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Helicobacter pylori protein response to human bile stress

Chunhong Shao,[1]† Qunye Zhang,[2]† Yundong Sun,[1] Zhifang Liu,[3] Jiping Zeng,[3] Yabin Zhou,[1,2] Xiuping Yu[1,2] and Jihui Jia[1,2]

[1]Department of Microbiology, School of Medicine, Shandong University, 44 Wenhuaxi Road, Jinan, Shandong 250012, PR China

[2]Key Lab for Experimental Teratology of Chinese Ministry of Education, School of Medicine, Shandong University, Jinan, Shandong 250012, PR China

[3]Department of Biochemistry, School of Medicine, Shandong University, Jinan, Shandong 250012, PR China

Correspondence
Jihui Jia
jiajihui@sdu.edu.cn

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The ability of *Helicobacter pylori* to tolerate bile is likely to be important for its colonization and survival in the gastrointestinal tract of humans. As bile can be acidified after reflux into the low pH of the human stomach, the inhibitory effect of fresh human bile with normal appearance on *H. pylori* before and after acidification was tested first. The results showed that acidification of bile attenuated its inhibitory activity towards *H. pylori.* Next, the protein profiles of *H. pylori* under human bile and acidified bile stress were obtained by two-dimensional electrophoresis. Protein spots with differential expression were identified using tandem matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The results showed that the changes in proteomic profiles under bile and acidified bile stress were similar when compared with that of normal *H. pylori.* Expression of 28 proteins was found to be modulated, with the majority being induced during bile or acidified bile exposure. These proteins included molecular chaperones, proteins involved in iron storage, chemotaxis protein, enzymes related to energy metabolism and flagellar protein. These results indicate that *H. pylori* responds to bile and acidified bile stress through multiple mechanisms involving many signalling pathways.

INTRODUCTION

The reflux of bile into the stomach post-prandially is a normal occurrence, especially in patients with gastroduodenal disease (Worku *et al.*, 2004). Although it has been established that *Helicobacter pylori* is bile-sensitive, the mechanism allowing it to colonize a bile-containing environment is unclear. It has been suggested previously that the 'acidification' of bile through reflux into the low pH of the stomach precipitates inhibitory bile acids, thereby allowing colonization by *H. pylori* (Hynes *et al.*, 2003). Many studies support the suggestion that the faecal–oral route is important for *H. pylori* entering the digestive tract (Cellini *et al.*, 1999; Stone, 1999; Kivi & Tindberg, 2006). During its transmission, *H. pylori* must tolerate and resist bile stress when traversing the bile-rich intestinal tract. Furthermore, *H. pylori* has been detected in bile, gall-bladder tissue and liver samples by PCR and Southern blot hybridization (Nilsson *et al.*, 2000; Fallone *et al.*, 2003; Neri *et al.*, 2005). All these points indicate that the ability of *H. pylori* to tolerate bile is likely to be important for its colonization and survival in the gastrointestinal tract of humans.

Sublethal bile concentrations can trigger a physiological adaptive response in bacteria, resulting in the employment of mechanisms similar to those used in other stress responses (Begley *et al.*, 2005). For example, a study by Bernstein *et al.* (1999) in *Escherichia coli* showed that genes including *micF*, *osmY* and *dinD*, which can protect DNA against damage and oxidative stress, are activated by bile salts. A DNA microarray assay in *Salmonella* showed that the AcrAB pump is required for bile resistance (Prouty *et al.*, 2004). However, proteome studies focus on the complete set of proteins encoded by the genome and thus complement transcriptional studies. Fox *et al.* (2007) used two-dimensional electrophoresis (2-DE) to compare protein expression profiles of *Campylobacter jejuni* grown with and without ox bile. They found that the bile could modulate proteins such as elongation factors, ferritin, chaperones, ATP synthase and others. A proteomic approach was also used to analyse the regulation of bifidobacterial proteins during growth in the presence of

†These authors contributed equally to this work.

