World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i32.4323

World J Gastroenterol 2012 August 28; 18(32): 4323-4334
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Screening *Helicobacter pylori* genes induced during infection of mouse stomachs

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Supported by Intramural Research Program of the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Disease; The Division of Intramural Research of the National Institute of Allergy and Infectious Diseases; An Inter-Agency Agreement (Y3-DK-3521-07) with the National Institute on Minority Health and Health Disparities
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Received: June 9, 2012 Revised: July 30, 2012
Accepted: August 3, 2012
Published online: August 28, 2012

Abstract

AIM: To investigate the effect of *in vivo* environment on gene expression in *Helicobacter pylori* (*H. pylori*) as it relates to its survival in the host.

METHODS: *In vivo* expression technology (IVET) systems are used to identify microbial virulence genes. We modified the IVET-transcriptional fusion vector, pIVET8, which uses antibiotic resistance as the basis for selection of candidate genes in host tissues to develop two unique IVET-promoter-screening vectors, pIVET11 and pIVET12. Our novel IVET systems were developed by the fusion of random Sau3A DNA fragments of *H. pylori* and a tandem-reporter system of chloramphenicol acetyltransferase and beta-galactosidase. Additionally, each vector contains a kanamycin resistance gene. We used a mouse macrophage cell line, RAW 264.7 and mice, as selective media to identify specific genes that *H. pylori* expresses *in vivo*. Gene expression studies were conducted by infecting RAW 264.7 cells with *H. pylori*. This was followed by real time polymerase chain reaction (PCR) analysis to determine the relative expression levels of *in vivo* induced genes.

RESULTS: In this study, we have identified 31 *in vivo* induced (*ivi*) genes in the initial screens. These 31 genes belong to several functional gene families, including several well-known virulence factors that are expressed by the bacterium in infected mouse stomachs. Virulence factors, vacA and cagA, were found in this screen and are known to play important roles in *H. pylori* infection, colonization and pathogenesis. Their detection validates the efficacy of these screening systems. Some of the identified *ivi* genes have already been implicated to play an important role in the pathogenesis of *H. pylori* and other bacterial pathogens such as *Escherichia coli* and *Vibrio cholerae*. Transcription profiles of all *ivi* genes were confirmed by real time PCR analysis of *H. pylori* RNA isolated from *H. pylori* infected RAW 264.7 macrophages. We compared the expression profile of *H. pylori* and RAW 264.7 coculture with that of *H. pylori* only. Some genes such as *cag*A, *vac*A, *lpx*C, *mur*I, *tlp*C, *trx*B, *sod*B, *tnp*B, *pgi*, *rbf*A and *inf*B showed a 2-20 fold upregulation. Statistically significant upregulation was obtained for all the above mentioned genes ($P < 0.05$). *tlp*C, *cag*A, *vac*A, *sod*B, *rbf*A, *inf*B, *tnp*B, *lpx*C and *mur*I were also significantly upregulated ($P < 0.01$). These data suggest a strong correlation between results obtained *in vitro* in the macrophage cell

line and in the intact animal.

CONCLUSION: The positive identification of these genes demonstrates that our IVET systems are powerful tools for studying *H. pylori* gene expression in the host environment.

Key words: *Helicobacter pylori*; *In vivo* expression technology; Virulence genes; Mice; Infection

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Singh A, Hodgson N, Yan M, Joo J, Gu L, Sang H, Gregory-Bryson E, Wood WG, Ni Y, Smith K, Jackson SH, Coleman WG. Screening *Helicobacter pylori* genes induced during infection of mouse stomachs. *World J Gastroenterol* 2012; 18(32): 4323-4334 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i32/4323.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i32.4323

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that infects a large percentage (50% to 90%) of the world's population and is a causative agent for gastritis, ulcer disease and some gastric cancers. To date, the mechanism of *H. pylori* pathogenesis is not completely understood. *H. pylori* can infect and survive in the stomachs of mice and macrophages. *H. pylori* infection can last for a lifetime, suggesting that the microbes successfully evade the host immune response to infection. Although previously considered an extracellular organism, several recent *in vitro* studies suggest that *H. pylori* may be a facultative intracellular bacterium[1]. The intracellular habitation offers a plausible explanation for the evasion of host immune response and thus a life-long persistence in the host.

