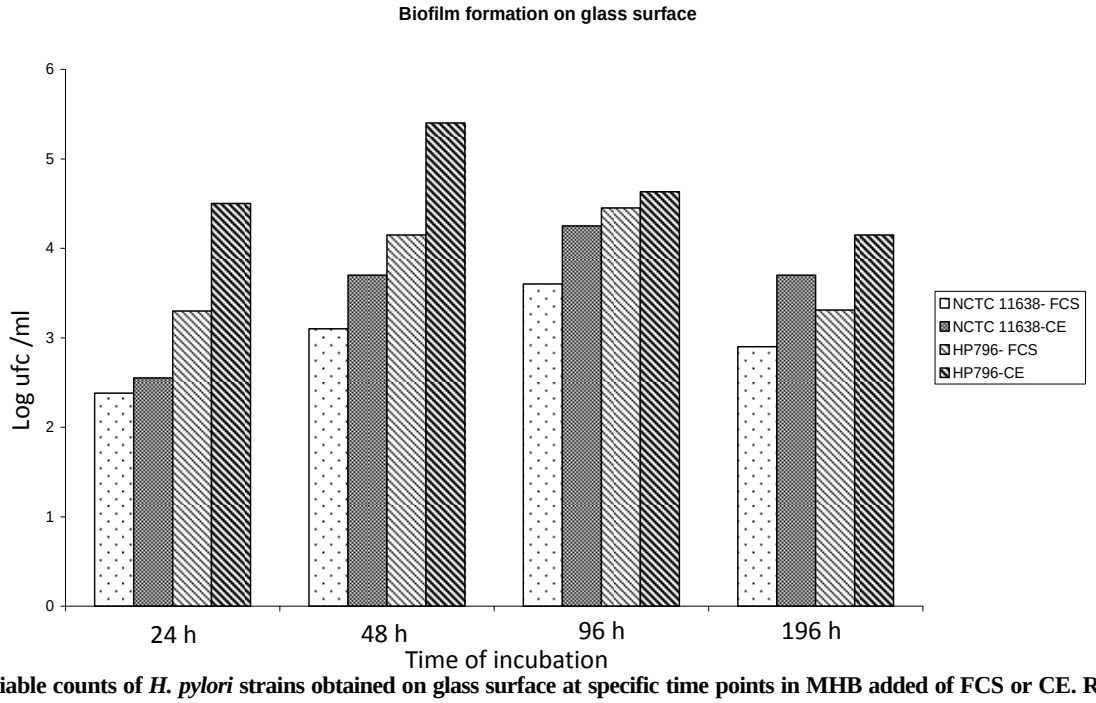

Fig. 1 Viable counts of *H. pylori* strains obtained on glass surface at specific time points in MHB added of FCS or CE. Results are the average of three independent experiments.

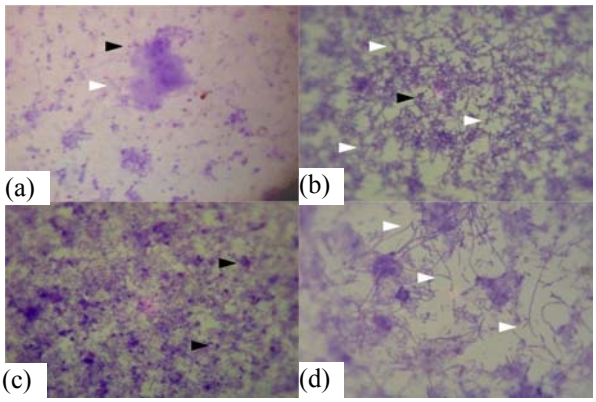

Fig. 2 Biofilms of *H. pylori* strains on glass surface. Bacteria were grown in (1) MHB-FCS and (2) MHB-CE. (a) *H. pylori* NCTC11638 (1), (b) *H. pylori* NCTC11638 (2), (c) *H. pylori* HP796 (1), (d) *H. pylori* HP796 (2). Biofilms were stained with CV after 196 h of incubation. White arrows indicate bacillary forms and black coccoids forms. Results are representative of three independent experiments.