Abbreviations: 2-DE, two-dimensional electrophoresis; IPG, immobilized pH gradient; MALDI-TOF/TOF, tandem matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry.

bile salts (Sánchez *et al.*, 2005). However, global changes in protein expression of *H. pylori* during bile and acidified bile exposure have not yet been reported.

The genomic sequencing of many micro-organisms, including *H. pylori*, has been completed and proteomic technologies have proved to be particularly useful for studying the physiological responses of bacteria to various environmental stresses (Tomb *et al.*, 1997). Using this information, we analysed protein expression changes of *H. pylori* under bile and acidified bile stress using comparative proteomics. In an attempt to reveal the possible molecular mechanisms underlying bile resistance in this important human pathogen and to simulate accurately the effects of human bile on *H. pylori*, fresh human bile of normal appearance was used in this study.

METHODS

Treatment of bile. Treatment of bile samples was conducted as described previously (Graham & Osato, 2000). Fresh, normal-looking human bile was collected from patients (28–65 years, five male and seven female) undergoing bile drainage for various indications without complicated chronic cholecystitis. The protocol was directed by the ethics committee and with the informed consent of patients. Bile samples were mixed, centrifuged, sterilized through a 0.22 μm pore-size membrane and separated into equal portions. Some of the bile was acidified to pH 2.0 by the addition of 1 M HCl after sterilization. The samples were then centrifuged at 8000 r.p.m. in a microfuge for 20 min to remove the precipitated bile salt, the supernatant was decanted and the pH was adjusted to pH 7.0 by the addition of 1 M NaOH for the acidified bile samples. Acidified and unacidified bile solutions were diluted 1 : 3 in Brucella broth containing 10 % fetal bovine serum and then serially diluted twofold up to a dilution of 1 : 768.

Bacterial strain and culture conditions. *H. pylori* strain 26695 was kindly provided by Dr Zhang Jianzhong (Chinese Disease Control and Prevention Center). The bacteria were cultured on Skirrow agar with 5 % (v/v) sheep blood for 48 h under microaerobic conditions (5 % O_2/10 % CO_2/85 % N_2, by vol.) at 37 °C. To examine the inhibitory activity of bile and acidified bile on *H. pylori*, plate-grown bacteria were scraped and resuspended in Brucella broth. Each test solution, comprising the various dilutions of acidified and unacidified bile, was inoculated with *H. pylori* cells (approx. 1×10^7 bacteria ml^{-1}). The control was Brucella broth containing 10 % fetal bovine serum without bile or acidified bile. These cultures were incubated at 37 °C under microaerobic conditions for 72 h. At the end of the incubation, 10 μl aliquots were removed from each test solution and used to inoculate Skirrow agar plates containing 5 % (v/v) sheep blood to determine whether growth was inhibited. The results were scored as growth or no growth. The maximum bile and acidified bile dilution with macroscopic growth of *H. pylori* was defined as the maximum growth dilution. *H. pylori* colonies were then counted at intervals of 12 h to determine the growth rate of *H. pylori* in the absence of human bile and at the maximum growth dilution of unacidified and acidified bile.

Preparation of *H. pylori* proteins. *H. pylori*, cultured in the absence of bile and in the maximum growth dilution of unacidified and acidified bile, was harvested by centrifugation at 5000 ***g*** for 10 min at 4 °C and washed three times with ice-cold PBS (pH 7.2). The pellet was then dissolved in lysis buffer containing 8 M urea, 2 M thiourea, 4 % CHAPS, 1 % DTT, 1 % Pharmalyte (pH range 3–10), 1 % protease inhibitor mix and 1 % nuclease mix (Amersham Biosciences). After sonication, the solution was centrifuged at 20 000 ***g*** for 60 min at 4 °C. The protein concentration was determined using the Bradford method and proteins were stored at −80 °C until 2-DE analysis.

2-DE and image analysis. Following a typical protocol, 100 μg total protein was adjusted to a total volume of 340 μl with rehydration buffer containing 8 M urea, 4 % CHAPS, 20 mM DTT, 0.5 % Pharmalyte and a trace of bromophenol blue. For isoelectric focusing, samples were loaded onto 18 cm immobilized pH gradient (IPG) strips (pH 3–10 NL) and placed on an IPGphor instrument (Amersham Biosciences). The strips were rehydrated for 12 h at 60 V and then run for 2 h at 100 V and 1 h at 500, 1000 and 5000 V; the final step was kept constant at 8000 V to a total of 80 kVh.