Characterization of microbial genes that are specifically induced during infection is critical to the understanding of the mechanism by which microbial pathogens cause disease. Many different techniques have been developed to study bacterial genes that are expressed during growth in specific niches[2-4]. A useful tool for identifying genes involved in virulence is *in vivo* expression technology (IVET)[5,6]. IVET was designed to identify genes of pathogens that are preferentially expressed during infection and has been used extensively[7,8]. It is a promoter trapping strategy used for identifying bacterial promoters that are upregulated in the host by using an auxotrophic mutant strain or with various reporter systems. This technique allows the identification of genes that may be expressed only under *in vivo* conditions. Such genes are difficult to identify during growth under laboratory conditions, but are likely to play an important role in survival inside the host. This technology has not been exhaustively utilized in *H. pylori* because of limitations imposed by the genetic intractability of this bacterium. Recently, recombination-based IVET has been utilized to identify *H. pylori* genes important for host colonization[9]. In this study, we developed an antibiotic-based IVET tool (a variant of IVET[10]) that is specific for screening *H. pylori* genes that are specifically expressed *in vivo* in mice and macrophage hosts.

MATERIALS AND METHODS

Bacterial strains and growth media

All bacterial strains used in this study are listed in Table 1. The *H. pylori* strains used in this study were: Sydney strain 1 (SS1)[11] and strain HP1061[12]. The strains were grown for 16 h to 18 h at 37 ℃ in a microaerophilic atmosphere in bisulfiteless Brucella broth (BLBB)[13] containing 5% fetal bovine serum (Hyclone, Logan, UT). For BLBB solid medium, 1.7% agar was added. Unless stated otherwise, the antibiotics used in BLBB solid or liquid medium were: kanamycin (kan) 15 mg/L, Glaxo Selective Supplement A (GSSA) (5 mg/L of Amphotericin-B, 20 mg/L of Bacitracin, 1.07 mg/L of Nalidixic acid, 0.33 mg/L of Polymyxin-B, and 10 mg/L of Vancomycin)[14]. BLBB 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates were supplemented with X-gal at 40 mg/L. *Escherichia coli* (*E. coli*) strains, TAM1λ pir and DH5α λ pir were grown in L broth (LB) medium[15].

Animal housing and diet

Mice were maintained in a National Institutesof Health (NIH) animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Rockville, MD).

They were maintained in a specific-pathogen-free animal care holding room and were confirmed to be free of the following microorganisms: cilium-associated respiratory bacillus, ectromelia, mouse rotavirus, mouse encephalomyelitis virus, lymphocytic choriomeningitis virus, murine cytomegalovirus, mouse hepatitis virus, mouse adenovirus, minute virus of mice, *Mycoplasma pulmonis*, parvovirus, polyomavirus, pneumonia virus of mice, reovirus, and Sendai virus. Mice were housed in 7.5- by 11.5- by 5-in. sterilized ventilated Thoren cages (Thoren Caging System, Inc., Hazleton, PA) on Tek Fresh bedding (Harlan Teklad, Madison, WI). Cages were changed weekly. The animal holding room was maintained under environmental conditions of 20 ℃, 40% to 70% relative humidity, 15 air changes/h and a 12-h/12-h light-dark cycle. Mice were fed an autoclaved pelleted rodent diet (rodent NIH-31 autoclavable NA; Zeigler Brothers, Inc., Gardners, PA) ad libitum and provided sterilized individual water bottles for an ad libitum water source. Upon arrival, the mice were acclimated for a minimum of 7 d prior to being used in the experiments. Mice were identified by numerical stainless steel rodent ear tags (National Band and Tag Co., Newport, KY). This study was

Table 1 Strains and plasmids used in this study

Strains or phenotype	Relevant genotype or plasmids	Source or reference
Helicobacter pylori		
SS1		Lee *et al*[11]
HP1061		Goodwin *et al*[12]
Escherichia coli		
DH5αλpir$^+$	*λpir*, *sup*E44, Δ*lac*U169(φ80*lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1	Stanley Maloy, San Diego State University
TAM1 λpir$^+$	*Λpir*, *mcr*A, Δ(*mrrhsd*RMS*mcr*BC), φ80*lacZ*ΔM15, Δ*lac*X74, *rec*A1, *ara*Δ139(*ara-leu*)7697, *gal*U, *rps*L, *end*A1, *nup*G	Active Motif, Inc., Carlsbad, CA 92008
Plasmids		
pIVET8		Slauch *et al*[52]
pIVET9		This study
pIVET10		"
pIVET11		"
pIVET12		"

SS1: Sydney strain 1.

reviewed and approved by the NIH Institutional Animal Care and Use Committee. All procedures and use of animals were in compliance with the Public Health Service Guide for the Care and Use of Laboratory Animals[16].

Isolation of DNA from bacterial strains

Plasmid DNA was isolated from *E. coli* using the QIAprep Miniprep or QIAfilter plasmid maxi kit (QIAgen, United States) in accordance with manufacturer's recommended protocols. Genomic DNA was extracted from *H. pylori* strains, SS1 and 1061 using the Wizard Genomic DNA Purification Kit (Promega, United States) as described by the manufacturer.

