Transcriptional Profiling of Type II Toxin–Antitoxin Genes of Helicobacter pylori under Different Environmental Conditions: Identification of HP0967–HP0968 System

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Helicobacter pylori is a Gram-negative bacterium that colonizes the human gastric mucosa and is responsible for causing peptic ulcers and gastric carcinoma. The expression of virulence factors allows the persistence of H. pylori in the stomach, which results in a chronic, sometimes uncontrolled inflammatory response. Type II toxin–antitoxin (TA) systems have emerged as important virulence factors in many pathogenic bacteria. Three type II TA systems have previously been identified in the genome of H. pylori 26695: HP0315–HP0316, HP0892–HP0893, and HP0894–HP0895. Here we characterized a heretofore undescribed type II TA system in H. pylori, HP0967–HP0968, which is encoded by the bicistronic operon hp0968–hp0967 and belongs to the Vap family. The predicted HP0967 protein is a toxin with ribonuclease activity whereas HP0968 is an antitoxin that binds to its own regulatory region. We found that all type II TA systems were expressed in H. pylori during early stationary growth phase, and differentially expressed in the presence of urea, nickel, and iron, although, the hp0968–hp0967 pair was the most affected under these environmental conditions. Transcription of hp0968–hp0967 was strongly induced in a mature H. pylori biofilm and when the bacteria interacted with AGS epithelial cells. Kanamycin and chloramphenicol considerably boosted transcription levels of all the four type II TA systems. The hp0968–hp0967 TA system was the most frequent among 317 H. pylori strains isolated from all over the world. This study is the first report on the transcription of type II TA genes in H. pylori under different environmental conditions. Our data show that the HP0967 and HP0968 proteins constitute a bona fide type II TA system in H. pylori, whose expression is regulated by environmental cues, which are relevant in the context of infection of the human gastric mucosa.

Keywords: H. pylori, HP0967, HP0968, toxin–antitoxin system, environmental cues
INTRODUCTION

*Helicobacter pylori* is a Gram-negative bacterium, member of the Epsilon proteobacteria that colonizes the human gastric mucosa (Marshall and Warren, 1984). A hallmark of *H. pylori* is its ability to survive in the hostile environment found in the stomach, which is attributed to the expression of various virulence factors, such as secretion systems, cytotoxins, flagella, and adhesins. In this regard, the *H. pylori* genome encodes a type IV secretion system, which is responsible for the translocation of the CagA effector protein into host epithelial gastric cells (Amieva and El-Omar, 2008; Tegtmeyer et al., 2011). In addition to CagA, *H. pylori* produces the vacuolating cytotoxin VacA, which is present in all *H. pylori* strains (Palframan et al., 2012). The toxin–antitoxin (TA) systems have emerged as important virulence factors in many pathogenic bacteria (Kedzierska and Hayes, 2016). At present, six types of TA systems have been described (Page and Peti, 2016) among which, the type II is highly prevalent in prokaryote genomes (Makarova et al., 2009). The functionality of these systems has been described to be beneficial in bacterial fitness, persistence, and virulence (Pandey and Gerdes, 2005; Lewis, 2007; Leplae et al., 2011; Kedzierska and Hayes, 2016). These systems are formed by a couple of TA proteins, where the toxin exerts a bacteriostatic effect on bacterial growth and the antitoxin neutralizes the toxin through a protein–protein interaction. A remarkable characteristic of the type II TA system is that the antitoxin acts as a repressor of its own transcription (Yamaguchi and Inouye, 2011; Yamaguchi et al., 2011). In terms of virulence, type II TA systems have been described to be important in the host–pathogen interactions, particularly in Gram-negative bacteria. In uropathogenic *Escherichia coli*, a type II TA module promotes the colonization of the mouse bladder or kidneys (Norton and Mulvey, 2012). The SehAB system was shown to be important for the survival of *Salmonella enterica* serotype Typhimurium in the mesenteric lymphoid nodes of mice (De la Cruz et al., 2013). Also, the RelBE TA system of *Vibrio cholerae* affects biofilm formation and intestinal colonization (Wang et al., 2015). Moreover, in both *Leptospira interrogans* and *Rickettsia* spp., type II TA mutants were affected in their induction of apoptosis in eukaryotic cells (Audoly et al., 2011; Komi et al., 2015). Thus, a growing body of evidence suggests that these emerging virulence factors display functional and regulatory roles in the expression of multiple virulence traits.

Three type II TA systems have been structurally and functionally characterized in *H. pylori* 26695: HP0315–HP0316, HP0892–HP0893, and HP0894–HP0895 (Han et al., 2011, 2013; Kwon et al., 2012; Pathak et al., 2013; Im et al., 2014). These TA systems belong to the Vap (virulence-associated protein) family, found in Bacteria and Archaea (Makarova et al., 2006). However, no transcriptional analyses have been reported for these TA systems in *H. pylori* under environmental conditions such as acidic pH or in the presence of urea, nickel, and iron, which affect the expression of virulence factors in this bacterium (Sachs et al., 2003; Haley and Gaddy, 2015; Keilberg and Otto, 2016).

In this work we identified and characterized a new type II TA system (HP0967–HP0968) in the genome of *H. pylori* strain 26695. Whilst HP0967 turned out to be a toxin with ribonuclease activity, HP0968 was found to work as an antitoxin that bound to its own regulatory region. Compared to the other three known type II TA systems, the *hp0968–hp0967* system was highly expressed when *H. pylori* 26695 was grown in *Brucella* broth, particularly during the early stationary phase. Interestingly, metals such as nickel and iron repressed the expression of *hp0968–hp0967*, while urea had a moderate positive effect. Transcription of *hp0968–hp0967* was highly induced in a mature biofilm and when *H. pylori* was interacting with AGS epithelial cells, whereas the other type II TA systems showed a weaker induction, suggesting that particularly this TA system might be activated in the environment of the host gastric mucosa prior to host colonization. The presence of antibiotics such as kanamycin and chloramphenicol dramatically boosted the transcription of *hp0968–hp0967*. The *hp0968–hp0967* genes were present in both clinical isolates and in *H. pylori* strains whose genomes are publicly available. Our data show that HP0967 and HP0968 proteins form a type II TA system whose expression activates known virulence factors of *H. pylori*.