After isoelectric focusing, the IPG strips were equilibrated with gentle shaking for 15 min each in buffer [50 mM Tris/HCl (pH 8.8), 6 M urea, 30 % glycerol, 2 % SDS, trace of bromophenol blue] with 0.5 % (w/v) DTT and 2 % (w/v) iodoacetamide. SDS-PAGE was performed on 13 % acrylamide gels using a PROTEAN IIxi two-dimensional cell (Bio-Rad) at 15 mA per gel for 30 min followed by 30 mA per gel until the bromophenol blue front reached the bottom of the gel. The gels were then silver-stained and scanned using ImageScanner II (Amersham Biosciences) at the 256 greyscale and 300 d.p.i. degree levels. A gel of *H. pylori* cultured without bile was used as a reference. The test gels were matched against the reference gel using ImageMaster 2D Elite v5.0 (Amersham Biosciences) in order to find differentially expressed protein spots (greater than twofold difference, $P<0.05$).

In-gel digestion and MALDI-TOF/TOF MS. Differentially expressed protein spots were excised, tryptically digested and identified by tandem matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) using a 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). Gel spots were excised manually and then digested and desalted with trypsin and a ZipPlate (Millipore). Digested peptides were mixed with α-cyano-4-hydroxycinnamic acid and spotted onto MALDI target plates. Peptide mass fingerprints were collected in the positive-ion mass spectrometry (MS) reflector mode. MS/MS was performed with air as the collision gas at 6×10^{-7} torr (800×10^{-7} Pa). The MS and MS/MS spectra were analysed with a 50 p.p.m. mass tolerance by GPS Explorer v2.0.1 and Mascot v1.9 based on NCBI SWISS-PROT and local *H. pylori* databases (updated April 2006). Background peaks from known trypsin autodigestion fragments and common contaminants were automatically excluded prior to database searches. Oxidation of methionine and carbamidomethylation of cysteines were allowed as variable modifications. Identifications with a GPS confidence interval of greater than 99 % were accepted.

RESULTS AND DISCUSSION

One of the originally described characteristics of *H. pylori* was that its growth is inhibited by ox bile (Marshall *et al.*, 1984). After reflux into the human stomach, bile is absorbed by the underlying mucosa and forms a concentration gradient that decreases from the luminal to the epithelial surface of the mucus layer. The study of Worku *et al.* (2004) showed that all bile acids are chemorepellents for *H. pylori*. Thus, the bile gradient may drive *H. pylori* into the epithelial surface. This suggests that *H. pylori* has to endure a certain amount of bile or acidified bile stress before colonizing the gastric mucosa epithelial cells.

Inhibitory influence of bile on *H. pylori* before and after acidification

To approximate the *in vivo* conditions of the human stomach, where *H. pylori* mainly exists, we examined the inhibitory activity on *H. pylori* of fresh, normal-looking human bile before and after acidification. The maximum dilution of unacidified bile in which *H. pylori* could grow was 1 : 192, whilst that of acidified bile was 1 : 48. Fig. 1 shows the growth rate of *H. pylori* cultured in medium without human bile and with 1 : 192-diluted bile and 1 : 48-diluted acidified bile. This demonstrated that the growth of *H. pylori* was remarkably inhibited by human bile. Furthermore, acidification of human bile reduced its inhibitory influence on *H. pylori* considerably, although a certain level of activity remained. However, *H. pylori* was still able to propagate at this level of sublethal bile and acidified bile stress.