Plasmid construction

Two novel *H. pylori* specific plasmids, pIVET11 and pIVET12 (Figure 1.) were constructed by modifying plasmid pIVET8[10]. pIVET8 is a suicide vector containing *oriR6K* origin that requires, in trans, a host-encoded Pi protein for replication[17-19]. It also contains an ampicillin resistance gene and a promoterless *cat* and *lacZY* gene fusion. The gene encoding kanamycin resistance was amplified by PCR from pCR Ⅱ (Stratagene), with primers KanF and KanR (Table 2). The amplified fragment was cloned at the *Aat* Ⅱ site of pIVET9. A *Bgl* Ⅱ restriction site in the kanamycin sequence was removed by Quick change site-directed mutagenesis kit (Stratagene). In so doing, we produced pIVET 10 which contained the unique *Bgl* Ⅱ cloning site immediately upstream of promoterless *cat* and *lacZY* genes. We produced pIVET11 by removing the mob RP4 sequence which accounted for the conjugal transfer functions necessary for mobilization. Finally, pIVET12 plasmid was generated by the removal of ampicillin gene by *Bsph1* restriction and the transfer of the kanamycin gene from the *Aat* Ⅱ site to a unique *Bam*HI site in pIVET11. The kanamycin gene sequence, inserted at the *Aat* Ⅱ site in pIVET11, disrupts *lacZ* gene without affecting its activity. *H. pylori* is sensitive to ampicillin and therefore we could not use this antibiotic in our system.

Construction of genomic library

H. pylori Sydney strain 1 (SS1) genomic DNA was isolated using the Wizard Genomic DNA purification kit (Promega, United States). The genomic DNA was partially digested with *Sau3*AI. After agarose gel electrophoresis, fragments of 1-3kb were purified using QIAquick Gel Extraction Kit (Qiagen, United States) and ligated into *Bgl* Ⅱ digested and dephosphorylated pIVET11 or pIVET12 using the Rapid DNA Ligation kit (Roche, United States). Ligation samples were transformed into DH5α λ pir competent cells which provided the Pi protein, in trans, for plasmid replication. Transformants were replica plated in a 6 × 8 pattern on LB agar plates containing kanamycin. Forty eight colonies from the replica plates were pooled in TE buffer, pH 8.0 and plasmid DNA purification was carried out using the QIAprep Miniprep kit (QIAGEN, United States).

Preparation of electro-competent H. pylori

An overnight-grown 100 mL *H. pylori* culture was chilled for 10 min on ice. The cells were pelleted at 4360 × *g* for 5 min. The centrifugations and all subsequent procedures were done at 4° C. The pellets were washed three times, twice with 40 mL ice cold water and finally with 40 mL ice cold water containing 5% glycerol. The washed pellets were placed on ice, covered with 2 mL ice cold 10% glycerol, incubated for 10 min and later resuspended in the same solution. 200 μL samples were placed in cold screw capped tubes and after quickly freezing on dry ice samples were stored at -80 ℃.

Electrotransformation of H. pylori and merodiploid selection

Electro-competent *H. pylori* strain 1061 was transformed with 0.5-2 μg recombinant plasmid DNA pools by electroporation (12.5 ms, 2.5 kV, 25 μF, 600 ohm, 0.4 cm gap, Bio-Rad gene pulser, United States). Electrotransformed *H. pylori* were screened for kanamycin resistance on BLBB GSSA Kan agar plates. Selection for kanamycin resistance requires the integration of the recombinant plasmids into the chromosome by homologous recombination, using the cloned Helicobacter DNA as the source of homology (wild type *H. pylori* does not have *cat* and *lacZY* genes). This integration event duplicates a small portion of the *H. pylori* genomic DNA leading to the generation of a merodiploid. Kanamycin resistant colonies/merodiploids were replica plated in a 6 × 8 pattern on BLBB GSSA Kan agar plates. After 72 h of microaerophilic incubation, forty eight colonies from the replica plates were pooled in 1 mL of BLBB, GSSA, and Kan. This suspension was inoculated in 10 mL BLBB, GSSA, Kan and grown under microaerophilic conditions. After 24 h of incubation, cultures were subcultured 1:100 in 10 mL of BLBB, GSSA,

