The role of C^5 -cytosine methyltransferase, hpyAVIBM in *Helicobacter pylori* associated virulence in mice

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Abstract:

Helicobacter pylori is a common human pathogen that causes gastroduodenal diseases. *H. pylori* genome consists of numerous restriction-modification (R-M) genes. It is established that N^6-adenine methylation plays a crucial role in bacterial gene regulation and virulence, but not much is known about the role of C^5-cytosine methylation. In this study, we examined the influence of an orphan cytosine methyltransferase, *hpyAVIBM* on gastric infection in mice and cultured cells. Histopathological staining showed that the deletion of *hpyAVIBM* in *H. pylori* strain SS1 had increased damaging hemorrhagic effects on the mice stomach. The gelatin-zymography result demonstrated that the mice infected with mutant SS1Δ*hpyAVIBM* had significantly up-regulated pro-MMP-9 than those infected with SS1. Additionally, ELISA results of pro-inflammatory cytokines proved that mutant strain caused significantly more inflammatory effect on mice stomach than its wild-type counterpart. The immunohistochemistry data showed that mutant strain caused attenuated epithelial cell damage. Co-culture studies of *H. pylori* with AGS (Human Gastric Adenocarcinoma cell line) cells revealed that SS1Δ*hpyAVIBM* instigated significantly more apoptotic death in the AGS cells compared to the wild-type strain. Our results indicated that DNA methylation by *hpyAVIBM* plays a crucial role in modulating virulence factors in bacterial cells and their interaction with the host cells.

Keywords: *H. pylori*, Restriction modification system, Virulence, IL-8, apoptosis

Introduction:

Helicobacter pylori is a microaerophilic, Gram-negative bacterium that chronically colonizes the human stomach of about 50% of the world's population and considered as a causative factor of chronic gastritis, peptic ulcer disease and gastric cancer [1,2]. The natural competency accompanied by high mutation rates and recombination frequencies make *H. pylori* as one of the most genetically diverse species [3]. Comparison of the first two *H. pylori* complete genome sequences suggested that the two strains are quite similar, with only about 7% of the genes are specific to each strain [4].

DNA methylation is regarded as one of the most general forms of DNA modification occurring in the genome of both prokaryotes and eukaryotes [5,6]. DNA methylation is carried out by DNA methyltransferases (MTases), and the majority of the DNA MTases are constituents of restriction-modification (R-M) systems [6]. R-M systems are renowned as rudimentary immune systems of bacteria [7]. The R-M systems are made of two enzymes exhibiting different activities. "R" symbolizes a restriction endonuclease enzyme that has the capability to cleave particular DNA sequences, whereas the "M" stands for a modification methyltransferase that makes these sequences resistant to cleavage [8]. More than 90% of analyzed bacterial genomes contain R-M systems and were primarily thought of as a defense mechanism against bacteriophage infection. Methylation may not affect the Watson-Crick pairing but can produce a signature motif that is recognizable by DNA interacting proteins. It has been found that the affinity of transcription factors for DNA can be enhanced or reduced by DNA methylation, thereby affecting gene expression and regulation [5]. By the position of the methyl group on the bases, prokaryotic MTases can be divided into two major groups – exocyclic MTases and endocyclic MTases. The exocyclic MTases are of two types – N^6

adenine methyltransferase and N^4 cytosine methyltransferase, whereas the endocyclic MTases methylate cytosine at the C^5 position [9,10].

H. pylori genome consists of a large number of R-M systems. The significance of having such a huge number of these genes is not clear yet. *H. pylori* can persist for decades in the gastric environment, and possession of numerous R-M systems might be a causative factor of its long persistence [11,12,13]. The majority of the DNA methyltransferases present in *H. pylori* are N^6 adenine methyltransferases [14]. There are several reports regarding the importance of the N^6 adenine methyltransferase in *H. pylori* physiology beyond the role of genome protection [15,16]. In comparison to N^6 adenine methyltransferase, there have been fewer reports about the other two types of methyltransferases. The role of C^5cytosine methylation in eukaryotic epigenetic regulation is already established [17].

In 26695 strain, *hpyAVIBM* is an orphan C^5 cytosine methyltransferase which remains functional [11]. It exists as an overlapping ORF with another methyltransferase *hpyAVIAM* [14]. These enzymes are assumed vestiges of non-operational R-M systems [18,19]. It has been found that plasmids expressing *h*pyAVIAM and *h*pyAVIBM are resistant to *Mnl*IA digestion independently [11]. It has been previously reported that *hpyAVIBM* consists of a stretch of ‘AG’ repeats, which ultimately takes part in phase variation [20]. The role of phase variation in bacterial virulence has been reported earlier [21], as it is used to evade host immunity. The requirement of phase variable R-M systems in *H. pylori* colonization has been already reported [2]. Previous report suggested that *hpyAVIBM* plays a crucial role in *H.pylori* mediated inflammation in *ex vivo*, as the deletion of this gene makes this bacterium more motile and capable of inducing more IL-8 secretion from AGS cells [14]. As *in vivo* studies have revealed the roles of host factors in disease pathogenesis [22], it is almost

mandatory to perform the *in vivo* study before confirming the role of a particular gene in bacterial virulence. We are unaware of any study that deals with the significance of the *hpyAVIBM* gene in *in vivo* conditions. Taking all these into considerations, our enthusiasm to uncover a role for phase variable R-M systems in *H. pylori* prompted us to examine the contribution of cytosine methylation by *HpyAVIBM* MTase in mice colonizing strain, SS1.

