Changes of proteome components of *Helicobacter pylori* biofilms induced by serum starvation

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Abstract. Biofilm is the adaptive living mechanism of *Helicobacter pylori* (*H. pylori*) during survival and propagation. Nutrient starvation is an environmental pressure for *H. pylori in vivo* and *in vitro*. Serum starvation effectively mimics the microenvironment in which *H. pylori* colonizes healthy humans who carry *H. pylori* and patients with chronic atrophic gastritis. In addition, it also mimics the *in vitro* environmental pressures of *H. pylori*. An *H. pylori* biofilm was successfully induced with serum starvation. To identify novel proteins associated with biofilm formation at the early stage in *H. pylori*, high-resolution 2-dementional gel electrophoresis was performed to obtain the proteome profiles of spiral *H. pylori* and early biofilm. Differential protein spots were identified using tandem matrix assisted laser desorption ionzation time of flight mass spectrometry, which revealed 35 proteins. These proteins are associated with various biological functions, including flagellar movement, bacterial virulence, signal transduction and regulation. To verify the results, the expression of cagA at the mRNA and protein levels was examined by fluorescence quantitative PCR and western blot analysis, respectively. This study indicates that *H. pylori* form biofilms by initiating multiple mechanisms involving a number of signaling pathways.

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Introduction

Helicobacter pylori (*H. pylori*) infection is a predominant cause of gastristis and peptic ulcer disease, as well as an early risk factor for gastric cancer. It is a globally epidemic pathogenic bacteria and more than half of the world's population has been infected with this bacterium. *H. pylori* infection is common; however, its precise mode of transmission has not yet been identified. The main reason for this is that the etiology of *H. pylori* has not been solved effectively. Therefore restrictng the formulation of prevention measures against *H. pylori*.

A biofilm is a multicellular layer of bacteria, which attaches to surfaces, interfaces or other cells and is embedded within a matrix of extracellular polymeric substances under stress conditions (1). Growth in a biofilm provides a number of advantages for bacteria, including enhanced power of resistance to environmental stresses as well as to host defenses, which leads to serious clinical problems, particularly chronic and refractory infection. The biofilm of *H. pylori* has been observed in the water system, including drinking water, groundwater and sewage (2,3) and also on the human gastric mucosal epithelium (4,5). A number of studies have been performed on *H. pylori* biofilms (6,7). Thus, biofilms are hypothesized to be another adaptive survival mode of *H. pylori*. In conventional experiments, *H. pylori* is cultured in a rich medium containing fetal bovine serum (FBS). However, this may not effectively imitate the *in vitro* and *in vivo* survival environment of *H. pylori*. Nutrient starvation is a common environmental pressure that *H. pylori* is faced with. Serum starvation effectively mimics the microenvironment *H. pylori* within healthy human carriers of *H. pylori* and patients with chronic atrophic gastritis (8) and also the *in vitro* pressure environment pressure that *H. pylori* is subjected to. The current study identified that *H. pylori* adhered to each other and formed a population structure similar to that of a bacterial biofilm by removing serum from medium. Williams *et al* (9) observed that serum significantly affects the movement, morphology and adhesion of *H. pylori* and may result in agglutination in 1% serum concentration. *Campylobacter jejuni* also agglutinate in nutrient-limiting conditions. Reeser *et al* (10) observed that the autoagglutinated structure is a biofilm by analyzing its extracellular polymer matrix.

The formation of a biofilm is a complex and dynamic process. Under adverse conditions, the bacteria induce a unique set of genes, adhere to each other and form structural groups or subgroups. During biofilm formation, each link may be regarded as a key point to treat infection and drug design target, particularly at the early stage. In the present study, proteomic technologies were used to analyze the proteomic expression profiles of *H. pylori* biofilms at an early stage, and aimed to reveal the proteins and signaling molecules involved in *H. pylori* biofilm formation and identify novel pathogenic factors and antibacterial targets.