This is the first report about regulation of transcription of *H. pylori* type II TA systems under different environmental conditions.

MATERIALS AND METHODS

**Bacterial Strains and Culture Conditions**

*Helicobacter pylori* 26695 (Tomb et al., 1997) was grown for 2 days on blood agar culture plates containing 10% defibrinated sheep blood at 37°C under microaerophilic conditions, and a bacterial suspension was prepared in *Brucella* broth and adjusted to an optical density of 0.1 at 600 nm. *H. pylori* was grown at 37°C for 24 h (exponential phase) and 48 h (stationary phase) in *Brucella* broth supplemented with 10% fetal bovine serum (FBS; Invitrogen). In addition, *Brucella* broth adjusted to pH 5.5 or containing either urea [5 μM CO(NH₂)₂], nickel [250 μM NiCl₂] or iron [150 μM (NH₄)₂Fe(SO₄)₂·6H₂O], were tested as previously described (Contreras et al., 2003; Wen et al., 2003; Vannini et al., 2014). In all cases, the bacteria were incubated at 37°C with shaking and samples were collected 48 h post-inoculation for RNA extraction. Fold-change transcription was determined by calculating the relative expression of TA genes under different environmental conditions as compared to bacteria growing in *Brucella* broth.

**Cloning and Purification of HP0967 and HP0968 Proteins**

For cloning of *hp0967* and *hp0968* coding regions, specific primers (Table 1) containing the *Nco* (5′)/*Hind*III (3′) restriction sites were used to obtain PCR products, which were digested with *Nco* and *Hind*III and then ligated into pBAD-Mycl-HisA previously digested with the same restriction enzymes. HP0967–Mycl-His₆ and HP0968–Mycl-His₆ proteins were purified...
TABLE 1 | Primers used in this study.

| Primer                  | Sequence (5′–3′)                  | Target gene | RE       |
|------------------------|----------------------------------|-------------|----------|
| For qPCR               |                                  |             |          |
| hp0315-5′              | CGGAGAACCCCTACAAATAGGCC          | hp0315      |          |
| hp0315-3′              | TCCTTATGCTGTTACCAAAACCC          |             |          |
| hp0316-5′              | CGGTATAGAATGACTTGTGC             | hp0316      |          |
| hp0316-3′              | ATGCCCAACAAACATCTC              |             |          |
| hp0892-5′              | AATGCTGACGGTGAACACAG            | hp0892      |          |
| hp0892-3′              | TTTAACACGGCTTTGAGTG             |             |          |
| hp0893-5′              | GCAAAAGCAACACAAACAGGA           | hp0893      |          |
| hp0893-3′              | GCGCTTTAATTGGAGTGCT             |             |          |
| hp0894-5′              | AATGCTGACCGATCCCAATTTCA         | hp0894      |          |
| hp0894-3′              | TAAAAGGCTGTGGTGAGATTA           |             |          |
| hp0895-5′              | GGCATGAGATCCCAACAACT            | hp0895      |          |
| hp0895-3′              | TCAAACCTCAATCAAAGCA             |             |          |
| hp0896-5′              | TAGCTTATGCGGCTGTAA             | hp0896      |          |
| hp0896-3′              | AGGATTTACAGTCTGCGTTT           | cagA        |          |
| cagA-3′                | AGCAAAAAGCGACCTTGAAA           |             |          |
| vacA-5′                | AGGCCATTTGCTCTTGAQ             | vacA        |          |
| vacA-3′                | AAGCAACATTGCTCTTGGAC           |             |          |
| 16S-5′                 | GTGTGGAGAGGATGAGTG             | 16S         |          |
| 16S-3′                 | GTTATGCGGCGGTGACTACCA         |             |          |
| For gene cloning       |                                  |             |          |
| HP0967-NcoI-5′         | AGTTACCATGGATACTGTAATTATTTTGATTTGAC | hp0967      | NcoI     |
| HP0967-HindIII-3′      | GGGAATCTTTAAAACACTTGATAGCCGATTAAATC |             | HindIII  |
| HP0968-NcoI-5′         | ATTTCCCAATGTAACCTTTAGAAATTTATGG | hp0968      | NcoI     |
| HP0968-HindIII-3′      | GGGAAGCTTCAACCCATGGCTTTGAGAATAAACAATTTC |             | HindIII  |
| For EMSA               |                                  |             |          |
| HP0968-5′              | GCCTTATGCGTCTTTTGAGTTGC         | hp0968      |          |
| HP0968-3′              | CAATATATATTCTAAAGCGGCGATG |             |          |

Italic letters indicate the respective restriction enzyme site in the primer. RE, restriction enzyme for which a site was generated in the primer.

with Ni-nitrilotriacetic acid resin. Briefly, E. coli BL21(DE3) carrying the pBAD-HP0967-Myc-His<sub>6</sub> and pBAD-HP0968-Myc-His<sub>6</sub> plasmids were grown to mid-logarithmic phase. L(+-)-arabinose (Sigma-Aldrich) was added at final concentration of 0.1%, and the bacteria were grown at 6 h at 30°C. The cells were then pelleted by centrifugation, resuspended in PBS buffer and ruptured by sonication. The suspension was centrifuged and the supernatant was filtered through a Ni-nitrilotriacetic acid agarose column (QIAExpress, Qiagen) pre-equilibrated with PBS buffer. After extensive washing with binding buffer containing 50 µM imidazole (100 ml), the bound protein was eluted with 500 µM imidazole. Fractions were analyzed by SDS-PAGE. Protein concentration was determined by the Bradford procedure. Aliquots of the purified protein were stored at −70°C.