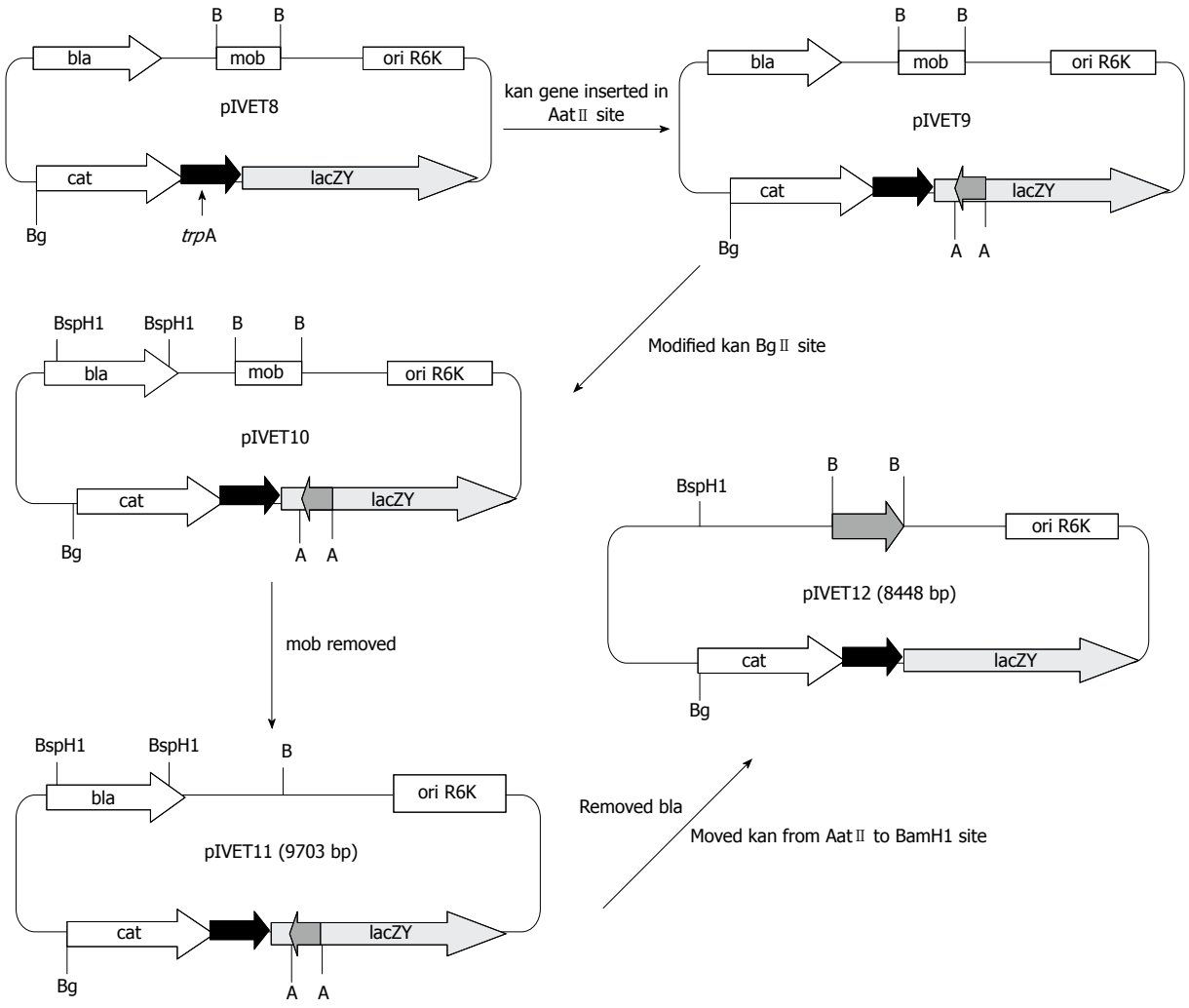

Figure 1 Construction of *Helicobacter pylori* specific antibiotic-based *in vivo* expression technology plasmids, pIVET11 and pIVET12. These plasmids are derivatives of plasmid pIVET8[10]. A: *Aat* II; B: *Bam*HI; Bg: *Bgl* II; bla: β-lactamase; kan: Kanamycin gene; mob: Plasmid mobilization; trpA: Tryptophan synthase α-subunit.

Kan and grown for 16-18 h under microaerophilic conditions. These resulting merodiploids were used to infect mice or macrophage cultures (see below).

Inoculation of mice with H. pylori merodiploids

Six to eight week old *Helicobacter* and pathogen-free female C57BL/6 mice (Jackson Laboratory Maine, United States) were used, in compliance with guidelines and protocol approved by the Animal Care and Use Committee of the National Institutes of Health, United States. Using a 20-gauge ball-point metal feeding tube (Harvard Apparatus, Inc., Holliston, MA), mice were inoculated intragastrically with 0.1 mL of *H. pylori* merodiploid pooled cultures (10^8 colony-forming-units per milliliter-CFU/mL) once a day every other day for a total of three inoculations. Each pair of mice was inoculated with a fresh 18 h culture. Control mice were inoculated with BLBB medium.