Materials and methods

Bacterial strain and culture conditions. The *H. pylor*i strain 26695 was provided by Dr Zhang Jianzhong from the Chinese Disease Control and Prevention Center. It was cultured in Brucella broth with 10% FBS with 120 rpm agitation under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2, v/v) at 37˚C. The same quantity of exponentially growing *H. pylori* was resuspended in Brucella broth with and without 10% FBS. Bacterial morphological changes were then observed using Gram staining at intervals of 2 h until the autoagglutinated structure formed. The initial time of biofilm formation was recorded. Subsequently, the planktonic *H. pylori* and the biofilm were fixed with 2.5% glutaraldehyde solution for >2 h. The critical-point dried sample was observed with a Hitachi S-520 scanning electron microscope (Hitachi Limited Company, Tokyo, Japan).

2-dimentional gel electrophoresis (2-DE) and image analysis. The planktonic *H. pylori* and its early biofilm cultured with and without 10% FBS for 4 h were harvested by centrifugation at 5000 x g for 10 min at 4˚C and washed three times with ice-cold PBS (pH 7.2). The pellets were solubilized in lysis buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 1% pharmalyte (pH range, 3-10), 1% protease inhibitor and 1% nuclease mix (Amersham Pharmacia Biotech, Piscatawway, NJ, USA). Following sonication, the solution was centrifugated at 20,000 x g for 60 min at 4˚C to remove cell debris. Protein concentrations were determined using the Bradford method and proteins were stored at -80˚C. The 2-DE analysis was performed as described previously (11). The SDS gels were silver-stained and the protein patterns were scanned using an ImageScannerII (Amersham Pharmacia Biotech). The gels were analyzed by ImageMaster 2D Elite 5.0 (Amersham Pharmacia Biotech) to determine differentially expressed protein spots whose vol% ratio increased >2-fold ($P<0.05$).

In-gel digestion and matrix assisted laser desorption ionzation time of flight mass spectrometry (MALDI-TOF-TOF MS). Differentially expressed protein spots were excised, tryptic digested and identified with a 4700 MALDI-TOF-TOF proteomic system (Applied Biosystems) as described previously (11). The MS and MS/MS spectra were analyzed with a 50 ppm mass tolerance by GPS Explorer V.2.0.1 and Mascot V1.9 based on the NCBI and SWISSPROT databases.

Quantitative PCR . Total RNA was isolated from three independent cultures of planktonic *H. pylori* and biofilm at early stages using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity was measured by absorbance at 260 nm. Subsequently, 4 μg total template RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase and random hexamer primer (MBI). The primers for PCR are listed in Table I. The 16S rRNA gene served as the endogenous control. PCR was performed as described previously (12). The operating conditions were as follows: One cycle at 50˚C for 2 min, one cycle at 95˚C for 10 min, 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Gene expression was quantified by the comparative threshold cycle (Ct) method and normalized to the quantity of 16S rRNA cDNA in each sample. The relative quantity of the target was calculated by the ΔΔCT-method.

Table I. Primers used in the study

Primers	Sequence (5'-3')
cagA Forward	GCTTACCGCCTGAAGCTAGG
cagA Reverse	CCTTTCTCACCACCTGCTATG
16S rRNA Forward	GCTCTTTACGCCCAGTGATTC
16S rRNA Reverse	GCGTGGAGGATGAAGGTTTT

Western blot analysis. Protein samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (8% SDS-acrylamide gels) and transferred to a nitrocellulose membrane. The membranes were incubated in blocking buffer [TBS containing 0.1% Tween-20 (TBST) and 5% non-fat powdered milk] for 1.5 h and immunoblotted for 1.5 h with antibodies against β-actin or C-CagA (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). Membranes were washed three times with the TBST solution and incubated with the corresponding antibodies conjugated with horseradish peroxidase for 50 min. The washed membranes were developed by the Chemilucent ECL Detection system (Millipore, Billerica, MA, USA).