Materials and Methods

H. pylori culture

H. pylori strains were cultured on brain heart infusion agar (BHIA) plates containing horse serum (7%, Invitrogen, Grand Island, NY, USA), IsoVitalex (0.4%, Becton Dickinson, San Jose, CA, USA), trimethoprim (5 µg/ml), vancomycin (6 µg/ml), polymyxin B (10 µg/ml) (all from Sigma Chemicals, MO, USA). These cultures were grown at 37°C in microaerophilic condition (85% N_2, 10% CO_2, and 5% O_2) for 3 to 6 days in a double gas incubator (Heraeus Instruments, Hanau, Germany). Transformation was carried out as described previously [14].

DNA extraction and PCR of *hpyAVIBM* allele

Genomic DNA was extracted from bacterial cultures by the cetyltrimethyl ammonium bromide extraction method as described elsewhere [23]. Each of the PCR reactions were carried out in a 25 µl reaction volume containing genomic DNA (50 ng), forward and reverse primers (25 pmol), each dNTPs (0.25 mM each, Roche, Berlin, Germany), Taq DNA polymerase (1 U, Genei, Bangalore, India), and $MgCl_2$ (1.5 mM) in a standard PCR buffer (Genei, Bangalore, India) for 35 cycles, generally under the following reaction condition: 95°C for 1 min, 55°C for 1 min, and 72°C for a time chosen based on the size of the expected

amplified fragment (1 min/kb) in a Master Cycler apparatus (Eppendorf, Hamburg, Germany). Positive and negative controls were taken in each assay. The primers used in this study [14] are listed in Table 1.

Construction of *ΔhpyAVIBM* mutant strain

By using primer 1 and 2 (Table 1) the 1064 bp long *hpyAVIBM*gene was amplified from the strain SS1by PCR with the help of Pfu polymerase. This amplified product was then used to construct the desired mutant strain as described previously [14].

***ex vivo* IL-8 Assay**

The bacterial strains (SS1 and SS1Δ*hpyAVIBM*) were cultured in 7% serum containing BHIA plates for a day at 37°C under microaerophilic conditions. To find out the *ex vivo* IL-8 secretion from gastric epithelial cells, AGS cells were cultured (2.5 X 10^5cells/ml) into 24 well plates for 24 h in the humidified atmosphere containing 5% CO_2. The wild-type and Δ*hpyAVIBM* mutant *H. pylori* strains were added to the cultured cells at multiplicity of infection (MOI) of 100. After 8 h of infection, IL-8 levels in the culture supernatant were assayed in duplicate. The experiments were conducted three times using a commercially available specific ELISA kit (Genetix, New Delhi, India) following the manufacturer's instructions.

Cell cycle analysis

To obtain the percentage of apoptotic cell death, AGS cells were cultured (1X10^6 cells/ml in each well) into 6 well plates for 24 h. These cells were then infected with *H. pylori* culture in log phase. After 24 h of infection cells were washed twice with PBS and fixed in 70% chilled ethanol. Cells were kept at 4°C for downstream analysis. Before the analysis 2% fetal

bovine serum (FBS) containing PBS (pH 7.4) were used to wash the cells followed by staining with propidium iodide (50µg/ml) containing DNase-free RNase (0.1mg/ml). Cells were then acquired on flow cytometer and the data was analyzed in FACS Diva software (Becton Dickinson, USA).

***ex vivo* Caspase-3 activity assay:**

The AGS cells were cultured for 24 h ($2.5X10^6$ cells/ml in each plate) into Petri plate (60 mm discs) to carry out this assay. The cells were then infected with 24 h old *H. pylori* culture at MOI of 100. After 24 h of infection the AGS cells were collected by centrifugation at 1,000 × g for 10 min at room temperature followed by washing twice with PBS. The cells were then resuspended in lysis buffer at a density of 10^7 cells/ ml and kept on ice for 15 min. The cell debris was removed by centrifugation at 10,000 × g for 5 min at 4°C, and the cell supernatant was used for the colorimetric assay of caspase-3 activity using commercially available kit (Abcam, Cambridge, UK). Protein concentrations were calculated using the Bio-Rad protein assay according to the manufacturer's instructions.

***H. pylori* Infection in C57BL/6 Mice:**

C57BL/6 mice bred in house with continuous access to food and water were used in all experiments. Experiments were performed in such a way which minimizes the animal suffering and we also tried to use the minimum number associated with valid statistical evaluation. Animals were anesthetized by ketamine (12 mg/kg b.w.) and then sacrificed by cervical dislocation. Animal experiments were carried out following the guiding principle of the animal ethical committee of the institute. The study approval number is BT/PR10407/BRB/10/604. Animals of both control and experimental groups (n=6) were kept

in different cages under standard controlled conditions and were fasted for 6 h with free access to water before each inoculation. Mice infection was done using a modification of the method as described previously [24]. Briefly, one day old bacterial cultures were harvested in 10 mM PBS. Inoculation of about 10^8 CFU/mouse/inoculation were given intragastrically to each groups of mice by orogastric gavages twice in a period of 3 days. Groups of mice inoculated with SS1, SS1Δ*hpyAVIBM*, and PBS were kept in different cages with free access of food and water. All the mice were sacrificed after 45 days of infection.

Histology:

After 45 days of infection, the body and the pyloric parts of control and infected mice stomach were used for histological studies. 10% formalin was used to fix the section and then these tissue sections were embedded in paraffin. The 5 µm sections were cut using microtome which was then followed by the staining with hematoxylin and eosin [25], and observed under an Olympus microscope (1X70). Camedia software (E-20P 5.0 Megapixel) was used to capture the images using at original magnification 10x10, 20X10, and 40x10 and processed in Adobe Photoshop version 7.0.