Bile acids constitute approximately 50 % of the organic components of bile. Before secretion, all bile acids are conjugated as *N*-acyl amidates with either glycine or taurine. The ratio of glycoconjugates to tauroconjugates in human bile is usually 3 : 1 (Begley *et al.*, 2005). The pK_a of glycine conjugates ranges between 4.3 and 5.2 and that of taurine conjugates between 1.8 and 1.9. Thus, whereas both glycine and taurine conjugates are soluble in neutral solutions, only taurine conjugates are soluble in acidic conditions. This is likely to be because glycine-conjugated bile acids are precipitated under acidic conditions and are removed by centrifugation. The reflux of bile into the stomach is a normal occurrence. Under the acidic conditions of the stomach where *H. pylori* mainly colonizes, glycoconjugates are precipitated and the inhibitory factors of bile will be markedly reduced.

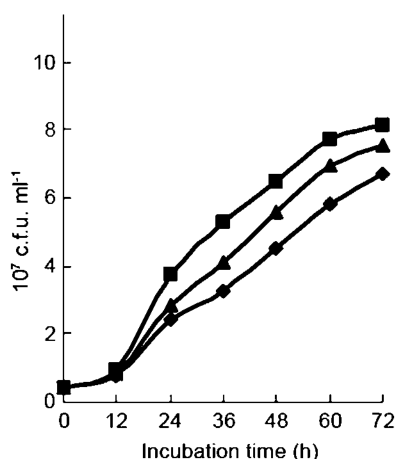

Fig. 1. Growth of *H. pylori* cultured in medium without human bile (■) and with 1 : 192-diluted bile (◆) and 1 : 48-diluted acidified bile (▲).

Proteomic profiles of *H. pylori* under bile and acidified bile stress

Global protein expression profiles of *H. pylori* strain 26695 grown in the absence of human bile or with 1 : 192-diluted bile and 1 : 48-diluted acidified bile were analysed using high resolution 2-DE. To confirm the data obtained, we repeated the 2-DE analysis with three sets of independently grown cultures. Fig. 2 shows the 2-DE maps of *H. pylori* under normal, bile and acidified bile stress conditions. In the present study, the expression of a protein was considered to have changed if the percentage volume of its spot in the gels between bacteria cultured with and without bile showed a twofold or greater difference ($P<0.05$). Protein spots that were regulated significantly were cut from the gel and further identified by MALDI-TOF/TOF MS.

We obtained information on 28 proteins whose expression changed under bile or acidified bile stress (Table 1). Among these, 23 proteins were upregulated and the other five proteins were downregulated by bile and acidified stress. These proteins are involved in various physiological functions such as molecular chaperones, iron storage, chemotaxis, flagellar mobility and the energy metabolism possibly necessary for enhanced flagellar motion required for chemotaxis. These observations indicated that *H. pylori* responds to bile stress through multiple mechanisms involving many signalling pathways (Fig. 3). What was interesting was that the changes in proteomic profile under bile and acidified bile were similar when compared with that of normal *H. pylori*, indicating that *H. pylori* resists bile and acidified bile through similar mechanisms.

In this experiment, three proteins involved in iron storage and metabolism were induced by bile and acidified bile stress. These proteins were non-haem iron-containing ferritin, neutrophil-activating protein (bacterioferritin) and NifU-like protein. A previous study reported that bile can chelate calcium and iron in bacterial cells resulting in iron starvation (Begley *et al.*, 2005). Iron is essential for maintaining the basic energy and redox metabolism in bacteria and also acts as a signal to regulate the expression of many genes via the ferric uptake regulator (Fur) protein. Bacteria have two classes of iron storage protein: ferritin and bacterioferritin. Like other pathogens, *H. pylori* has developed several iron-acquisition systems including its Fur protein (Dhaenens *et al.*, 1997). *H. pylori* non-haem iron-containing ferritin is a major component of iron storage as well as iron distribution in the cells. It can protect the bacteria from metal toxicity (Bereswill *et al.*, 2000). It has been reported that this protein is down-regulated by Fur to secure the availability of free iron when iron is scarce or titrated out by other metals (Bereswill *et al.*, 2000). Neutrophil-activating protein is important for the pathogenesis of *H. pylori* because it can promote neutrophil and monocyte infiltration into the gastric mucosa. Recent studies have shown that this protein is regulated by environmental iron levels and may play a role