Results and Discussion

In recent years, increasing evidence has indicated that bacterial biofilms are important in infectious diseases. Biofilms effectively protect bacteria from external environmental damage and are an effective strategy to adapt to environmental pressures *in vivo* and *in vitro*. As one of the predominant pathogens affecting humans, *H. pylori* was subjected to various types of adverse conditions, including nutrient starvation. To produce the survival environment *H. pylori* generated when under starvation stress, exponentially growing *H. pylori* 26695(Fig. 1A) was inoculated into medium without FBS. Following 4 h, the bacteria began to adhere to each other and formed a subgroup structure (Fig. 1B). Typical community structures were observed following 12 h starvation and a number of *H. pylori* changed to a dormant coccoid form (Fig. 1C). Thus, a biofilm of *H. pylori* was successfully induced with serum starvation.

To identify novel proteins associated with *H. pylori* biofilm formation, high-resolution 2-DE was performed to obtain the

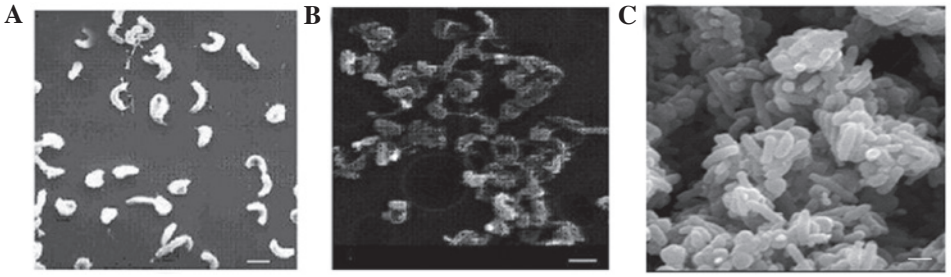

Figure 1. Scanning electron microscopy of floating unicellular *H. pylori* and its biofilm. (A) Floating unicellular *H. pylori* cultured with 10% FBS; (B) *H. pylori* biofilm cultured without FBS for 4 h and (C) *H. pylori* biofilm cultured without FBS for 12 h. Scale bar, 1 μm. *H. pylori, Helicobacter pylori*; FBS, fetal bovine serum.

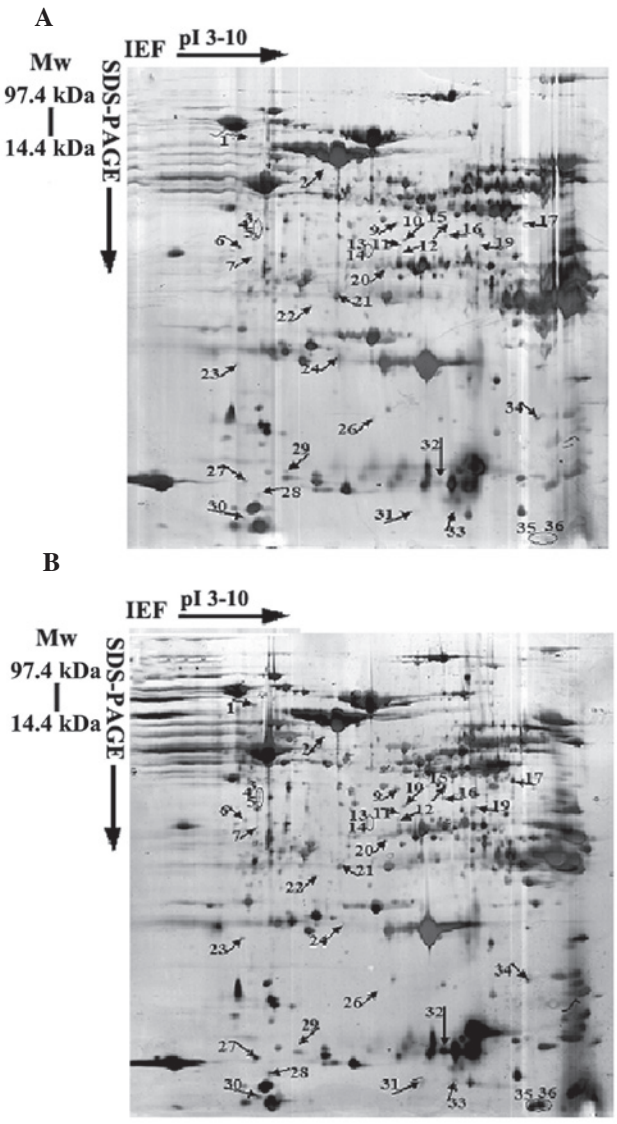

Figure 2. 2-DE profiles of whole cell proteins from (A) floating unicellular *H. pylori* and (B) its early biofilm following serum starvation for 4 h. *H. pylori, Helicobacter pylori*.