Tissue Extraction:

The body and the pyloric parts of stomach were suspended in PBS containing protease inhibitors (Sigma Chemicals, MO, USA) followed by mincing and incubation for 10 min at 4°C. The supernatant was collected as PBS extracts after centrifugation at 12,000 X g for 15 min. This pellet was then extracted in lysis buffer (10mM Tris-HCl, pH 8.0, 150mM NaCl, 1% Triton X-100, and protease inhibitors) and centrifuged at 12,000 X g for 15 min to get Triton X-100 extracts. Both PBS and Triton X-100 extracts were stored at -70 °C for

downstream studies [25].

Gelatin zymography:

Tissue extracts were electrophoresed in a 8% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Sigma Chemicals, MO, USA) under non-reducing conditions for assaying MMP-9 activity. The gels were then washed twice in 2.5% Triton X-100 (Sigma Chemicals, MO, USA) followed by incubation in calcium assay buffer (40 mMTris-HCl, pH 7.4, 0.2 M NaCl, 10 mM $CaCl_2$) for 18 h at 37 °C. 0.1% Coomassie Blue was used for staining purpose which was followed by destaining. The zones of gelatinolytic activity appeared as negative staining. Standard MMP-9 enzyme was supplied by Chemicon, Hampshire, UK. Densitometric analysis of zymographic bands was done by using Lab Image software [25].

IL-6 and IL-1β assay:

100 pg protein from each tissue lysate was used to assay the IL-6 and IL-1β secretion. Commercially available specific ELISA kits were used to detect these pro inflammatory cytokines according to manufacturer's protocol (Invitrogen, CA, USA).

Immunohistochemistry:

3 µm tissue sections were stained with polyclonal rabbit antiactive Caspase-3 antibody (Cell Signaling Technology, MO, USA). Antigen retrieval was done in citrate buffer (10 mM, pH 6.0). After antigen retrieval, antibodies were incubated for overnight. Visualizations were performed using diaminobenzidine (DAB) as chromogen.

Statistical analysis:

Each experiment was carried out minimum of three times in duplicate and results expressed

as mean ± standard error of the mean (SEM). Statistical analysis was done by Student's *t* test, using Graph Pad Prism software (version 5, Graph Pad Software Inc, La Jolla, CA, USA), *P* values <0.05 were considered to be significant.

Results:

100 *H. pylori* strains were isolated from a total of 160 subjects who underwent endoscopy. On the basis of their clinical symptoms as described previously [23], the biopsies were divided into two groups: duodenal ulcer (DU) and non-ulcer dyspepsia (NUD). The strains were isolated from 61 DU patients (male 51%, median age 61 years, range 27-85 years) and 39 NUDs (male 52%, median age 65 years, range 25-84 years).

On the basis of PCR based genotyping, it was found that *hpyAVIBM* gene was present among 59% of studied population (59/100), and there was significant difference in the distribution pattern of this gene among DUs (80.3%, 49/61) and NUDs (25.64%, 10/39) [data not shown], which is in accord with our previous results [14].

Mutant strain causes significantly more apoptosis *ex vivo*

The cell cycle analysis with propidium iodide reveals allocation of cells in three major phases of the cell cycle (G1 vs. S vs. G2/M) and makes it possible to distinguish unhealthy cells with fractional DNA content from the healthy ones. The sub-G0 phase cells represent the apoptotic cells. In fig.1, the gates P3, P4, P5, and P6 stand for sub-G0, G1, S, and G2/M phases respectively. After co-culturing the AGS cells with *H. pylori* for 24 h, a significant ($P< 0.05$) higher amount of apoptotic cell death was noticed when the cells infected with SS1Δ*hpyAVIBM* strain (45.43 ± 1.85%) in comparison to the cells infected with its wild type counterpart (27.1 ± 2.43%, Fig. 1A & B).

Deletion of *hpyAVIBM* causes more induction of caspase-3 in AGS cells:

Next we assessed the effects of *hpyAVIBM* deletion regarding apoptosis via the extent of caspase-3 activity as caspase-3 is one of the most important factors in apoptosis machinery. Activation of Caspase-3 by *H. pylori* was assessed by measuring cleavage of the colorimetric substrate DEVD-pNa. As shown in Fig. 2, caspase-3 activity was significantly higher ($P < 0.05$) after 24 h of infection in SS1Δ*hpyAVIBM* strain infected AGS cells compared to the cells infected with its wild type counterpart.

SS1Δ*hpyAVIBM* strain exacerbates tissue damage in gastric tissue of mice 45 days post-infection:

As *H. pylori* infection causes inflammation in gastric tissues as well as degradation of extracellular matrix (ECM) of stomach, we were encouraged to inspect the likely changes in tissue integrity. Each groups of mice (n=6) were separately inoculated with PBS, SS1 strain, and SS1Δ*hpyAVIBM* strain of *H. pylori* and were sacrificed after 45 days of inoculation. Histological studies showed that infection with either wild type or mutant strain was responsible for inflammation in gastric cells along with epithelial mucosal damage (Fig. 3). Epithelial mucosal disruption along with hemorrhagic damage was more distinct in SS1Δ*hpyAVIBM* than in SS1 infected mice compared with the control.

Deletion of *hpyAVIBM* causes a substantial up-regulation of pro-MMP-9 activity in mice:

Matrix metalloproteinases (MMPs) are causative factors for the degradation of a variety of ECM molecules. We examined whether colonization with SS1 and SS1Δ*hpyAVIBM* bacteria had any differential effect on pro-MMP-9 activity. Fig. 4A & B revealed significant up

regulation of pro-MMP-9 activity at secretion level after 45 days of infection in mutant strain colonized mice tissue.