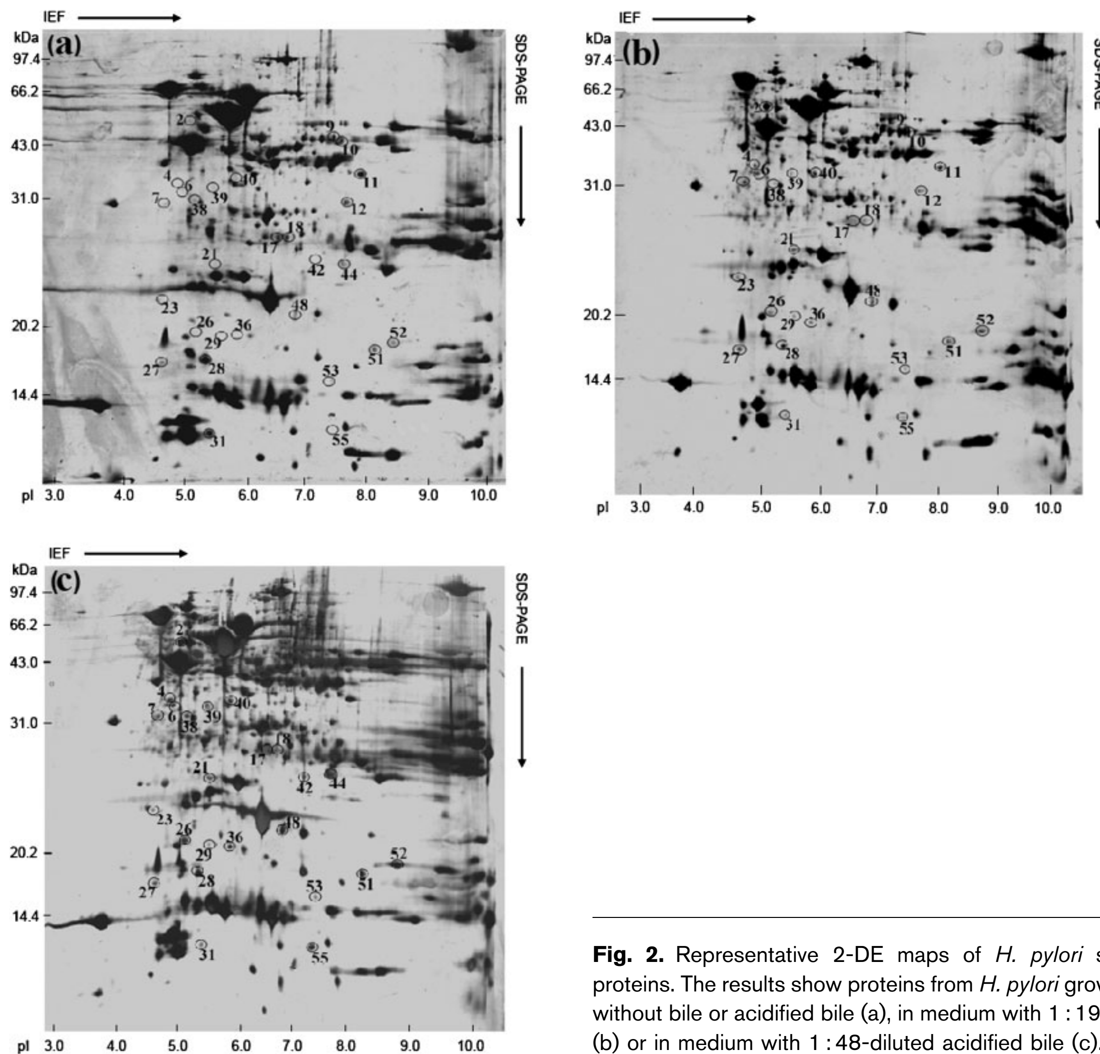

Fig. 2. Representative 2-DE maps of *H. pylori* strain 26695 proteins. The results show proteins from *H. pylori* grown in medium without bile or acidified bile (a), in medium with 1 : 192-diluted bile (b) or in medium with 1 : 48-diluted acidified bile (c).