**Electrophoretic Mobility Shift Assay (EMSA)**

Electrophoretic mobility shift assay (EMSA) experiments were performed as previously described (De la Cruz et al., 2007, 2013). Specific primers (Table 1) were used to amplify the hp0968–hp0967 promoter region by PCR. The PCR product (100 ng) was mixed with increasing concentrations of HP0968-Myc-His<sub>6</sub> at room temperature in the presence of the binding buffer 10X (400 µM HEPES, 80 µM MgCl<sub>2</sub>, 500 µM KCl, 10 µM DTT, 0.5% NP40, and 1 µg/ml BSA). The reactions were incubated during 30 min at room temperature and then separated in 6% SDS-PAGE gels in Tris-Borate-EDTA buffer. The DNA bands were visualized with ethidium bromide staining.

**Ribonuclease Activity Assay**

HP0967-Myc-His<sub>6</sub> protein (0, 0.6, and 1.2 µM, respectively) was incubated with 1 µg of total H. pylori RNA in the presence of 50 µM Tris (pH 8.0) and 10 µM MgCl<sub>2</sub> in a final reaction volume of 20 µl. When necessary, HP0968-Myc-His<sub>6</sub> protein was added to analyze the antagonistic effect of the antitoxin on the toxin activity. The reaction was allowed to proceed for 30 min at 25°C, and the samples were then loaded in a bleach agarose gel 2% as previously described (Aranda et al., 2012). RNA was visualized with ethidium bromide staining.
RNA Isolation and Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the different culture conditions using the hot phenol method (Jahn et al., 2008). Purification of RNA, and qRT-PCR were performed as previously reported (Ares et al., 2016). Briefly, DNA was removed with TURBO DNA-free (Ambion, Inc.) and the quality of RNA was assessed using a NanoDrop (ND-1000; Thermo Scientific) and a bleach agarose gel (2%) as previously described (Aranda et al., 2012). cDNA was prepared using 1 µg of RNA, random hexamer primers (0.2 µg/µl), and M-MulV-RT (20 U/µl) reverse transcriptase of Moloney Murine leukemia Virus (Thermo Scientific). Specific primers were designed with the Primer3Plus software and are listed in Table 1. The absence of contaminating DNA was controlled by lack of amplification products after 35 qPCR cycles. Control reactions with no template (water) and minus-Reverse Transcriptase were run with all reactions. 16S rRNA (HPPrnA16S) was used as a reference gene for normalization and the relative gene expression was calculated using the 2^−ΔΔCt method (Livak and Schmittgen, 2001).

TA Gene Expression during H. pylori Infection of AGS Cells

AGS gastric epithelial cells were grown to about 75% confluence in RPMI-1640 medium containing 10% FBS, and washed thrice with PBS before adding fresh RPMI media with 10% FBS. H. pylori 26695 was grown in Brucella broth for 24 h, suspended in RPMI, and added to the AGS cell culture at a multiplicity of infection (MOI) of 100. Infected cells were incubated at 37°C under microaerophilic conditions for 0 and 6 h, and bacteria were recovered. RNA was extracted from bacteria to determine gene expression. Fold-change in gene transcription was determined by calculating the relative expression of TA genes with respect to bacteria at time 0 of infection.

Biofilm Formation Assay on Abiotic Surface and TA Gene Expression

Adhesion to abiotic surface (polystyrene) was analyzed using 96-well plates as previously described (Ares et al., 2016). H. pylori 26695 was grown for 2 days on blood agar culture plates containing 10% defibrinated sheep blood at 37°C under microaerophilic conditions, and a bacterial suspension was prepared in Brucella broth supplemented with 10% decomplemented FBS and adjusted to an optical density of 0.1 at 600 nm. Two-hundred µl of the bacterial suspensions were added per well into 96-well plates in octuplicate and incubated at different times at 37°C under microaerophilic conditions. Unbound bacteria were removed by washing the wells three times with PBS, and bound bacteria were stained with 1% Crystal Violet for 20 min. Wells were thoroughly rinsed thrice with PBS and the dye was solubilized in 100 µl of ethanol 70%. Finally, the amount of extracted Crystal Violet was determined by measuring color absorbance at OD600 using a spectrophotometer plate reader (Thermo Scientific).

For determination of TA gene expression, the biofilm assay was performed in 6 well plates (3 ml) and supernatant (planktonic) and adhered (sessile) bacteria were recovered for RNA extraction. Fold-change in gene transcription was determined by calculating the relative expression of TA genes within biofilms (sessile bacteria) as compared to the planktonic bacteria.

Transcription of TA Genes in the Presence of Antibiotics

Helicobacter pylori was grown in Brucella broth supplemented with 10% decomplemented FBS at 37°C for 48 h (stationary phase), with gentle shaking under microaerophilic conditions. The antibiotics ampicillin (Ap, 100 µg/mL), kanamycin (Km, 50 µg/mL), chloramphenicol (Cm, 30 µg/mL), or tetracycline (Tc, 10 µg/mL) were added to the liquid culture and incubated for 1 h as previously described (Christensen-Dalsgaard et al., 2010). Antibiotics concentrations were used as reported for E. coli and Salmonella enterica (Christensen-Dalsgaard et al., 2010; Maisonneuve et al., 2011; Silva-Herzog et al., 2015; Li et al., 2016). Fold-change in gene transcription was determined by calculating the relative expression of TA genes in the presence of each antibiotic as compared to bacteria growing in Brucella broth without antibiotics.