Similarly, *H. pylori* HP796 biofilm developed for 196 h on glass surface, examined by SEM, showed predominant bacteria with coccoid shapes and U forms using MHB-FCS and filamentous aggregates that conserved the characteristic spiral morphology with MHB-CE (Fig. 5).

3.2 Gene Expression

The expression of *lux*S, *fla*A, *omp*18, *ure*A, *lpx*D and 16S rRNA genes of *H. pylori* strains in planktonic and sessile cells on glass surface, at specific time points (48, 96 and 196 h) using MHB-FCS and MHB-CE media are shown in Fig. 6. The expression of the same genes in cells attached to polypropilene surface at 196 h is also shown in Fig. 6.

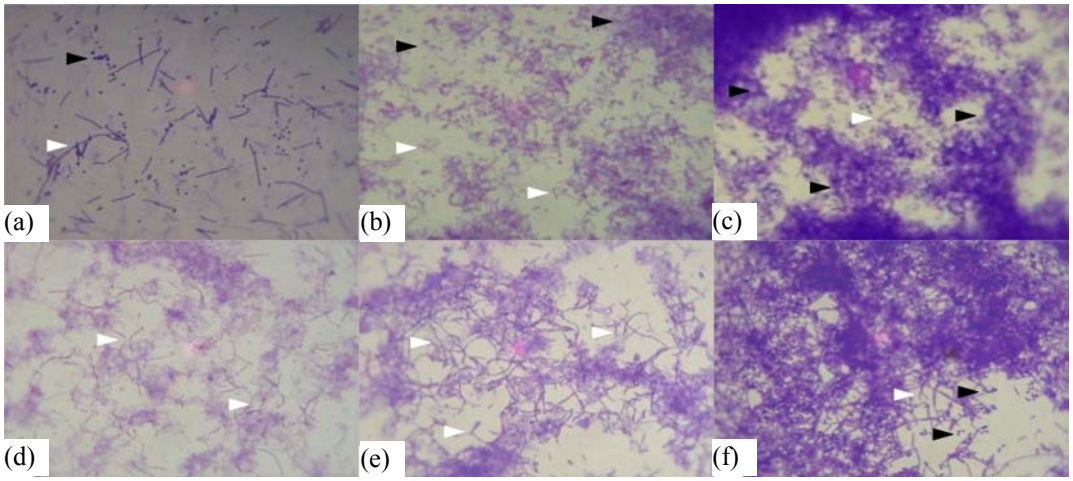

Fig. 3 Biofilm evolution of HP796 strain on PP surface. Bacteria were grown in MHB-FCS and MHB-CE media and stained with CV. (a), (b) and (c): MHB-FCS; D, (e) and (f): MHB-CE after 48, 96 and 196 h of incubation respectively. White arrows indicate bacillary forms and black arrows indicate coccoid forms. Results are representative of three independent experiments.