as bacterioferritin in the response of *H. pylori* to iron changes (Leakey *et al.*, 2000; Cooksley *et al.*, 2003; Lee *et al.*, 2004). Non-haem iron-containing ferritin and neutrophil-activating protein (bacterioferritin) were both induced by bile and acidified bile stress in our experiment. Although the mechanism by which they are regulated is not clear, we believe it is related to the change in iron levels caused by bile. The expression of these two proteins was also shown to be upregulated in *H. pylori* under acid stress (Toledo *et al.*, 2002; Merrell *et al.*, 2003). NifU-like protein is a component participating in iron–sulfur cluster biosynthesis. This protein was also upregulated by bile and acidified bile. In short, these three proteins may play important roles in the response of *H. pylori* to bile stress.

Mobility has been shown to play an important role in the pathogenicity of *H. pylori.* The flagellum, its principal mobile apparatus, is a complex system. The presence of bile is an unfavourable condition and bile is a chemorepellent for *H. pylori.* Thus, the flagellar system of *H. pylori* should exert its function in response to bile stress. Under bile and acidified bile stress, one flagellar protein, the flagellar motor switch protein, was upregulated. This protein is a component of the switch complex on the rotor of the bacterial flagellum, which modulates the direction of rotation of the flagellar motor (Toker & Macnab, 1997). Additionally, the expression of one chemotaxis protein, CheW, was downregulated by bile. This is consistent with a previous study on *C. jejuni* (Fox *et al.*, 2007). When the bacterial cell senses the presence of bile, its flagellar switch modulates its direction of mobility in response to the environmental information received through the chemotaxis signal transduction pathway (Toker & Macnab, 1997). Previous studies have reported that acid shock also influences the expression of a large number of genes that encode components of the flagellar apparatus and chemotaxis protein in *H. pylori* (Merrell *et al.*, 2003; Wen *et al.*, 2003). Moreover, the upregulation of ATP synthase, isocitrate dehydrogenase and glutamate dehydrogenase may contribute to the energy needs of the enhanced flagellar motion required for chemotaxis.

Many molecular chaperones are affected by bile stress, as well as other types of stress. In our study, the expression of

Table 1. Summary of protein spots showing altered expression under bile and acidified bile stress

Function class	Spot no.*	Protein (gene)	TIGR ORF no.†	NCBI no.	Top score	Sequence coverage (%)	Percentage volume ratio‡	
							Bile	Acidified bile
Molecular chaperones	4	Chaperone and heat-shock protein 70 (*dnaK*)	Hp0109	2313191	74	20	3.13	4.01
	6	Hydrogenase expression/formation protein (*hypE*)	Hp0047	2313121	143	46	4.68	5.85
	17	Co-chaperone (*groES*)	Hp0011	2313085	93	64	0.48	0.53
	18	Co-chaperone (*groES*)	Hp0011	2313085	75	42	0.46	0.59
	31	Thioredoxin (*trxA*)	Hp0824	2313958	150	72	0.18	0.12
	39	Chaperone and heat-shock protein (*groEL*)	Hp0010	2313084	130	45	10.5	23.3
Iron storage	7	NifU-like protein	Hp0221	2313312	40	9	7.43	8.48
	29	Non-haem iron-containing ferritin (*pfr*)	Hp0653	2313771	128	55	13.0	11.4
	36	Neutrophil-activating protein (bacterioferritin) (*napA*)	Hp0243	2313332	94	67	4.17	9.44
Metabolism	9	Isocitrate dehydrogenase (*icd*)	Hp0027	2313104	86	19	2.05	0.83
	10	Glutamate dehydrogenase (*gdhA*)	Hp0380	2313483	86	31	3.20	2.03
	11	Aspartate-semialdehyde dehydrogenase (*asd*)	Hp1189	2314350	345	78	0.44	1.31
	12	Aldo-keto reductase	Hp1193	2314358	72	33	0.36	1.41
	40	Cysteine synthetase (*cysK*)	Hp0107	2313190	227	51	3.91	5.05
	44	3-Ketoacyl-acyl carrier protein reductase (*fabG*)	Hp0561	2313678	79	30	0.79	3.45
	51	ATP synthase F1, subunit delta (*atpH*)	Hp1135	2314286	105	47	1.70	2.42
Mobility	28	Purine-binding chemotaxis protein (*cheW*)	Hp0391	2313492	150	20	0.24	0.50
	38	Flagellar motor switch protein (*fliG*)	Hp0352	2313453	101	35	1.91	7.70
Proteolysis	26	ATP-dependent Clp protease proteolytic component (*clpP*)	Hp0794	2313923	99	60	10.4	27.4
Virulence	48	Cag pathogenicity island protein (*cag24*)	Hp0545	2313660	107	53	2.07	5.05
Translation	2	Translation elongation factor EF-Tu (*tufB*)	Hp1205	2314366	184	55	15.3	13.1
	53	Ribosome-releasing factor (*frr*)	Hp1256	2314423	93	42	2.70	3.24
Cell division	39	Rod shape-determining protein (*mreB*)	Hp1373	2314543	76	37	10.5	23.3
Unknown function	21	Conserved hypothetical protein	Hp0813	2313945	105	49	3.48	6.67
	23	*H. pylori* predicted coding region HP0406	Hp0406	2313513	106	64	2.82	2.00
	27	*H. pylori* predicted coding region HP0697	Hp0697	2313821	78	47	2.80	2.72
	42	Modulator of drug activity (*mda66*)	Hp0630	2313748	78	48	0.83	18.7
	52	*H. pylori* predicted coding region HP0721	HP0721	2313852	48	40	1.97	5.21
	55	*H. pylori* predicted coding region HP0902	Hp0902	2314040	81	80	17.5	60.8