Mutant strain shows significantly higher IL-6 and IL-1β secretion *in vivo*:

In order to observe the outcome of *hpyAVIBM* deletion on the prospective of *H. pylori* strains with respect to IL-6 and IL-1β secretion in mice gastric tissue, each groups of mice (n=6) were separately fed with PBS, SS1 strain, and SS1Δ*hpyAVIBM* strain of *H. pylori* and were sacrificed 45 days post infection as described in materials and methods. After 45 days of infection IL-6 and IL-1β measurement from PBS extracts were done as described previously. It was found that both the IL-6 and IL-1β secretion was significantly ($P< 0.05$) higher from those tissues which were infected with the mutant strain (768.9 ± 19.81 pg/ml, 548.8 ± 12.35 pg/ml respectively) than the mice infected with the wild type strain (365.7 ± 19.02 pg/ml, 227.7 ± 13.8 pg/ml respectively, Fig. 5A & B).

Increased caspase-3 activation by SS1Δ*hpyAVIBM*:

Degree of apoptosis was determined *in situ* using immunohistochemical stains for caspase-3 antibody. Caspase-3 epithelial staining was increased in mice infected with the mutant strain than the SS1 infected tissues (Fig. 6). Immunohistochemistry results also confirmed the histopathology results and showed increased apoptosis by SS1Δ*hpyAVIBM* compared to the wild-type strain.

Discussions:

Although *H. pylori* colonizes the gastric epithelium of about 50% of the world population, 15–20 % of infected individuals develop gastric, or duodenal ulcer (DU) and less than 1% develop gastric adenocarcinoma [26]. So, there might be some strain-specific attributes that

make some strains more capable of causing diseases. In the *H. pylori* genome, the majority of the methyltransferase genes are components of restriction and modification systems [4,27]. About 2% of the total number of genes in *H. pylori* is made up of these R-M system genes [4,28,29], which is much higher as compared to other bacteria [30]. About 30 R-M genes are present in *H. pylori* sequenced genomes, whereas other bacterial genomes have 4.3 R-M systems per genome [31]. These large numbers of genes represent more than 50% of the strain-specific genes [32,33], and the role played by this large number of genes is not properly understood.

In spite of the growing interest in the understanding of the role played by the DNA methylation in the host-pathogen interaction, there is still an inadequacy of reports that uncover the importance of cytosine methyltransferase of *H. pylori* in disease outcomes. Our PCR-based analysis showed the difference in the distribution pattern of *hpyAVIBM* among the DUs and NUDs in our studied population, which indicates that this gene might have been associated with disease outcomes.

In *H. pylori* mediated infection, the inflammatory response is one of the vital pathophysiological events. In many cases, chemokines are considered as potential mediators to act as a signal for the emigration of blood cells. Accumulation of granulocytes is mostly mediated by the C-X-C cytokines [34]. It has been shown previously that the level of the chemokine IL-8, one member of this cytokine family is increased in the gastric biopsy samples of *H. pylori*-associated infection [35]. It has been already said in the previous report that the *hpyAVIBM* gene is involved in the modulation of the IL-8 secretion *ex vivo*.

IL-8 secretion and inflammation as a result of *H. pylori* infection ultimately lead to epithelial

cell damage or apoptosis. Apoptosis is an energy-dependent method in which dying cells turn on a genetically encoded cell-death program and is characterized by some distinct molecular and morphological characters. Microbial pathogens or their products can turn on apoptosis in a cytokine-mediated manner, which plays an important role in pathogenesis [23,36]. The caspase family of cysteine proteases is necessary for the process of apoptosis [37]. It was found that the mutant strain caused significantly ($P < 0.05$) higher level of apoptosis as well as cleavage of caspase-3 as compared to its wild-type counterpart in AGS cells (Fig. 1 & 2). Therefore, it might be said that *hpyAVIBM* is involved in the induction of *ex vivo* apoptosis in cytokine-mediated manner.

A comprehensive understanding of the pathogenicity of a microbe must consist of in-depth knowledge of the patterns and regulation of virulence gene expression during the itinerary of infection in a host. To investigate this, the C57BL/6 mice were independently infected with SS1 and its deletion mutant of *hpyAVIBM* as described in materials and methods.

The role of matrix metalloproteinases (MMPs) as one of the key regulators in inflammatory processes is well known [38]. Increased expression of matrix metalloproteinase-9 (MMP-9) is already reported as one of the significant markers of gastric cancer [39]. It has been suggested that there is an up-regulation of MMP-9 in *H. pylori*-infected mouse gastric tissues [40]. MMP-9 has the substrate specificity for the basement membrane, which is usually composed of type IV collagen [40]. Therefore, the extent of MMP-9 activity could be correlated with the degree of *H. pylori* virulence. In this study it was found that after 45 days of infection, SS1Δ*hpyAVIBM* strain exhibited about 170% higher pro-MMP-9 activity compared to the wild-type SS1 strain-infected tissue, indicating that the infection with the mutant strain was related with increased tissue damage *in vivo* (Fig. 4). As per our knowledge, it is the first

demonstration that the deletion of *hpyAVIBM* increases the host pro-MMP-9 secretion.