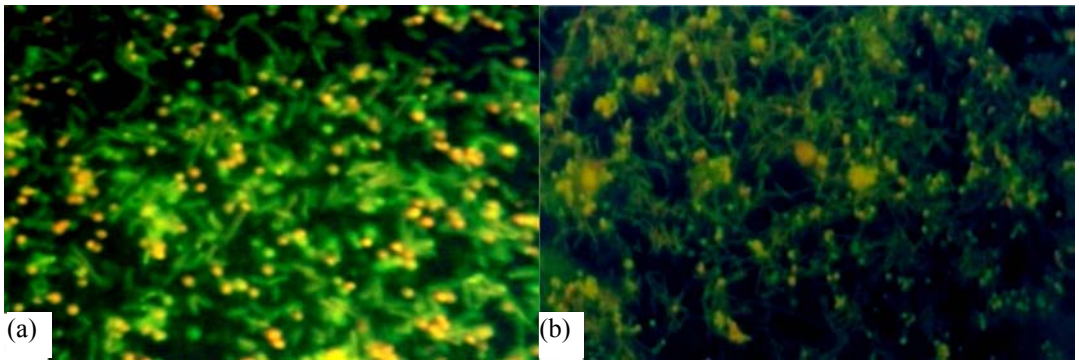

Fig. 4 Images of *H. pylori* NCTC11638 biofilm formed with (a): MHB-FCS and (b): MHB-CE. Bacteria were labeled with the Bacto Live/Dead viability stain after 96 h of incubation, wherein live bacteria fluoresce green and dead bacteria fluoresce red. Results are representative of three independent experiments.

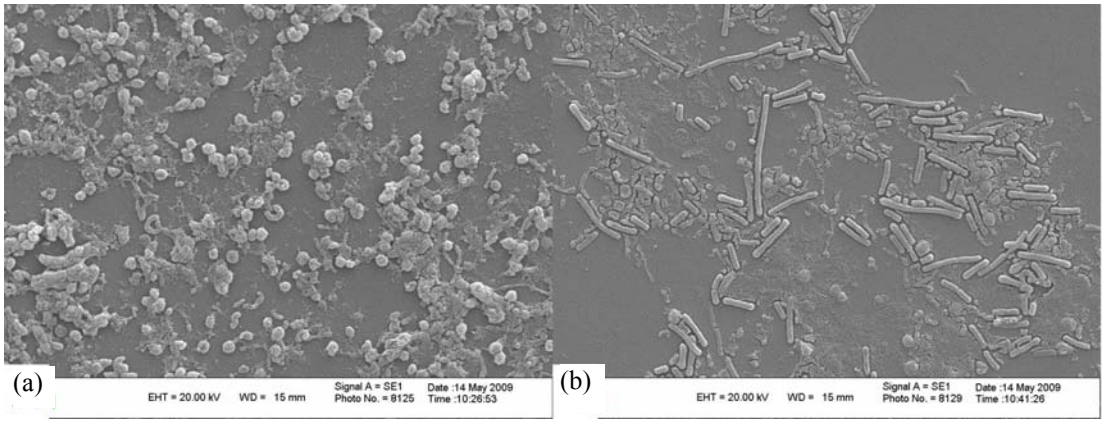

Fig. 5 Scanning electronic micrographs of *H. pylori* HP796 cells attached to coverslips after growth for 196 h with (a): MHB-FCS and (b): MHB-CE, showing aggregated bacteria with coccoid shape and U forms in (a) and aggregated bacteria with filamentous characteristic morphology in (b). Scale bar represents 2 µm. Results are representative of three independent experiments.