In silico Identification of TA Modules

Analyzed sequence data and loci annotations of 260 H. pylori genomes were retrieved from the NCBI database by a series of custom Perl scripts. In addition, the genomes of 57 Helicobacter non-pylori strains were included in the comparative analysis.

We also selected the sequences from known toxin and antitoxin modules that were classified into super-families as stated previously (Leplae et al., 2011) and compared their homology with previously referred Helicobacter genomes. Briefly, each TA protein pair was queried using PSI-BLAST (Altschul et al., 1997) under the following parameters: matrix = BLOSUM62, word size = 3, PSI-BLAST threshold = 0.005, expect threshold = 10, and without filtering low complexity regions. Hits were carefully examined and selected according to their functional annotation.

Furthermore, we employed the Toxin–Antitoxin Data Base (TADB3) (Shao et al., 2011) to extend our findings.

Heatmap Construction

In order to illustrate the presence of every toxin and antitoxin module in all Helicobacter genomes, a gene content matrix (“heatmap” function) was built using the R program v3.2.4. Next, these paired loci were hierarchical clustered (“hclust” function, “ward.D” method) according to their loci-content similarity to build a sidelong dendrogram.

References

1http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/
2http://ftp.ncbi.nlm.nih.gov/genomes/genbank/bacteria/
3http://202.120.12.135/TADB2/index.php
4http://www.R-project.org/
RESULTS

The hp0968–hp0967 H. pylori Genes Code for a New Type II TA System

Three pairs of type II TA proteins have been described in H. pylori: HP0315–HP0316, HP0892–HP0893, and HP0894–HP0895 (Han et al., 2011, 2013; Kwon et al., 2012; Im et al., 2014). Searching for TA-encoding homolog genes of different families, we identified hp0967, a gene that codes for a type II toxin belonging to the VapD protein family. Of note, an intergenic region was identified between hp0967 and hp0969 genes and was designated herein as antitoxin gene hp0968. In contrast, by analyzing the genome sequence of H. pylori J99 strain, a homolog of hp0968 (jhp0902) antitoxin gene was found. The analysis of the H. pylori 26695s genome revealed a stop codon in the coding region of hp0968 gene resulting in a peptide of 19 aminoacids. However, by comparing the nucleotidic sequences of all H. pylori strains deposited in the GenBank, no stop codon was found in this gene, suggesting a problem of annotation in the strain 26695. To demonstrate that hp0968 antitoxin gene is present and transcribed in 26695 strain, we performed RT-PCR experiments using primers that hybridize with TA genes as shown in Figure 1A. We found that both hp0968 and hp0967 genes were transcriptionally expressed and as expected, they presented a single mRNA similar to other type II TA systems. Genes were transcriptionally expressed and as expected, they were poorly expressed in this growth condition. Similarly, expression of cagA and vacA was found increased during stationary growth phase (Figure 2B), in agreement to previously reported data (Thompson et al., 2003; Boonjakuakul et al., 2005).

Of note, the effect of the environmental cues such as pH, urea, nickel, and iron in transcription of type II TA systems has not been reported for H. pylori, although, they are known to affect the expression of other virulence factors (Sachs et al., 2003; Haley and Gaddy, 2015; Keilberg and Ottemann, 2016). We found that during stationary growth phase type II TA genes showed different levels of expression in the presence of the tested cues. While nickel repressed the expression of hp0316–hp0315 (fivefold) and hp0895–hp0894 (threefold) (Figures 2C,E), all conditions tested triggered expression of hp0893–hp0892 TA genes (Figure 2D). Expression of hp0968–hp0967 was repressed in the presence of nickel (20-fold) and iron (10-fold), while urea caused a twofold increase in its expression (Figure 2F). As reaction controls, the transcription of cagA and vacA genes was determined under the same growth conditions. We found that transcription of both virulence genes was differentially regulated: urea slightly boosted transcription (1.5-fold), while acidic pH (2.2-fold), nickel (2.7-fold), and iron (2.7-fold) repressed them (Figure 2G).

Environmental Cues Differentially Regulate Expression of H. pylori Type II TA Systems

In order to elucidate the environmental cues that may affect expression of type II TA systems we performed qRT-PCR experiments. Relative expression was determined during both exponential (24 h) and stationary phase (48 h). Interestingly, expression of all the type II TA genes was higher during stationary phase than in exponential phase (Figure 2A); although hp0316–hp0315 and hp0968–hp0967 showed the highest transcriptional levels (Figure 2A), and hp0893–hp0892 and hp0895–hp0894 were poorly expressed in this growth condition. Similarly, expression of cagA and vacA was found increased during stationary growth phase (Figure 2B).

Biofilm Formation and Contact with Gastric Epithelial Cells Stimulate the Expression of hp0968–hp0967

Type II TA systems are reported to influence biofilm formation (Wang and Wood, 2011; Wen et al., 2014; Kedzierska and Hayes, 2016). We quantitatively analyzed the biofilm formation of H. pylori on a polystyrene surface at different time points measuring uptake of Crystal Violet. After 72 h of incubation, a mature biofilm was observed (Figure 3A). Thus, in order to evaluate the expression of type II TA genes in a mature biofilm, we determined their expression in both H. pylori planktonic and sessile cells at 72 h. Expression of all type II TA genes was found to be induced in biofilms-associated H. pylori, as compared to planktonic bacteria (Figure 3B). It should be noted that hp0968–hp0967 showed the highest induction in this environment (500-fold). In a mature biofilm, vacA transcription was significantly increased (23-fold), whereas cagA expression was not affected (Figure 3B). On the other hand, the contact with AGS cells significantly increased transcription of type II TA genes. Of note,
hp0968–hp0967 genes presented the highest levels of expression over the other TA systems, even higher than expression of cagA and vacA (Figure 3C). These data strongly suggest that hp0968–hp0967 genes might be expressed in the host during stomach colonization.