*Spot number refers to the proteins labelled in Fig. 2.
†The Institute for Genomic Research (TIGR) ORF numbering follows the nomenclature of *H. pylori* strain 26695.
‡Percentage volume ratio for each protein is derived from *H. pylori* cultured in medium without human bile compared with the protein derived from *H. pylori* cultured in medium with maximum growth dilution of bile and acidified bile.

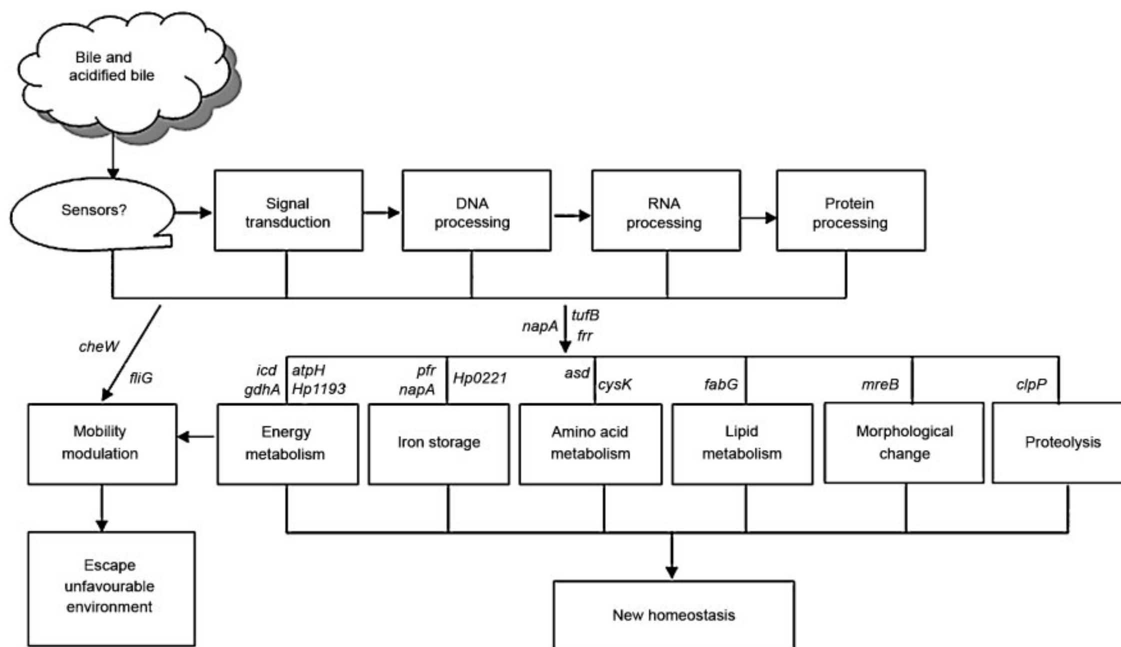

Fig. 3. A simple model of the bile stress response in *H. pylori*. *H. pylori* can perceive bile and acidified bile stress signals by putative sensors and transmit them to the cellular machinery by signal transduction to regulate protein expression. Through regulation of DNA, RNA and protein processing, *H. pylori* thus escapes an unfavourable environment by mobility modulation or establishing a new cellular homeostasis by changing the abundance and activities of functional proteins involved in iron storage, morphological change, proteolysis and metabolism of energy, amino acids and lipids. These processes might work cooperatively under bile and acidified bile stress.

chaperone and heat-shock protein 70 and chaperone and heat-shock protein GroEL increased, whilst that of co-chaperone GroES decreased under bile and acidified bile stress. In addition, the intensity of protein spot 2, identified as translation elongation factor EF-Tu, increased in gels of *H. pylori* cultured with bile and acidified bile. This factor was also induced by ox-bile stress in a previous report on *C. jejuni* (Worku *et al.*, 2004). EF-Tu can act as a molecular chaperone participating in protein folding and protection from stress (Caldas *et al.*, 2000).

In this experiment, we found that the expression of other proteins was altered under bile stress. Rod shape-determining protein has been shown to be required for maintaining the rod-like shape in many bacteria (Jones *et al.*, 2001; Kruse *et al.*, 2003; Figge *et al.*, 2004). This protein was shown to be induced by bile and acidified bile stress in our study. A previous study has shown that *H. pylori* changed from its primary rod-like shape to a spherical shape with blebs on the cell surface when exposed to bile (Itoh *et al.*, 1999). These results indicate that this protein plays an important role in the morphological changes of *H. pylori*. Expression of the gene encoding this protein was reproducibly altered by a shift to a low pH in *H. pylori* (Ang *et al.