Gastric inflammation is a characteristic feature of *H. pylori* disease pathogenesis where mucosal epithelial cells take part in bacterial colonization. Histological examination of mouse gastric tissues showed that the inflammation along with the disruption in the gastric mucosal cells is more prominent in SS1Δ*hpyAVIBM* than in SS1-infected mice compared with the control (Fig. 3). It has been already reported previously that the inflammatory response of the gastric mucosa by *H. pylori* infection is the key to understanding the biochemical mechanisms for variability in the clinical symptoms [41,42]. The inflammatory effects as a result of *H. pylori* infection could be studied in the intragastrically infected mice. The role of cytokines in the up-regulation of MMP expression under pathological conditions has been already established [43]. It has been shown earlier that there is an increase in the induction of IL-6 and IL-1β in *H. pylori*-infected mouse gastric tissues [44,45]. Consistently, Our results show that the levels of secreted IL-6 and IL-1β were significantly ($P < 0.05$) higher in SS1Δ*hpyAVIBM* infected mouse gastric tissues than in SS1-infected tissue compared with control after 45 days of infection, indicating the more virulence potential of SS1Δ*hpyAVIBM* strain (Fig. 5A& B).

In *ex vivo* study, we showed that increased secretion of proinflammatory cytokines is associated with increased caspase-3 activation, which ultimately leads to increased apoptosis. To ascertain the effect of the *hpyAVIBM* gene on apoptosis, we inoculated separate groups of C57BL/6 mice with PBS, SS1, and SS1Δ*hpyAVIBM* strain. Our immunohistochemistry data showed elevated expression of caspase-3 in mouse gastric tissue infected by the mutant strain than the wild type as compared with control (Fig. 6) which is in accord with our *ex vivo* data.

This study shows that *hpyAVIBM* regulates the virulence potential of *H. pylori* both *ex vivo* and *in vivo*, which may affect the clinical outcome of *H. pylori* mediated infection. Our study shows the multidimensional effects of cytosine methylation on the virulence potential of this bacterium. The methyl group addition on the cytosine base is an important form of DNA modification, which in turn affects the interaction between DNA transacting proteins and DNA [14]. It has been described previously in an *ex vivo* experiment, that methylation by the HpyAVIBM of the promoter of alcohol oxidase coding *AOXI* hinders the binding of Mxr1p (methanol expression regulator 1) which plays a vital role in methanol metabolism of yeast *Pichia pastoris* [46]. *AOX1* promoter has the recognition sequence for HpyAVIBM.

H. pylori is one of the most genetically diverse species and its genotype varies geographically. This diversity stems from differences among strains in gene content, arrangement, point mutations and recombination between divergent lineages, all without apparent selection for any one or few genotypes that are most suitable for all people [47,48]. The genome analysis of various *H. pylori* strains reveals that the sequence variation is significantly higher at the nucleotide level than at the amino acid level and as the nucleotide variation occurs mostly in the third position of a coding triplet, therefore the primary sequence of the encoded protein remains highly conserved. Contrary to this finding, a tremendous variety of the genes associated with virulence has been found, and many of them have different allelic structure. However, this nucleotide difference ultimately leads to the differential distribution of the R-M system recognition sequence between *H. pylori* strains [14,49]. This differential pattern of recognition sequence might result in different methylation pattern, which ultimately results in variation of virulence gene expression.

H. pylori can sustain itself for decades in the ever-changing gastric niche of the host by the

continuous alteration of its genome as a result of point mutations and horizontal gene transfer. Our data shows that the cytosine methylation might play a significant role in regulating the expression of genes involved in virulence and thus its interaction with the host. Therefore, it can be hypothesized that the methylation by HpyAVIBM alters the interaction between various regulatory factors and associated recognition sites on the promoters of target genes. The mutant strain showed more inflammation as well as apoptosis than its wild type counterpart suggesting differential interaction with host both *in vivo* and *ex vivo*. For a successful colonizer like *H. pylori*, it is necessary to maintain a balance between virulence and avirulence [14]. The true attributes of a successful pathogen lie in its ability to evade the inflammatory eradication programs initiated by the host and thus, it may evolve strategies that allow it to go unnoticed. For the successful colonization inside the host, a balancing mechanism must have been developed by *H. pylori* by which it can modulate its virulence to an extent [50]. It is quite amazing that in spite of having strong immunogens like CagA, VacA, etc., *H. pylori* can survive inside the majority of the host without turning on the strong host immune response. Additionally, it has developed certain mechanisms to counteract the host immune response, and catalase and arginase protein could be pivotal in this host-pathogen interaction [14].

In conclusion, this study highlights the regulatory role of cytosine methylation mediated by *HpyAVIBM* in *H. pylori* virulence. *HpyAVIBM* can alter the methylation landscape in *H. pylori* possibly by phase variation and thus, modulate its virulence. Further studies are required to determine the mechanism by which *hpyAVIBM* manifests its action.

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Author Contributions

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Figure legends

Figure 1:SS1Δ*hpyAVIBM*strain causes more apoptosis in AGS cells compared to its wild type counterpart. A) Apoptosis of AGS cells (i worked as control) co-cultured with SS1 wild type (ii) and its isogenic mutant of *hpyAVIBM* strains (iii) for 24 h (MOI is 100), stained with propidium iodide and analysed by flow cytometry. These figures are representative profile of at least three experiments. **B)** Graphical representation of % apoptotic cells (Sub G0 phase) infected with similar strains are expressed as mean ± SEM. *P<0.05 (Student's *t*

test) as compared between groups.

Figure 2: Deletion of *hpyAVIBM* causes more induction of caspase-3 in AGS cells. The caspase 3 activity in the AGS cells after bacterial infection for 24 h is measured by OD values from a specific colorimetric assay Data are expressed as mean ± standard error of mean (SEM) of 3 experiments in duplicates. *P<0.05 (Student's t test) as compared between groups.