Isolation and analysis of DNA from fecal pellets

Five days post-infection, fecal pellets were collected from the infected and control mice. DNA was isolated from fecal pellets (2 pellets per mouse) by using the QIAamp DNA stool mini kit (QIAGEN, United States). DNA samples extracted from the fecal pellets were analyzed by PCR as done previously[20].

Selection for chloramphenicol resistant H. pylori in mice

Seven days post-infection, mice were treated with chloramphenicol. 100 μL chloramphenicol (0.9 g/L, apple flavored, Foer's Pharmacy, United States) was given to each mouse by oral gavage twice a day for three days. Chloramphenicol was also added to the water in the mouse cages to a concentration of 0.1 g/L. Following the last chloramphenicol treatment, the mice were sacrificed, and the stomachs were excised.

H. pylori IVET selection in mouse stomachs

The excised stomach was cut into two longitudinal sections. One half was added to 1 mL BLBB medium containing 20% glycerol and frozen at -80 ℃. The other half of the stomach was added to 1 mL BLBB Kan medium

Table 2 Primers used in this study

Genes	Primer sequences 5'-3'
HP1	CTGGAGAGACTAAGCCCTCC
HP2A	AACATTACTGACGCTGATTG
Kan AatⅡ F	GATTTAGACGTCTCAGGGCGCAAGGGCT
Kan AatⅡR	TTCCTTTGACGTCTCAGAAGAACTCGTCAA
Kan BglⅡ F (Mut)	AGG GGA TCA AGG TCA GAT CAA GAG A
Kan BglⅡ R (Mut)	TCT CTT GAT CTG ACC TTG ATC CCC T
MCS BamHI F (Mut)	GTC GAC CGA CCC CAA GCT TCT AGA GGT ACC G
MCS BamHI R (Mut)	CGG TAC CTC TAG AAG CTT GGG GTC GGT CGA C
Kan BamHI F (pIVET12)	GAT TTA GGA TCC TCA GGG CGC AAG GGC T
Kan BamHI R (pIVET12)	TTC CTT TGG ATC CTC AGA AGA ACT CGT CAA
CAT	CAA CGG TGG TAT ATC CAG
MCAT	GCC ATT GGG ATA TAT CAA CGG TGG TAT ATC C
NMCAT	CTC CAT TTT AGC TTC CTT AGC TCC TGA AAA TCT CG
CagA F	GATGGCGTGATGTTTGTTGATT
CagA R	CGTAGCACCCACACCCTTATC
CysS F	TGTTATTCCCCCACCATGAAA
CysS R	GCAAGCTCCACGCCAAAG
Flhf F	TGTTGCCCTGAAGATTTGAATTT
Flhf R	TAGCGCGCGGCTAATTTAG
FliA F	AAGCAACAACACCACCATCAAG
FliA R	GCTGGGCAAGCGCTCTT
FliM F	CGCCAGGTGATGCAAATTT
FliM R	AGCGACGATTTGGACCACAT
FucT F	TGAATGTGCATGATTTCAACAACT
FucT R	GCTTTCCCATCAAGGGTGTTT
HsdM F	CTGGGACATTCAAATCAATTATGG
HsdM R	CGTCTTGCAAGCGTTTAAGATCT
InfB F	GAGTCGCTCACACGGAAGCT
InfB R	CAATGAAAGACACCCATTTGTCA
LpxC F	CAAGCCCATCATTTTGTCTTTAGTAA
LpxC R	CAAGTAATTCACTTCTTGCAAAAACC
MinC F	TGCGCAAACACCAGCTTTT
MinC R	ATGGCGCTCATAAATCGTTGT
MurC F	GGCTCATGGAAAGAGCAGTATCA
MurC R	GCCCCAATAATCGCTCCAA
MurD F	GCGGCACTAACGGGAAAA
MurD R	CCCACTCACAGCCTTAAAATCTTC
MurI F	AAAAGCGACGATTCAATCCAA
MurI R	AAGTAGCTAAATGCGAAATGTTCAAA
Nth F	TGAATTATTGGTGGCGACCAT
Nth R	TTGGGCGTTATTTGATTCACTCT
Omp26 F	CAAATCGGCACCGTTACCA
Omp26 R	CATGAAATCCCGCTAGAAGCA
Pgi F	AGCCCACCACAGGGTGTTT
Pgi R	CTCCAATATTGCTTGGTGAAATCTT
PriA F	GGAGGAGCGCTAGGCAAAT
PriA R	AGTTCGCACCTTTCTTGCAATT
RbfA F	GCGAGTTGAATTCTTTAAGCGTTAC
RbfA R	GACCCTCAGCTTTTTTCAATTTAGA
RecG F	GCGATCAACAAAACGCCATTA
RecG R	GCACCCCACATCGCCTATAA
SodB F	TGCTAAAGACAGCATGGGAGATT
SodB R	TTCACATAAGTTTGATGGTGTTTCC
TlpC F	TGGTTAGCGCGATTTATACGAA
TlpC R	GGGTAGGCGATTTTTGAATCC
TnpB F	TTGGTGTTTAGAATGCGGGTATAG
TnpB R	TTTCCTACCCCAAAAAGACTTAACC
TrxB F	CGCCATTGCTTTGTGCAA
TrxB R	TGATGCGGCTGAATTCTTTTT
Type Ⅱ R / M F	TTCTATAACAGCACCGCTGACATT
Type Ⅱ R / M R	CGCGTATATTTGTTAGAAGTGATGAAA
VacA F	GGGTTATGCCAGACAAATGATTG
VacA R	TCTTATGCTCTAAACTGGCTATGTTGTT
VirB4 F	GACCATAGCCCTTATTTGTTTAATTTTC
VirB4 R	CTCCTATAATCATGGTATGTCCCACTAC
Ycf5 F	AGCGCTATCAATGAAATGAGCAT
Ycf5 R	CCACACCCCGCCTAAGAAA
HP0423 F	TCGCTCCTTAAGGTTACACGATT
HP0423 R	TCAAAGCCACCATCAATAACAAA
HP0424 F	CGCTGTTTTTAGTTTTAGAGGCTTTC
HP0424 R	GGCTAGGGAAGTGGCTCAAA
HP0426 F	TGCGGTATAGGCTTTCATGAACT
HP0426 R	AAGGTGTTCAAAGACAGCAAAAAA
HP0427 F	TTGCGGTGTGGGTTAATGAA
HP0427 R	GCAACGCTACCATACTTTTATCACTT