The Presence of Antibiotics Affects the Transcription of H. pylori Type II TA Genes

We analyzed the expression of type II TA genes in the presence of antibiotics, and found that kanamycin and chloramphenicol (Km/Cm) increased the expression of hp0316–hp0315 (6.2/9.9-fold), hp0893–hp0892 (3.1/9.2-fold), and hp0895–hp0894 (4.34/9-fold) systems (Figure 4). In particular, chloramphenicol, kanamycin, and tetracycline stimulated the expression of hp0968–hp0967 genes (50-, 12-, and 2.5-fold, respectively) while ampicillin repressed them (2.8-fold) (Figure 4D). Interestingly, similar transcriptional profiling was found between the hp0893–hp0892 and hp0968–hp0967 genes in presence of chloramphenicol, kanamycin, and tetracycline (Figures 4B,D). Concerning the effect of antibiotics on the expression of cytotoxin genes, only vacA expression was boosted in the presence of kanamycin (10-fold) and chloramphenicol (23-fold) (Figure 4E). These results suggest that type II TA genes may have an important role in the response of H. pylori to the presence of antibiotics by H. pylori, particularly to chloramphenicol and kanamycin.

The hp0968–hp0967 Genes Are Highly Prevalent among Helicobacter Strains

We performed PCR experiments to identify type II TA genes in a collection of 114 clinical H. pylori isolates. These bacteria were isolated from patients with diverse pathologies, such as non-atrophic gastritis, premalignant lesions, gastric ulcer, and gastric cancer. Whereas hp0968–hp0967 was the most prevalent type II TA system in these isolates (in over 70% of all isolates),
hp0895–hp0894 genes were the least prevalent (in less than 50%) (Figure 5A). In addition, a Blast search in sequences deposited in GenBank using sequences of different TA families (included Vap), revealed the presence of type II TA genes in other H. pylori strains. TA genes of the Vap family were highly prevalent, presenting high identity and constituting a genetic signature inside H. pylori species (Figure 5B). In agreement with our findings in the clinical isolates, the hp0968–hp0967 genes presented the highest prevalence as compared to the other type II TA genes (Figures 5A–C). Interestingly, in both groups of H. pylori clinical and sequenced strains, the hp0968 antitoxin gene was more conserved than hp0967 toxin gene (Figures 5A–C).

Since the number of type II TA systems has been associated to bacterial pathogenicity (Georgiades and Raoult, 2011; De la Cruz et al., 2013), we studied the presence of the type II TA systems of different families in other Helicobacter species, and found that the presence of type II TA genes varied from one species to another. Species phylogenetically related to H. pylori such as H. acinonychis (big cats) and H. cetorum (marin mammals), contain the four type II TA systems (hp0316–hp0315, hp0893–hp0892, hp0895–hp0894, and hp0968–hp0967) and show high identity to the H. pylori sequences (Figures 5B,C). The presence of TA genes marked a separation of H. acinonychis and H. cetorum from the rest of Helicobacter species and they were grouped together with the H. pylori strains.

DISCUSSION

Helicobacter pylori produces a number of virulence-associated proteins such as secretion systems, flagella, adhesins, and cytotoxins that enable successful colonization of its human host. Expression of virulence factors also allows for the survival
FIGURE 3 | Expression of type II TA genes in biofilm formation and in AGS cells. (A) Quantification of biofilm formation of *H. pylori* 26695 by measuring Crystal Violet uptake at different times. Fold-change expression (qRT-PCR) of the type II TA genes and *cagA/vacA* in a mature biofilm after 72 h (B) and in contact with AGS cells (C). Data represent the mean of at least three independent experiments (mean ± SD). ns, not significant; statistically significant ***p < 0.001; **p < 0.01; *p < 0.05.

and persistence of *H. pylori* in the hostile environment found in the stomach (Salama et al., 2013). This persistence is mediated by a myriad of bacterial strategies to evade the immune response, such as detoxifying enzymes for reactive species of oxygen and nitrogen, DNA repair pathways, and natural competence (Wang et al., 2006; Dorer et al., 2011, 2013). In this sense, type II TA systems have emerged as potential virulence factors, not only affecting pathogenicity, but they are also involved in biofilm formation and persistence (Gerdes and Maisonneuve, 2012; Wen et al., 2014; Kedzierska and Hayes, 2016). In this work, we describe the transcriptional profiling of type II TA genes present in the genome of *H. pylori* 26695: *hp0316–hp0315, hp0893–hp0892, hp0895–hp0894*, and *hp0968–hp0967*, since the *hp0968–hp0967* system had not been identified and it was characterized in this work. These genes are transcriptionally organized as bicistronic operons, which is a hallmark of the type II TA systems. Type II toxins present different biochemical activities such as ribonucleases, kinases, adenylyl- and acetyl-transferases, and act also as inhibitors of protein synthesis and DNA supercoiling (Jiang et al., 2002; Mutschler et al., 2011; Yamaguchi et al., 2011; Harms et al., 2015; Cheverton et al., 2016; Rocker and Meinhart, 2016). In contrast, type II antitoxins are DNA-binding proteins that regulate their own expression and directly interact with their cognate toxins (Yamaguchi and Inouye, 2011; Chan et al., 2016). Despite having more specific functions, antitoxins show more complexity by possessing two functional domains: a DNA-binding domain and a toxin-interaction domain. Our
FIGURE 4 | Effect of antibiotics on type II TA expression. Fold-change expression (qRT-PCR) of the type II TA genes (A–D) and cagA/vacA (E) in presence of different antibiotics concentrations such as: Ap (Ampicillin, 100 µg/ml), Km (Kanamycin, 30 µg/ml), Cm (Chloramphenicol, 30 µg/ml), and Tc (Tetracycline, 10 µg/ml) by 1 h. Data represent the mean of at least three independent experiments (mean ± SD). ns, not significant; statistically significant ∗∗∗p < 0.001; ∗∗p < 0.01; ∗p < 0.05.

caracterization of both H. pylori HP0967 and HP0968 proteins revealed that they display ribonuclease and DNA-binding activities, respectively. HP0967 and HP0968 proteins belong to the Vap family, which is the type II TA pair most widespread in Bacteria and Archaea (Pandey and Gerdes, 2005; Sevin and Barloy-Hubler, 2007; Arcus et al., 2011; Jin et al., 2015).