*, 2001; Merrell *et al.*, 2003). The modulator of drug activity encoded by *mda66* also changed its expression in the presence of acidified bile. The exact function of this protein in *H. pylori* is unclear; however, it is possible that acidified bile acts on *H. pylori* as a drug because of its antimicrobial actions, which induced upregulation of the modulator of drug activity. Proteolysis is essential for quick adaptation to stress and regulates important developmental processes. Clp-mediated proteolysis is also required for disease progression and virulence of several bacterial pathogens, favouring survival in the host or modulating the activity of genuine virulence factors (Gaillot, 2004). In this study, one ATP-dependent Clp protease proteolytic component was upregulated by bile and acidified bile. In *Bacillus subtilis*, the deletion mutant of this protein could not grow under several stress conditions, most severely during starvation and at high temperatures (Tomoyasu *et al.*, 2002). Finally, there were several proteins with unknown function that were influenced by bile and acidified bile stress. One of these was *H. pylori* predicted coding region Hp0721. This protein has been isolated as a sialic acid-specific lectin from *H. pylori*, which implies that it is involved in sialic acid-specific adhesion (Bennett & Roberts, 2005).

In conclusion, this work focused on the global proteomic changes and possible molecular mechanisms of *H. pylori* in response to bile and acidified bile stress, and indicated that at least 28 proteins were shown to be influenced by the presence of bile and acidified bile. These proteins are related

to various kinds of biological function such as iron storage, chemotaxis, flagellar mobility and the energy metabolism possibly necessary for enhanced flagellar motion required for chemotaxis. These results indicate that *H. pylori* responds to bile stress through multiple mechanisms involving many signalling pathways. These findings may provide valuable information in understanding the interactions between *H. pylori* and human bile and acidified bile, as well as the survival mechanism of this bacterium in humans.

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