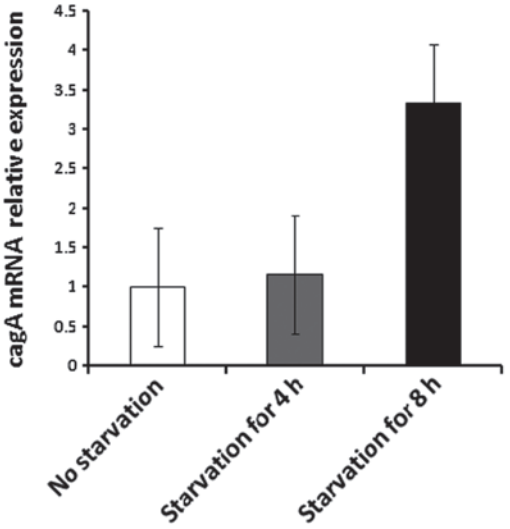

Figure 3. Expression of cagA in *H. pylori* cultured with 10% FBS and without FBS for 4 h and 8 h observed through quantitative PCR. *H. pylori, Helicobacter pylori*; FBS, fetal bovine serum.

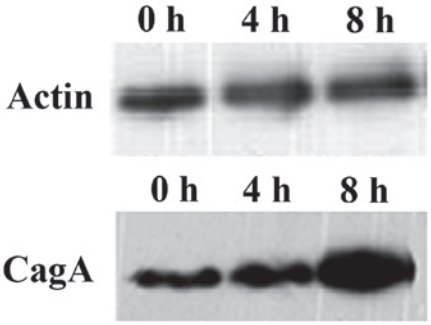

Figure 4. CagA protein expression in *H. pylori* cultured with 10% FBS and without FBS for 4 h and 8 h detected by western blot analysis. *H. pylori, Helicobacter pylori*; FBS, fetal bovine serum.

proteome profiles of spiral *H. pylori* and its early biofilm, which was induced by 4 h of starvation (Fig. 2). 2-DE analysis was repeated in triplicate using independently grown cultures. The comparison of the protein patterns of these two forms of *H. pylori* revealed that 36 spots exhibited different levels of expression (>2-fold, P<0.05). These spots were subjected to MALDI-TOF-TOF MS analysis to determine their putative genes and infer their functions. A total of 35 proteins with different functions were identified (Table II); of which 29 were upregulated and six were downregulated in the early biofilm of *H. pylori*. These proteins belong to diverse functional classes, including movement, virulence, energy metabolism, regulator and chaperone proteins. This suggests that *H. pylori* invokes a multi-mechanism response to adapt to morphological changes under starvation stress.

Table II. Summary of protein spots showing altered expression between planktonic *H. pylori* and its early biofilm.