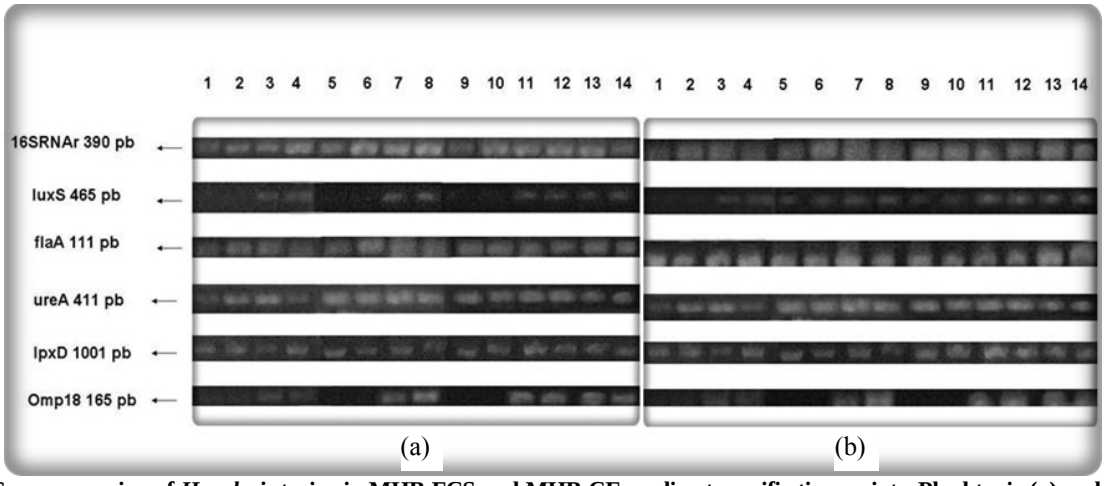

Fig. 6 Gene expression of *H. pylori* strains in MHB-FCS and MHB-CE media at specific time points. Planktonic (p) and sessile (s) cells on glass (g) and PP surfaces were assessed by PCR for 16S rRNA, *lux*S, *ure*A, *fla*A, *omp*18, *lpx*D genes. (a) Reference strain; (b) HP796 *H. pylori* strain. Lines 1, 5 and 9: p cells in MHB-FCS; lines 2, 6 and 10: p cells in MHB-CE; lines 3, 7, 11 s cells in MHB- ;FCS; lines 4, 8, 12 s cells in MHB-CE; lines 13, 14 s cells in MHB-FCS and MHB-CE respectively; lines 1-12: g surface; lines 13, 14: PP surface; lines 1-4: 48 h; lines 5-8: 96 h; lines 9-12: 196 h; lines 13 and 14 s cells attached on PP at 196 h. Results are representative of three independent experiments.

The relative increase in gene expression of *H. pylori* strains forming biofilm on glass surface in relation to that of planktonic cells using MHB-FCS and MHB-CE media at specific time points is shown in Table 2.

The *lux*S gene expression of the HP796 strain supplemented with CE was 1.7 fold higher while the reference strain 0.9 fold inferior after 48 h of biofilm evolution.

The expression of *H. pylori* HP796 *fla*A gene increased 1.5 fold using MHB-CE meanwhile with MHB-FCS the expression was 0.7 fold inferior than planktonic cells at initial stage of biofilm evolution (48 h). At this stage *H. pylori* HP796 strain showed higher *fla*A expression ($p \leq 0.05$) than the reference strain. No significant differences were observed in the expression of this gene between strains and nutritional supplements at other biofilm stages. The *ure*A gene expression increased 1.2 fold and 1.5 fold for reference and HP796 strains respectively at initial stage of biofilm evolution with MHB-FCS and then diminished at later stages. While, with MHB-CE *ure*A gene expression remained constant along biofilm evolution (Table 2).

No significant differences were observed in *lpx*D expression during biofilm evolution with respect to planktonic cells, using both supplements. The most remarkable increase in gene expression ($P \leq 0.05$) was observed with the *omp*18 gene at 196 h. For the HP 796 and reference strain, 4.6 fold and 2.4 fold increases resulted using the MHB-CE medium, values that were higher than that obtained in the biofilm developed with the MHB-FCS medium with 3.1 fold and 1.9 fold increases respectively.

The gene expression of biofilms evolving on the PP surface for the strains showed similar results to those obtained on glass surface. Apart from *omp18* gene there were no statistical differences in gene expression between both supplements and strains (Table 3). However, for this gene, a clear tendency of higher expression was observed for the HP796 strain using the MHB-CE medium ($P = 0.3$).

4. Discussion

Epidemiological studies performed in a wide variety of natural habits show that bacteria live in complex communities usually attached within a structured biofilm ecosystem and not as planktonic cells [28].

4.1 Biofilm Formation

H. pylori has the ability to form biofilms *in vitro* on different surfaces and on the human gastric mucosa [2, 29, 30].

H. pylori strains were able to form biofilms on glass and polypropilene surfaces growing in MHB-FCS or MHB-CE media. Large aggregates of predominant filamentous forms were characterized in a mature biofilm of 48 h, independently of the surface and nutritional supplement used. However, for the same

Table 2 Relative gene expression of *H. pylori* strains forming biofilm on glass surface in relation to planktonic cells, using MHB-FCS and MHB-CE media at specific time points.