Figure 3: Histology of mouse gastric tissues 45 days post infection by SS1 and SS1Δ*hpyAVIBM* strain. Different groups of mice (n=6) were intragastrically inoculated with SS1 and SS1Δ51 strain and were sacrificed on 45th day of post infection, and stomachs were sectioned for histological analysis. Control mice were fed with PBS and kept separately under the similar conditions. Histological appearances of control (A), SS1- infected (B), and SS1Δ*hpyAVIBM*-infected (C) gastric tissues stained with hematoxylin and eosin and were observed at 20 x 10 magnification. Mice infected with isogenic mutant show more epithelial border destruction and hemorrhagic damage.

Figure 4: Increased up-regulation of MMP-9 activities in mouse gastric tissues infected with strain SS1Δ*hpyAVIBM* compared to its wild type counterpart. Different sets of C57BL/6 mice were intragastrically inoculated with wild type and mutant strains independently and were sacrificed on day 45 post infection. A) Gelatin zymography was carried out for the detection of the activities of secreted MMP-9 in the gastric tissues exposed to wild type and mutant strains. Identical amounts (80µg of protein) of PBS extracts of gastric tissues were electrophoresed in 8% non-reducing SDS-polyacrylamide gel containing 1 mg/ml gelatin. MMP-9 activity was detected by gelatinolytic activity as stated under materials & methods. B) Histographic representation of secreted MMP-9 activities plotted

from the above zymogram and three other representative zymograms from independent experiments. Activities were determined by using Lab Image densitometry program. *P<0.05 (Student's *t* test) as compared between groups.

Figure 5: Deletion of *hpyAVIBM* gene causes increased secretion of IL-6 and IL-1β from mice gastric tissues. *In vivo* **A)** IL-6, and **B)** IL-1β production were determined from mice gastric tissues infected with SS1 and SS1Δ*hpyAVIBM* strains for 45 days. IL-6 and IL-1β from protein extracts was measured using ELISA as described in materials and methods. Data are expressed as mean ± standard error of mean (SEM) of 3 experiments in duplicates. *P<0.05 (Student's *t* test) as compared between groups.

Figure 6: Immunohistochemical study with caspase-3 antibody of mice gastric tissues 45 days post infection by SS1 and SS1Δ*hpyAVIBM* strains. Control mice were fed with PBS. Histological appearances of- A) Control, B) SS1 infected, and C) SS1Δ*hpyAVIBM* infected tissues. Mice infected with isogenic mutant show higher expression of caspase-3. The arrows are showing the epithelial border destruction.

Table 1: Primers used in this study

S. No.	Primer	Sequence 5´ to 3´
1	HP0051F1	GGATCCATGAATTATAAAATTTTA
2	HP0051R1	CTCGAGTCATTTTCTTAAGCTTTT
3	HP0051F2	GATTTATTTTGTGGGGCTGGG
4	HP0051R2	TTGACATGGAGGCCCGCCAA