Spot number[a]	Protein (gene)	TIGR ORF number[b]	Gi	Top score	%vol ratio[c]
1	Cag pathogenicity island protein (cag26)	Hp0547	2313664	139	2.89
2	Flagellar capping protein(fliD)	Hp0752	2313869	85	2.21
3	Aconitase B (acnB)	Hp0779	2313908	105	2.36
4	Aconitase B (acnB)	Hp0779	2313908	133	2.13
6	Type I restriction enzyme R protein (hsdR)	Hp0846	2313977	46	2.05
6	Urease β-subunit (urea amidohydrolase) (ureB)	Hp0072	2313153	45	2.05
6	Arginase (rocF)	Hp1399	2314565	50	2.05
7	*H. pylori* predicted coding region HP0013	Hp0013	2313087	64	2.02
9	Transaldolase (tal)	Hp1495	2314674	175	2.04
10	Holliday junction DNA helicase (ruvB)	Hp1059	2314203	161	2.04
10	GTP-binding protein, fusA-homolog (yihK)	Hp0480	2313589	75	2.04
11	UDP-N-acetylenolpyruvoylglucosamine reductase (murB)	Hp1418	2314592	69	2.15
12	Chaperone and heat shock protein (groEL)	Hp0010	2313084	106	7.45
13	Poly E-rich protein	Hp0322	2313421	64	2.14
14	DNA-directed RNA polymerase, β-subunit (rpoB)	Hp1198	2314357	64	2.26
15	GTP-binding protein (obg)	Hp0303	2313401	128	3.24
15	UDP-N-acetylenolpyruvoylglucosamine reductase (murB)	Hp1418	2314592	61	3.24
16	Delta-aminolevulinic acid dehydratase (hemB)	Hp0163	2313250	111	2.13
17	Aspartate-semialdehyde dehydrogenase (asd)	Hp1189	2314350	257	2.31
19	Aspartyl-tRNA synthetase (aspS)	Hp0617	2313739	65	3.16
20	*H. pylori* predicted coding region HP0958	Hp0958	2314102	90	4.76
21	Conserved hypothetical protein	Hp1588	2314773	73	0.32
21	3-oxoadipate coA-transferase subunit A (yxjD)	Hp0691	2313815	68	0.32
22	Response regulator (ompR)	Hp0166	2313252	105	2.07
23	*H. pylori* predicted coding region HP0406	Hp0406	2313513	131	2.01
24	Alkyl hydroperoxide reductase (AhpC)	Hp1563	2314747	146	0.31
26	Cag pathogenicity island protein (cag24)	Hp0504	2313660	77	0.44
26	Translation elongation factor EF-Tu (tufB)	Hp1205	2314366	45	0.44
27	Conserved hypothetical protein	Hp1046	2314193	76	2.57
27	Hemolysin secretion protein precursor (hylB)	Hp0599	2313716	49	2.57
28	Riboflavin synthase beta chain (ribE)	Hp0002	2313079	121	2.66
29	Nonheme iron-containing ferritin (pfr)	Hp0653	2313771	82	0.03
30	Hydrogenase expression/formation protein (hypA)	Hp0869	2313996	104	4.37
31	*H. pylori* predicted coding region HP1377	Hp1377	2314555	111	2.55
32	Co-chaperone (groES)	Hp0011	2313085	82	5.08
34	*H. pylori* predicted coding region HP1029	Hp1029	2314185	142	2.41
35	Thioredoxin	Hp1458	2314636	97	81.74
36	Thioredoxin	Hp1458	2314636	126	2.07

[a]Spot numbers refer to the proteins labeled in Fig. 2. [b]Nomenclature of the *H. pylori* strain 26695. [c]Percentage volume ratio for each protein derived from floating unicellular *H. pylori* with respect to the protein derived from the early biofilm. *H. pylori, Helicobacter pylori*.

The adhesion of bacteria is the early stages of biofilm formation and the flagellar movement are important in this process. The present study identified that the flagellar filament capping protein (HAP2) was increased in the early biofilm of *H. pylori*. This protein is encoded by the fliD gene, constitutes the flagellar hook together with HAP1 and HAP3, and is involved in the flagellar assembly of *H. pylori*. Martin *et al* observed that proteins associated with flagella exhibited higher expression in the biofilm of *Campylobacter* and that the deletion of flagellar genes may seriously affect the biofilm formation of bacteria (13,10). In *Escherichia coli* (*E. coli*), flagellar motility may significantly affect the three-dimensional structure of the biofilm (14). In addition, aconitase was observed to be upregulated in *H. pylor*i early biofilms. The structural analysis identified that this protein contained a domain similar to the HEAT domain, which mediates the interaction between

proteins. In *E. coli* and *Bacillus subtilis*, the HEAT protein has been confirmed to have a dual function and regulate flagellar movement as a post-transcriptional regulator under iron and oxygen conditions (15,16). Under serum starvation conditions, the overexpression of aconitase in *H. pylori* is hypothesized to be involved in biofilm formation by the promotion of flagellar movement. However, this requires further study to be confirmed.