In the stomach, H. pylori is exposed to frequent changes in pH, for example to acidic pH when present in the lumen and to neutral pH as it crosses the mucus to interact with
FIGURE 5 | Prevalence of type II TA genes. (A) The clinical isolates tested (n = 114) comprised strains of *H. pylori* with different pathologies. Bars show the prevalence of type II TA genes in clinic (black) and sequenced (gray) strains. The heatmaps and hierarchical clustering of selected genomes for *H. pylori* and *Helicobacter* species showing both the presence (B) and identity (C) of toxin–antitoxin genes were determined in R (version 3.2.4) using the hclust function with the "ward.D" method. The red and blue colors indicate the presence of the toxin and the anti-toxin gene.
epithelial cells. Presumably, these changes alter the expression of genes related to colonization and persistence in its human host. We found that an acidic pH boosted the expression of \textit{hp0893–hp0892}, while no effect was observed for the other type II TA systems, highlighting the importance of this system in the response to the acidic pH, which \textit{H. pylori} frequently finds prior to colonization of the stomach mucosa. Previous reports regarding the effect of acidic pH on the expression of the most thoroughly studied virulence factors \textit{cagA} and \textit{vacA} have been controversial (Karita et al., 1996; Allan et al., 2001; Merrell et al., 2003a; Bury-Mone et al., 2004; Vannini et al., 2014). Our results support the observation that an acidic pH represses both \textit{cagA} and \textit{vacA}, in agreement to the notion that \textit{CagA} and \textit{VacA} are produced when \textit{H. pylori} is in close contact to epithelial cells, where the pH is neutral (Salama et al., 2013). Urea, nickel, and iron are components present in the human stomach and it has been suggested that they act as regulatory networks whose purpose is the survival and persistence of \textit{H. pylori} (Haley and Gaddy, 2015). Urea and nickel are interconnected cues because transcription/activity of urease is nickel-dependent (Contreras et al., 2003; Khan et al., 2009). While high concentrations of nickel repress virulence genes (Haley and Gaddy, 2015), the presence of urea is essential for the survival and colonization of \textit{H. pylori} (Nakazawa, 2002; Sachs et al., 2003). We found that urea strongly stimulated the expression of \textit{hp0893–hp0892}, while it had a mild effect in the expression of \textit{hp0895–hp0894} and \textit{hp0968–hp0967}. With the exception of \textit{hp0893–hp0892}, nickel and iron repressed the type II TA systems in \textit{H. pylori}. Our results suggest that the high concentration of urea and low concentrations of both nickel and iron present in the stomach, would induce transcription of type II TA systems in preparation for successful colonization and persistent infection. Our study represents the first report on the transcriptional analysis of type II TA genes in \textit{H. pylori} under several environmental conditions that mimic those prevailing in the gastric mucosa.

Contrasting data have been reported concerning metal regulation of \textit{vacA} and \textit{cagA} (Merrell et al., 2003b; Ernst et al., 2005; Pich et al., 2012; Noto et al., 2013; Vannini et al., 2014). Iron exposure is associated with repression of numerous factors involved in the repertoire of virulence in \textit{H. pylori} (Haley and Gaddy, 2015). The role of nickel on the expression of \textit{vacA} and \textit{cagA} genes has not been previously studied. Transcriptional data showed that both, nickel and iron repressed both \textit{cagA} and \textit{vacA} genes, supporting the notion that the presence of both metals affect the virulence of \textit{H. pylori}.

Biofilm formation is a bacterial strategy to counteract the stress caused by the limitation of nutrients and the effect of antibiotics. It is well-established that type II TA systems are required for the biofilm formation (Wang and Wood, 2011; Wen et al., 2014; Kedzierska and Hayes, 2016). Since environmental and clinical strains of \textit{H. pylori} produce biofilm to persist and colonize both abiotic and biotic surfaces (Cammarota et al., 2012; Percival and Suleman, 2014), we studied the expression of the four type II TA genes in \textit{H. pylori} growing in a mature biofilm. In this condition, the four type II TA systems were induced, supporting the notion that these systems are expressed and may be required for the biofilm formation. In contact with AGS gastric epithelial cells, transcription of \textit{hp0968–hp0967} was considerably stimulated, presenting values higher than \textit{cagA} and \textit{vacA} cytotoxin genes. These results would support a probable role of \textit{hp0968–hp0967} genes in the context of infection of \textit{H. pylori}.

Kenn Gerdes's lab has clearly shown that antibiotics such as chloramphenicol, positively affect the expression of TA genes (Christensen et al., 2001; Christensen-Dalsgaard and Gerdes, 2006; Jorgensen et al., 2009; Winther and Gerdes, 2009; Christensen-Dalsgaard et al., 2010). We found that the presence of both chloramphenicol and kanamycin boosted transcription of all type II TA systems in \textit{H. pylori}. In the presence of both kanamycin and chloramphenicol, bacterial translation is inhibited and proteases such as Lon specifically degrades the type II antitoxins, generating an increase in the transcription of TA genes due to the lack of repression of the antitoxins on their own expressions (Christensen et al., 2001; Winther and Gerdes, 2009; Christensen-Dalsgaard et al., 2010). We found that the presence of both kanamycin and chloramphenicol, increased expression of type II TA genes, suggesting a probable role of type II TA systems in the bacterial fitness during biofilm formation and in the presence of antibiotics. In agreement, recent reports show that type II TA modules are necessary for the development of persister cells in the presence of antibiotics (Maisononneuve et al., 2011; Wen et al., 2014; Kedzierska and Hayes, 2016). Current experiments in our group are aimed to further analyze the expression of type II TA systems in persister \textit{H. pylori}.