and homogenized with a sterile motorized Polytron homogenizer (Kinematica AG, United States). The resulting homogenate was spread on BLBB, GSSA, Kan plates at 10, 100 and 1000 fold dilutions. The plates were incubated under microaerophilic conditions at 37 ℃ for 3-4 d. Kanamycin resistant colonies were replica plated in a 6 × 8 pattern on BLBB GSSA Kan agar plates. Forty eight colonies from the replica plates were pooled and grown overnight for the second round of mice infections.

H. pylori IVET selection in macrophages

The IVET selection in macrophage was performed as described previously with some modifications[10]. The mouse macrophage cell line, RAW 264.7 was grown in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) supplemented with 10 % heat-inactivated fetal calf serum (FCS; Gibco-BRL) and GSSA antibiotics at 37 ℃ in a 50 mL/L CO_2 humidified atmosphere. The day before each *in vivo* selection assay RAW 264.7 cells were seeded in 24-well tissue culture plates to 2×10^5 cells per well. *H. pylori* merodiploid pools were grown for 16 h to 18 h under microaerophilic conditions at 37 ℃ in BLBB Kan medium. 30 μL of the bacterial cultures were added per 1 mL of DMEM medium. The monolayers were infected with 1 mL of the bacteria suspension and centrifuged for 5 min at 600 r/min to synchronize bacterial contact with the monolayers[21,22]. The infected macrophage monolayers were incubated for 2 h at 37 ℃ in a microaerophilic atmosphere. The monolayers were washed three times with phosphate buffered saline (PBS) and then were incubated with 2 mL of DMEM containing 100 mg/L gentamicin for 2 h at 37 ℃ in a microaerophilic atmosphere to kill extracellular bacteria. Following extracellular killing, the monolayers were washed three times with 2 mL of PBS and then the infected cells were incubated overnight in DMEM containing 1 mg gentamicin/L and 20 mg chloramphenicol/L. After the incubation period the macrophage-monolayers were washed three times with PBS and then lysed by adding 1 mL of sterile water per well. The resulting lysate was spread on BLBB, GSSA, Kan plates at 10, 100 and 1000 fold dilutions. The plates were incubated under microaerophilic conditions at 37 ℃ for 3-4 d and the kanamycin resistant colonies were pooled and grown

overnight for the second round of infections.

Screening of chloramphenicol resistant H. pylori for β-galactosidase expression in vitro

Kanamycin resistant colonies recovered from the stomach homogenates and RAW 264.7 cell lysates were replica plated in a 6 × 8 pattern on BLBB GSSA Kan X-gal plates. These plates were then incubated under microaerophilic conditions at 37 ℃ for 24 h, and screened for blue or white color. White colonies were used to inoculate BLBB, GSSA medium for preparing genomic DNA.