5	HP0051F3	ACATTCTCAATCGCAATAAA
6	HP0051R3	ATCTATGCCTTGCCTTTTAGC

Figures

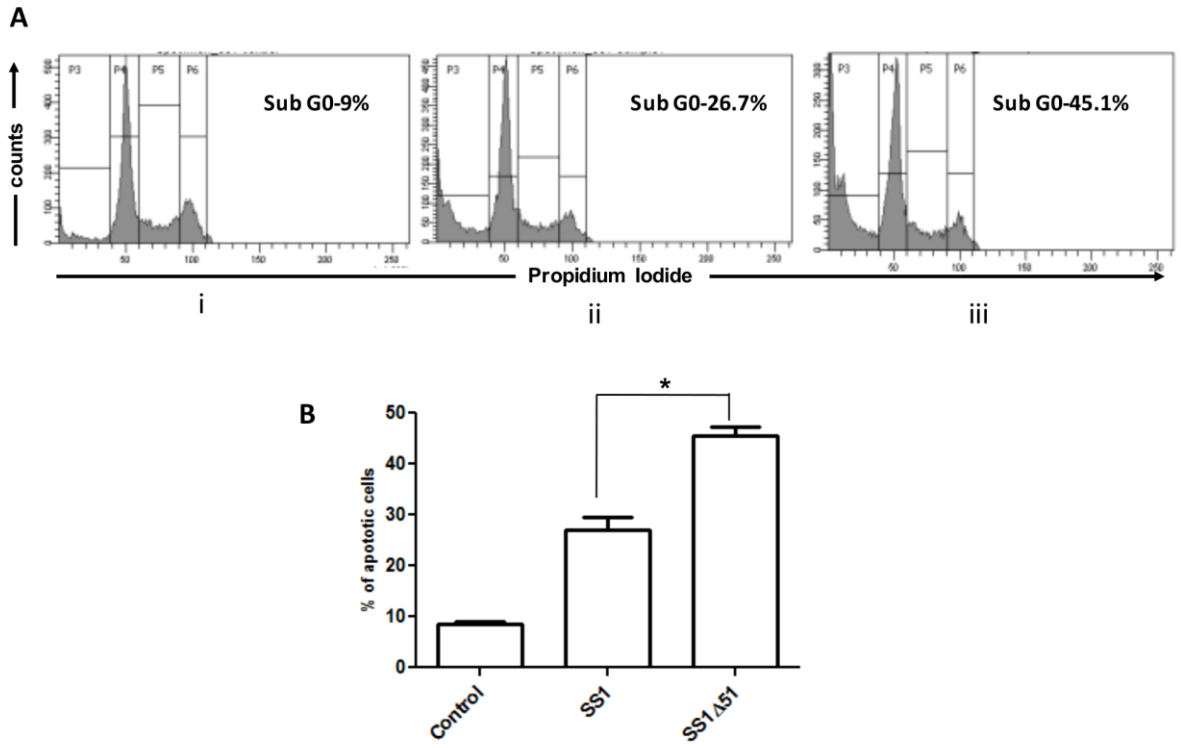

Figure 1

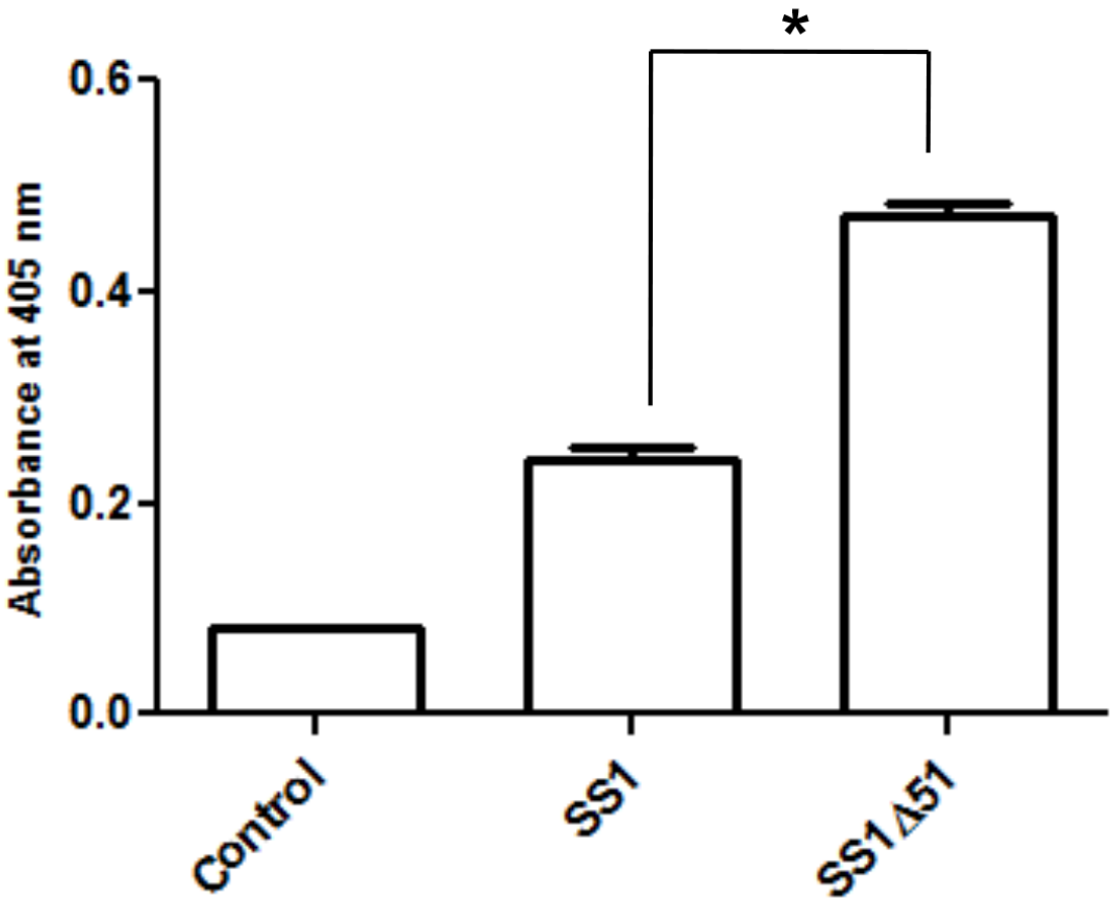

Figure 2

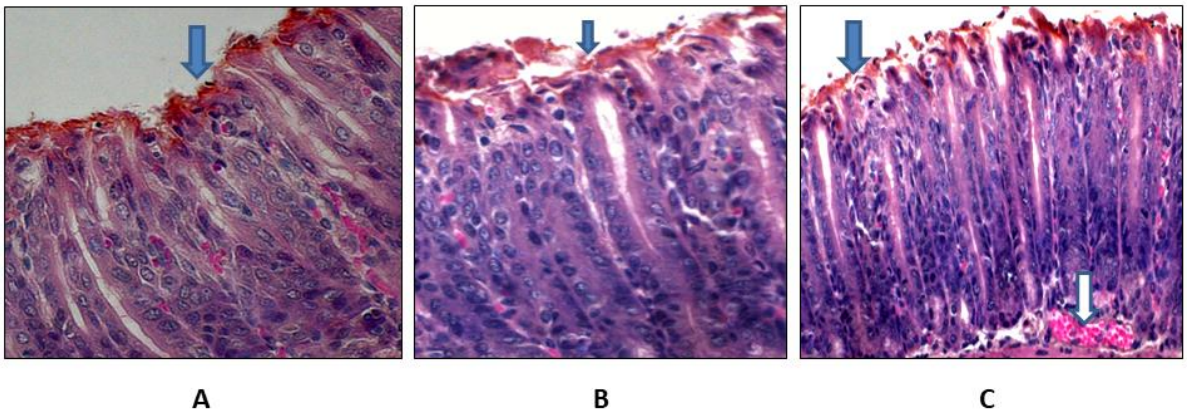