In most reports, expression of \textit{cagA} and \textit{vacA} has been found to be co-regulated, corroborating the concept that both cytotoxins are required and co-expressed in the context of infection (Yokoyama et al., 2005; Singh et al., 2012; Raghwan and Chowdhury, 2014). However, we found that in a mature biofilm and in the presence of kanamycin or chloramphenicol, transcription of \textit{vacA}, but not \textit{cagA} was significantly increased. In contrast to \textit{CagA} which is translocated into host cells by the type 4 secretion system, \textit{VacA} is secreted by a type 5 secretion system (autotransporter) and it can also be located on the bacterial surface (Ilver et al., 2004). Based on the above notions, we suggest that \textit{VacA} may have a role in bacterium-bacterium interactions, as it occurs during biofilm formation.

In terms of prevalence, \textit{hp0968–hp0967} system was the most prevalent in both clinical and sequenced \textit{H. pylori} strains. Moreover, the \textit{hp0968–hp0967} system was also widely distributed in all \textit{Helicobacter} species. It was recently reported the retention of the antitoxin gene but not the toxin gene in TA modules of \textit{Xanthomonas} genomes (Martins et al., 2016). Both clinical and sequenced \textit{H. pylori} strains revealed the same profile, loss of toxin and retention of antitoxin genes. In \textit{H. pylori} and non-\textit{H. pylori} genomes deposited in GenBank, \textit{hp0968} antitoxin gene was much more prevalent than \textit{hp0967} toxin. The retention of antitoxin genes in bacterial genomes has been postulated due to the toxin-independent functions of type II antitoxins such as MqsA and DinJ (Wang and Wood, 2011; Hu et al., 2012).

The presence and homology of type II TA genes in \textit{Helicobacter} strains generated two main clusters: \textit{H. pylori} and other \textit{Helicobacter} species, showing that these TA systems represent a DNA signature for \textit{H. pylori}. Moreover, species
phylogenetically related to *H. pylori* such as *H. acinonychis* and *H. cetorum* were separated from the rest of *Helicobacter* species and grouped with *H. pylori* strains, supporting the concept that TA genes are part of the prokaryotic mobilebome and that they are involved in the bacterial evolution (Koonin and Wolf, 2008). Searching type II TA genes by using Toxin–Antitoxin Data Base (TADB) we found two TA systems for the *H. pylori* strains 26695 and J99 (HP0892–HP0893 and HP0894–HP0895). In the case of other *Helicobacter* species, *H. hepaticus* was the only species showing one TA system (HH0272–HH0273), which clearly indicates the need for an update and construction of a new database that integrates more bacterial genomes.

It is worth noticing that hp0968–hp0967 genes presented the same transcriptional profile than cagA and vacA under growth in urea, nickel, iron and in contact with AGS cells. These findings together with the high prevalence of this TA system in *H. pylori* strains, suggest that the hp0968–hp0967 may represent a very important virulence factor. We cannot discard a probable virulence role for the other type II TA system, since these genes presumably display redundant and specific functions. The generation of null mutants will help to elucidate the role of type II TA genes in different phenotypes such as biofilm formation, persistence and mainly in the virulence of *H. pylori*.

**AUTHOR CONTRIBUTIONS**

Conceived and designed the experiments: MDC. Performed the experiments: MC-M, MA, LP, SP. Analyzed the data: MDC, MC-M, MA, MC-P, JG, JT. Wrote the paper: MDC, JG, JT.

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**REFERENCES**

Allan, E., Clayton, C. L., McLaren, A., Wallace, D. M., and Wren, B. W. (2001). Characterization of the low-pH responses of *Helicobacter pylori* using genomic DNA arrays. Microbiology 147, 2285–2292. doi: 10.1099/0221287-147-8-[/bre ak]2285

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Amieva, M. R., and El-Omar, E. M. (2008). Host-bacterial interactions in Helicobacter pylori infection. *Gastroenterology* 134, 306–323. doi: 10.1055/s-0033-1190040

Aranda, P. S., Lajoie, D. M., and Jorcyk, C. L. (2012). Bleach gel: a simple agarose gel for analyzing RNA quality. *Electrophoresis* 33, 366–369. doi: 10.1002/elps.201100335

Arcus, V. L., Mckenzie, J. L., Robson, J., and Cook, G. M. (2011). The PIN-domain ribonucleases and the prokaryotic VapBC toxin-antitoxin array. *Protein Eng. Des.* 24, 33–40. doi: 10.1093/protein/gpz081

Ares, M. A., Fernandez-Vazquez, J. I., Rosales-Reyes, R., Jarillo-Quijada, M. D., Von Bargen, K., Torres, J., et al. (2016). H-NS nucleoid protein controls virulence features of *Klebsiella pneumoniae* type 3 pili and the capsule polysaccharide. *Front. Cell. Infect. Microbiol.* 6:13. doi: 10.3389/fcimb.2016.00013

Audoly, G., Vincentelli, R., Edouard, S., Georgiades, K., Mediannikov, O., Gimenez, G., et al. (2011). Effect of rickettsial toxin VapC on its eukaryotic host. *PLoS One* 6:e26528. doi: 10.1371/journal.pone.0026528

Boonjakuakul, J. K., Canfield, D. R., and Solnick, J. V. (2005). Comparison of *Helicobacter pylori* virulence gene expression in vitro and in the *Rhesus macaque*. *Infect. Immun.* 73, 4895–4904. doi: 10.1128/IAI.73.8.4895-4904.1[\bre ak]2005