Identification of ivi gene fusions

The genomic DNA samples prepared from white colonies were tested for the presence of *H. pylori* 16S DNA[20]. The presence of pIVET11/pIVET12 in the genome of co-integrate strains was confirmed by PCR analysis using KanF and KanR primers. To sequence regions of genomic DNA flanking the inserted plasmid, we performed Vectorette PCR according to the manufacturer's instructions (Sigma-Genosys). Genomic DNA from the co-integrate strains was digested separately with *Eco*RI, *Bam*HI and *Hin*DⅢ. Following this, compatible vectorette linkers were ligated to the ends of the genomic DNA fragments and PCR was then performed using a primer (MCAT) homologous to the 5' end of *cat* gene in pIVET11/pIVET12 and a primer unique to the vectorette linker. The resulting PCR products were sequenced using MCAT primer or cloned into pSC-A-amp/kan, PCR AU cloning vector (Stratagene, United States) and sequenced subsequently.

RNA isolation

RAW 264.7 macrophages were infected with *H. pylori* as described above and incubated for 2 h at 37 ℃ in a microaerophilic atmosphere. An identical amount of *H. pylori* was added to a flask without RAW 264.7 cells and incubated in the same way as the *H. pylori*-RAW 264.7 cell coculture. A non-infected flask of RAW 264.7 cells served as a negative control for RNA isolation to ensure that no contaminating signals derived from eukaryotic RNA were present. After 2 h of incubation, the *H. pylori*-RAW cell coculture was washed three times with 2 mL of PBS to remove extracellular *H. pylori* cells. Finally, the *H. pylori* infected RAW cell cultures were incubated in 2 mL of medium containing 100 mg/L gentamicin for 2 h at 37 ℃ in a microaerophilic atmosphere to kill extracellular bacteria. Following extracellular killing, the coculture was washed three times with 2 mL of PBS and then the infected cells were treated with the TRIzol reagent for RNA isolation as described by the manufacturer (Invitrogen, United States). Further RNA purification was performed with an RNeasy mini kit (Qiagen, United States). The culture containing only *H. pylori* was centrifuged and washed twice with phosphate buffered saline, and TRIzol was directly applied to the pellet and the preparation was subsequently treated in the same fashion as *H. pylori* and RAW cells coculture described above for RNA purification.

Separation of eukaryotic and prokaryotic mRNA

H. pylori RNA from coculture was enriched by removal of the eukaryotic 18S and 28S rRNAs and polyadenylated mRNAs using the MICROBEnrich kit (Ambion) according to manufacturer's instructions.

Real time PCR

Primers were designed for 100-150 bp regions of *in vivo* induced genes obtained after sequencing (Table 3). Primer design was aided by Primer Express 3.0 software (Applied Biosystems, United States). Standard PCR was performed with *H. pylori* SS1 genomic DNA as the template to check that all the primer pairs resulted in the amplification of a single product. RNA was reverse transcribed using Superscript Ⅲ first strand synthesis system for RT-PCR (Invitrogen, United States). Real-time PCR was done using Power SYBR Green PCR Master mix (Applied Biosystems, United States). 16s RNA was used in each set of reaction for normalization. Each reaction was repeated thrice with three independent RNA samples in an Applied Biosystems 7500 real time PCR system. Melt curve analysis was done to confirm the specificity of the amplified product. Relative expression levels were determined using the 2-deltadelta Ct method[23]. Results were expressed as fold induction of expression in *H. pylori* and RAW 264.7 coculture as compared to *H. pylori* only.

Statistical analysis

Data were presented as mean ± SE in Microsoft Excel. Differences between the *H. pylori* alone, and *H. pylori* and RAW 264.7 co-culture group means were analyzed by the Student's *t* test. The threshold significance level for the mean difference between groups was $P < 0.05$.

RESULTS

Construction of H. pylori specific IVET vectors and vector validation with known H. pylori promoter

As a means to identify Helicobacter promoters specifically expressed under defined conditions, we have successfully developed two promoter trap vectors, pIVET11 and pIVET12 (Figure 1). These vectors are suicide vectors containing *oriR6K* origin that requires a host-encoded Pi protein, in trans, for replication. They contain two promoterless reporter genes, one encoding the *cat* gene and the other *lacZY* genes. These two genes are organized into a single transcription unit located immediately downstream from a unique *Bgl*Ⅱ site, allowing for the cloning of promoter libraries. In Figure 2, we summarized an overview of the IVET strategy used in these studies. To validate the *H. pylori* specific promoter trap vector system, we placed the *cat-lacZY* fusion of pIVET11 under the control of the *H. pylori vacA* promoter. We transformed the resulting plasmid, pIVET11/vacA, through electroporation into electro-competent *H. pylori* strain 1061. Transformed *H. pylori* were screened