	NCTC11638			HP796		
Time (h)	48	96	196	48	96	196
(a) Using FCS as supplement						
*lux*S	0.9	1.2	1.0	1.0	1.0	1.5
*fla*A	1.1	1.1	0.7	0.7	0.8	0.9
*ure*A	1.2	0.9	0.7	1.5	0.8	1.1
*lpx*D	0.8	0.8	0.9	0.7	0.8	1.4
*omp*18	1.0	1.6	1.9	1.1	1.8	3.1*
(b) Using CE as supplement						
*lux*S	0.9	1.4	1.3	1.7	1.1	1.5
*fla*A	0.7	0.9	0.8	1.5	1.0	1.1
*ure*A	0.7	0.9	1.0	1.0	1.1	1.2
*lpx*D	0.7	1.0	0.9	1.1	1.5	1.2
*omp*18	0.8	2.3	2.4	1.3	3.7	4.6*

*: $P \leq 0.05$.

Table 3 Relative gene expression of *H. pylori* strains forming biofilm on polypropylene surface in relation to planktonic cells using MHB-FCS and MHB-CE media at 196 h.

Genes	NCTC11638		HP796	
	FCS	CE	FCS	CE
*lux*S	1.0	1.9	1.3	1.4
*fla*A	0.7	1.1	1.2	1.6
*ure*A	0.6	1.0	0.8	0.9
*lpx*D	0.8	1.2	1.0	1.1
*omp*18	2.0	3.1	3.1	3.8*

*: $P = 0.3$.

time of incubation *H. pylori* biofilm developed with Brucella broth supplemented with 2% FCS presented cells with a predominant coccoid morphology [11].

During the process of biofilm formation the proportion of coccoid to spiral forms clearly varied with the supplement used. *H. pylori* conserved the characteristic bacillary morphology and viability with CE which correlated with a major attachment rate in relation to that with FCS regardless of strains or surface. These results can be attributed to the different nutrient potential provided by the two supplements.

The virulent strain HP796, Cla- and Mtz-resistant, had higher ability to form biofilm in vitro compared with the susceptible reference strain. For this strain the viable cell count significantly ($p \leq 0.05$) increased with both surfaces and nutrition supplements. Relation not found between resistant and susceptible virulent genotypes and the ability to form biofilm by Cellini et al. [11]. In this sense, the study of a greater number of *H. pylori* strains is required to improve the knowledge of biofilm formation by resistant and virulent strains of epidemiological importance.

While the attachment of *H. pylori* cells increased with incubation time, a difference of two logarithmic units was observed between total and viable counts at 196 h of biofilm formation, indicating a loss in the culturability status of the bacteria in the biofilm wich was associated with morphological changes.

The increase of coccoid cells considered as VBNC (viable but nonculturable state) has been observed during dynamic process of biofilm formation [31, 32]. The results obtained showed that the morphological condition of *H. pylori* did not affect its ability to surface attachment. In fact *H. pylori* may present a patchy distribution forming cluster of bacillary and coccoid cells as a strategy of preservation during colonization in the host [2]. The presence of these dormant cells in the bacterial biofilm can be considered one cause of treatment failure by antibiotics.

The fluorescent dyes of the Live/Dead kit with the Syto9/PI reagents [32-34] allowed the characterization of cell viability and the distinction of different morphotypes present in the biofilms. The use of this technique demonstrated that CE was superior to FCS as nutrient supplement in maintaining the viability of *H. pylori* within the biofilm structure, according to the biofilm viable counts obtained. A higher proportion on nonviable coccoid forms was present with FCS in glass biofilms of 196 h evolution. Observations of biofilm with SEM confirmed that the agglomerates were mostly constituted by coccoid or U-shaped bacteria in contrast to the spiral morphology predominant in biofilms developed with CE. As far as the bacillary form is associated with a more infective state [35], the maintenance of spiral morphology for longer periods of time indicates that CE can provide both essential nutrient and protection against adverse environmental conditions outside the human host.