Bury-Mone, S., Thibere, J. M., Conterras, M., Matournam, A., Labigne, A., and De Reuse, H. (2004). Responsiveness to acidity via metal ion regulators mediates virulence in the gastric pathogen *Helicobacter pylori*. *Microb. Mol. Biol. Rev.* 63, 623–638. doi: 10.1128/MMBR.135.4.4137-0.1111

Cammarota, G., Sanguinetti, M., Gallo, A., and Posteraro, B. (2012). Review article: biofilm formation by *Helicobacter pylori* as a target for eradication of resistant infection. *Aliment. Pharmacol. Ther.* 36, 222–230. doi: 10.1111/j.1365-2036.2012.05165.x

Chan, W. T., Espinosa, M., and Yeo, C. C. (2016). Keeping the wolves at Bay: antitoxins of prokaryotic type II toxin-antitoxin systems. *Front. Mol. Biosci.* 3:9. doi: 10.3389/fmolb.2016.00009
Haley, K. P., and Gaddy, J. A. (2015). Metalloregulation of Helicobacter pylori physiology and pathogenesis. *Front. Microbiol.* 6:911. doi: 10.3389/fmicb.2015.00911

Han, K. D., Ahn, D. H., Lee, S. A., Min, Y. H., Kwon, A. R., Ahn, H. C., et al. (2013). Identification of chromosomal HP0892-HP0893 toxin-antitoxin proteins in *Helicobacter pylori* and structural elucidation of their protein-protein interaction. *J. Biol. Chem.* 288, 6004–6013. doi: 10.1074/jbc.M111.322784

Han, K. D., Matsuura, A., Ahn, H. C., Kwon, A. R., Min, Y. H., Park, H. J., et al. (2011). Functional identification of toxin-antitoxin molecules from *Helicobacter pylori* 26695 and structural elucidation of the molecular interactions. *J. Biol. Chem.* 286, 4842–4853. doi: 10.1074/jbc.M110.097840

Harms, A., Stanger, F. V., Scheu, P. D., De Jong, I. G., Goepfert, A., Glatter, T., et al. (2015). Adenylylation of gyrase and topo IV by FicT toxins disrupts bacterial DNA topology. *Cell Rep.* 12, 1497–1507. doi: 10.1016/j.celrep.2015.07.056

Hu, Y., Benedik, M. J., and Wood, T. K. (2012). Antitoxin DufJ influences the general stress response through transcript stabilizer CapE. *Environ. Microbiol.* 14, 669–679. doi: 10.1111/j.1462-2920.2011.02618.x

Ilver, D., Barone, S., Mercati, D., Lupetti, P., and Telford, J. L. (2004). *Helicobacter pylori* toxin VacA is transferred to host cells via a novel contact-dependent mechanism. *Cell. Microbiol.* 6, 167–174. doi: 10.1111/j.1462-2920.2003.tb00349.x

Im, H., Jang, S. B., Pathak, C., Yang, Y. J., Yoon, H. J., Yu, T. K., et al. (2014). Crystal structure of toxin HP0892 from *Helicobacter pylori* with two Zn(II) at 1.8 Å resolution. *Protein Sci.* 23, 819–832. doi: 10.1002/pro.2465

Jahn, C. E., Charkowski, A. O., and Willis, D. K. (2008). Evaluation of isolation methods and RNA integrity for bacterial RNA quantitation. *J. Microbiol. Methods.* 75, 318–324. doi: 10.1016/j.mimet.2008.07.004

Jiang, Y., Pogliano, J., Helinski, D. R., and Konieczny, I. (2002). ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of *Esherichia coli* gyrA. *Mol. Microbiol.* 44, 971–979. doi: 10.1046/j.1365-2958.2002.02921.x

Jin, G., Pavelka, M. S. Jr., and Butler, J. S. (2015). Structure-function analysis of VapB4 antitoxin identifies critical features of a minimal VapC4 toxin-binding module. *J. Bacteriol.* 197, 1197–1207. doi: 10.1128/JB.02508-14

Jorgensen, M. G., Pandey, D. P., Jaksolska, M., and Gerdes, K. (2009). HicA from *E. coli* defines a novel family of translation-independent mRNA interferases in bacteria and archaea. *J. Bacteriol.* 191, 1191–1199. doi: 10.1128/JB.01013-08

Karita, M., Tummurri, M. K. W., and Blaser, M. J. (1996). Effect of growth phase and acid shock on *Helicobacter pylori* cagA expression. * Infect. Immun.* 64, 4501–4507.

Kedzierska, B., and Hayes, F. (2016). Emerging roles of toxin-antitoxin modules in bacterial pathogenesis. *Molecules* 21:E790. doi: 10.3390/molecules2107E790

Kellberg, D., and Ottemann, K. M. (2016). How *Helicobacter pylori* senses, targets and interacts with the gastric epithelium. *Environ. Microbiol.* 18, 791–806. doi: 10.1111/1462-2920.13222

Khan, S., Karim, A., and Iqbal, S. (2009). Helicobacter urease: niche construction at *Helicobacter pylori* Cárdenas-Mondragón et al. Expression of Type II TA Genes in *Khan, S., Karim, A., and Iqbal, S. (2009). Helicobacter urease: niche construction at *Helicobacter pylori* Cárdenas-Mondragón et al. Expression of Type II TA Genes in *Khan, S., Karim, A., and Iqbal, S. (2009). Helicobacter urease: niche construction at *Helicobacter pylori* Cárdenas-Mondragón et al. Expression of Type II TA Genes in *Khan, S., Karim, A., and Iqbal, S. (2009). Helicobacter urease: niche construction at *Helicobacter pylori* Cárdenas-Mondragón et al. Expression of Type II TA Genes in