In present study, the cagA protein encoded by cag pathogenicity islands was identified to be induced in *H. pylori* biofilms. As an important virulence factor of *H. pylori*, this protein is injected into gastric mucosa epithelial cells through the type IV secretion system when the cagA positive *H. pylori* infect the individual. CagA is then localized in the inner surface of the plasma membrane and results in a series of changes, including excessive proliferation and phenotypic change, such as extended 'hummingbird' morphology (17). To confirm the results, the expression of cagA was examined at the mRNA and protein levels by quantitative PCR and western blot analysis, respectively. The results showed that the expression of cagA was not significantly changed following 4 h of starvation. This may be due to higher resolution of the 2-DE compared with 1-D SDS. However, after 8 h cagA expression was upregulated significantly (Figs. 3 and 4). Thus the upregulation of this protein was hypothesized to aid in the improvement of the viability of *H. pylori* under adverse environments.

UspA is a conservative and integral promoter of a number of stress-associated proteins in a variety of bacteria. The β-subunit of RNA polymerase positively regulates the expression of UspA under a number of environmental pressures, including starvation. Thus, a variety of stress-related proteins show higher expression levels and bacterial biofilms have strong resistance to the outside pressure (18). In the current study, the β-subunit of RNA polymerase and two chaperone protein GroES and GroEL of *H. pylori* were observed to be overexpressed under serum starvation. In addition, two oxidative stress-related proteins, alkyl hydroperoxide reductase and thioredoxin, were identified to have a high level of expression in the biofilm of *H. pylori*. This is consistent with the biofilms of *E. coli* and *Campylobacter* (13,19).

The two-component system is a common signal transduction mechanism in bacterial responses to various stresses. This system is composed of two types of proteins, a histidine kinase and a response regulator. These two components exchange signals by phosphate transfer. Two-component systems are involved in the biofilm formation of *E. coli*, *Pseudomonas aeruginosa* and other bacteria (20,21). For example, the EnvZ/OmpR two-component system in *E. coli* promotes biofilm formation by regulating the expression of proteins associated with bacterial fimbriae to enhance its adhesion (22). In the present study, the response regulator Hp0166 was observed to be induced in *H. pylori* biofilms. A previous study also demonstrated that the Ars two-component system is involved in *H. pylori* responses to acid stress by regulating the expression of a variety of genes (23).

In addition, the current study also identified that the expression of other proteins changed in *H. pylori* biofilms. The expression of urease and arginase was upregulated. These two proteins generate ammonia in the process of amino acid metabolism, which is essential to biofilm formation to maintain the pH balance. The accessory protein of urease, ureE, is highly expressed in *Staphylococcus aureus* biofilms (24). Peptidoglycan is a predominant component of the cell walls of bacteria, UDP-N acetylene alcohol acid glucosamine reductase is an enzyme that is required in peptidoglycan biosynthesis. It catalyzes the reduction of UDPN GlcNAc to UDPN acetyl muramic acid (25). This protein was overexpressed in *H. pylori* biofilms and it is consistent with a previous study of *Staphylococcus aureus* (14). The function of peptidoglycan remains unclear, but it was observed that fracture of the peptidoglycan may promote the formation of bacterial biofilms (26). In addition, this study also showed that the expression of a number of other unknown proteins, including Hp1377 and Hp1029, changed in *H. pylori* biofilm formation. However, this also requires further study.

In the present study, *H. pylori* biofilm formation was induced successfully by serum starvation and its proteome was analyzed using proteomic methods. This study showed that ≥35 proteins are involved in biofilm formation. These proteins are associated with a number of types of biological functions, including flagellar movement, bacterial virulence, signal transduction and regulation. Such results showed that *H. pylori* biofilms are formed through multiple mechanisms involving numerous signal pathways. These findings may provide valuable information in understanding the survival mechanism of this bacterium in animals and humans.

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