4.2 Gene Expression

The development of a mature biofilm is achieved through a number of sequential steps, each of which is marked by changes in gene expression in response to environmental cues and cell-cell signaling [36], indicating that a biofilm is a highly regulated developmental biological system.

Depending on the bacterium, the autoinductor AI-2 encoded by the *lux*S gene, plays a role in motility, pathogenicity and biofilm formation [37].

The QS system, normally associated with the regulation of virulence factors, could also regulate the various phases of biofilm development from the initial adhesion to the final detachment of cells [38, 39].

H. pylori upon adhesion to gastric epithelial cells change only a short proportion of 1,542 genes analyzed with 22 up and 21 down regulated genes [16]. As adhesion is the first step in biofilm formation it was of interest to analyze the gene expression of QS *lux*S, virulence *ure*A, *fla*A, adhesion *lpx*D, *omp*18 and housekeeping 16S rRNA genes of *H. pylori* in the development of a mature biofilm on abiotic surfaces.

The QS *lux*S gene expression of the HP796 strain supplemented with CE was 1.7 fold up regulated while the reference strain 0.9 fold down regulated after 48 h of biofilm evolution. The virulence *ure*A gene, responsible for the ureasa enzyme required for colonization and maintenance of the organism in hostile environments, showed similar levels of expression in biofilm and planktonic cells. The HP796 strain increased 50% the *ure*A gene expression at the initial state of the biofilm using both nutrient supplements.

Flagella are necessary for colonization of the gastric mucosa and play an important role in biofilm formation suppressing repulsive forces of hydrophobic surfaces [3, 13]. The expression of *fla*A gen was strain and supplement dependent. The *fla*A gene of the HP796 strain, with higher ability to form biofilm, was 1.5 fold up regulated in comparison to planktonic cells while the reference strain was 0.7 fold down regulated at the early stage of biofilm formation using the MHB-CE medium. The *lpx*D gene with MHB-CE was up regulated with both strains and surfaces in biofilms of 196 h evolution.

The most remarkable increase in gene expression was observed with the *omp*18 gene with the CE supplement producing the most effective induction, indicating the important participation of outer membrane proteins in cell aggegation and biofilm establishment. Both strains up regulated the expression of *omp*18 gene, however the strain HP796, ClaR and MtzR, and genotype virulent cagA +, vacA+ and iceA1, showed similar, and even greater expression than the reference strain.

The soluble metabolites present in the CE increased biofilm formation, viability and gene expression of *H. pylori*. The CE was produced as a dry powder using concentrated biomass of the filamentous cyanobacterium *Nostoc* sp., heat treated with subsequent lyophilization of the soluble material released. The high protein content of CE [24] could stimulate the initial attachment of *H. pylori* cells to abiotic surfaces as far as its adherence is a protein mediated process [27]. Although quite different in procedure a similar release of soluble and non-soluble matter into the surrounding medium can be produced growing dense populations of filamentous cyanobacteria in enriched water lake, followed by cells collapse and liberation of fresh organic material, with an expected positive response of heterotrophic bacteria, ciliates and nanoflagellates [21].

5. Conclusions

The main contribution of this work was to demostrate the effect of a cyanobacterial extract on *H. pylori* biofilm formation. CE markadely increased the survival, gene expression and maintenance of *H. pylori* cells bacillary forms in the biofilm. The effect was superior to that obtained using FCS. The effect of the cyanobacterial extract favouring biofilm formation of *H. pylori* with retained pathogenicity can be considered a fact of possible occurence in water bodies where cyanobacteria are present that might constitute a novel source of transmission.

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