Figure 3

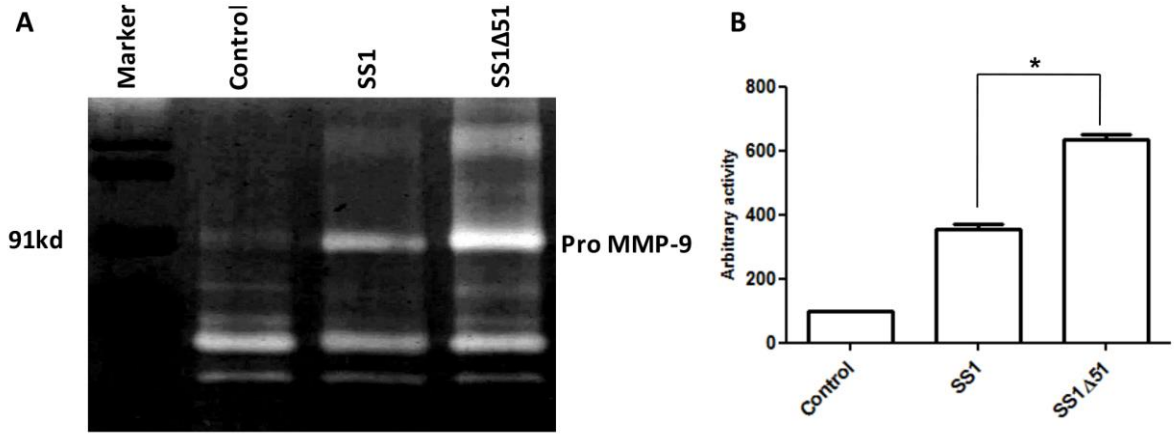

Figure 4

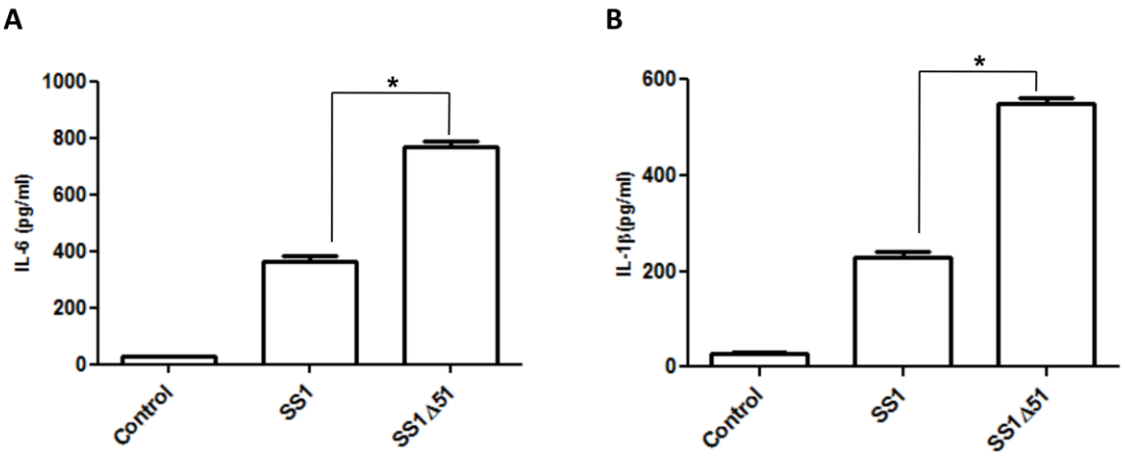

Figure 5

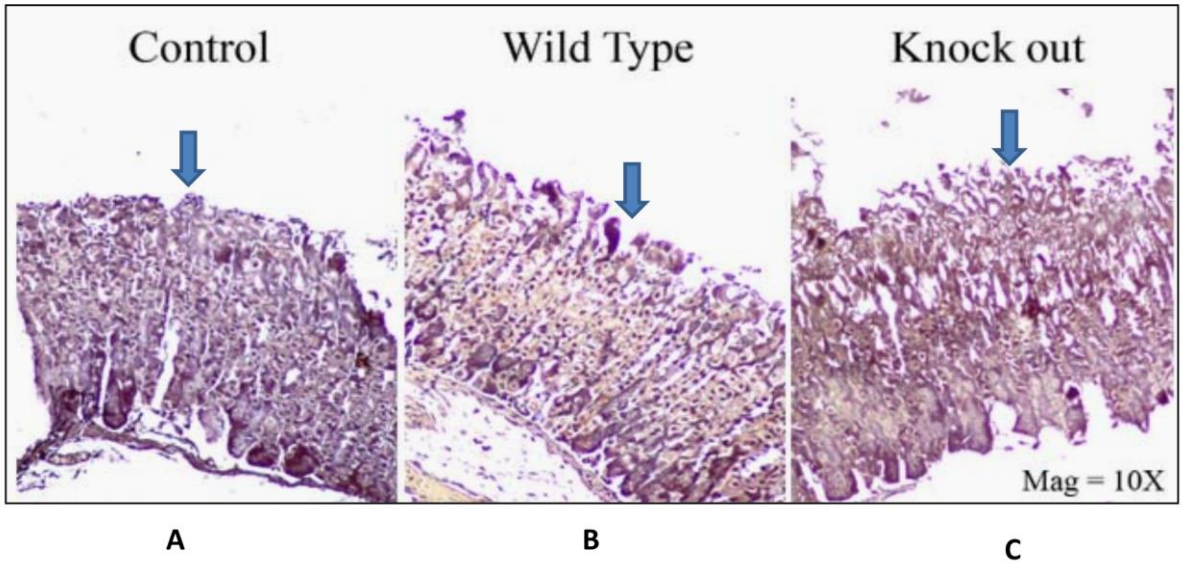

Figure 6

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