Table 3 *Helicobacter pylori in vivo* induced genes

	Locus	Function or enzyme	Role in virulence
ivi-232	flhF	Regulation of flagella biosynthesis	Motility
ivi-122	fliA	Flagellar motor switch protein	
ivi-121	fliM	Flagella motor switch protein	
ivi-364	tlpc	Methyl-accepting chemotaxis protein	
ivi-11	trxB	Thioredoxin reductase	Oxidative stress protection
ivi-134	nth1	Endonuclease Ⅲ	
ivi-322	ycf5	Cytochrome C biogenesis protein	
ivi-352	sodB	Iron-dependent superoxide dismutase	Acid resistance
ivi-161	hsdM	DNA methyltransferase	Nucleic acid metabolism
ivi-162	hsdM/R	Type II restriction enzyme R and M protein	
ivi-123	recG	ATP-dependent DNA helicase	
ivi-351	priA	Primosome protein replication factor	
ivi-361	tnpB	IS606 Transposase	
ivi-171	lpxC	UDP-3-O-acyl N- acetylglucosamine deacetylase	Cell envelope structures
ivi-172	minC	Septum site directing protein	
ivi-1722	murD	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthase	
ivi-1721	murC	UDP-N-acetylmuramoyl alanine-D-glutamate ligase	
ivi-363	murI	Glutamate racemase	
ivi-321	fucT	Alpha 1, 3-fucosyltransferase	
ivi-351	omp26	Outer membrane protein	
ivi-112	pgi	Glucose-6-phosphate isomerase	Sugar metabolism
ivi-1132	rbfA	Ribosome binding factor A	Translation and regulation
ivi-1131	infB	Translation initiation factor IF-2	
ivi-2721	cysS	Cysteinyl-tRNA synthetase	Protein and peptide synthesis
ivi-110	vacA	Vacuolating toxin	Virulence factors
ivi-362	cagA	Cytotoxin-associated gene	
ivi-192	virB4	Type IV secretion system	Transport cagA protein
ivi-241		Predicted coding region HP0426	*H. pylori* hypothetical protein
ivi-242		Predicted coding region HP0427	*H. pylori* hypothetical protein
ivi-3101		Predicted coding region HP0423	*H. pylori* hypothetical protein
ivi-3102		Predicted coding region HP0424	*H. pylori* hypothetical protein

H. pylori: *Helicobacter pylori*.

for kanamycin resistance (See materials and Methods). Selection for kanamycin resistance requires the integration of the recombinant plasmid into the chromosome by homologous recombination, where the source of homology is the cloned helicobacter DNA *vacA* promoter sequence. The integrated sequences partially duplicate the *H. pylori* genomic DNA, leading to the generation of a merodiploid. With this merodiploid, one copy of the promoter drives the expression of *cat-lacZY* fusion, while the other promoter copy drives the expression of a wild-type *ivi* gene. Kanamycin resistant merodiploids were used to infect mice and macrophages. After infection, choramphenicol resistant colonies were recovered and subjected to blue or white screening to analyze β-galactosidase expression *in vitro*. We identified 728 cm^R white colonies and analyzed their genomic DNA. The region of genomic DNA flanking the inserted plasmid was identified using the universal vectorette system as described by Sigma-Genosys. Sequence analysis of the PCR product showed the presence of the *vacA* promoter upstream of *cat-lacZY* fusion. These results indicate that the *vacA* promoter is capable of driving the expression of promoterless *cat-lacZY* genes *in vivo*. The expression results also serve to validate the efficacy of our promoter trap systems to detect and identify *H. pylori* promoters expressed *in vivo*.

IVET selection in mouse model

We generated IVET vectors that contained a library of *Sau3*AI digested *H. pylori* chromosomal DNA. These IVET vectors were transformed into *H. pylori* 1061 to generate a library of merodiploid (co-integrated) strains. These strains are plasmid recombinants characterized by integration into different loci of the *H. pylori* genome through homologous recombination. The recombinant strains along with wild type *H. pylori* strain were used to infect mice. Infected mice were then subjected to chloramphenicol treatment. Chloramphenicol effectively kills the intragastric wild type *H. pylori* strain. The surviving chloramphenicol resistant *H. pylori* are merodiploid which were recovered from the stomachs of mice infected with these strains. We pooled the resistant colonies and repeated the second round of infection in mice. Of a total of 702 merodiploid strains that survived chloramphenicol challenge, 38 (approximately 6%) were found to be negative for β-galactosidase activity during *in vitro* screens. In the pre-selection pool, 15% (86/596) of the *H. pylori* genomic DNA *cat-lacZY* fusions were $LacZ^+$ *in vitro* (light blue) and 85% (510/596) were $LacZ^-$ (white). In contrast, after two rounds of the antibiotic selection, 94% (664/702) were $LacZ^+$, and 6% (38/702) were $LacZ^-$. These $LacZ^-$ strains presumably carried gene fusions that expressed chloramphenicol transacetylase *in vivo* in order

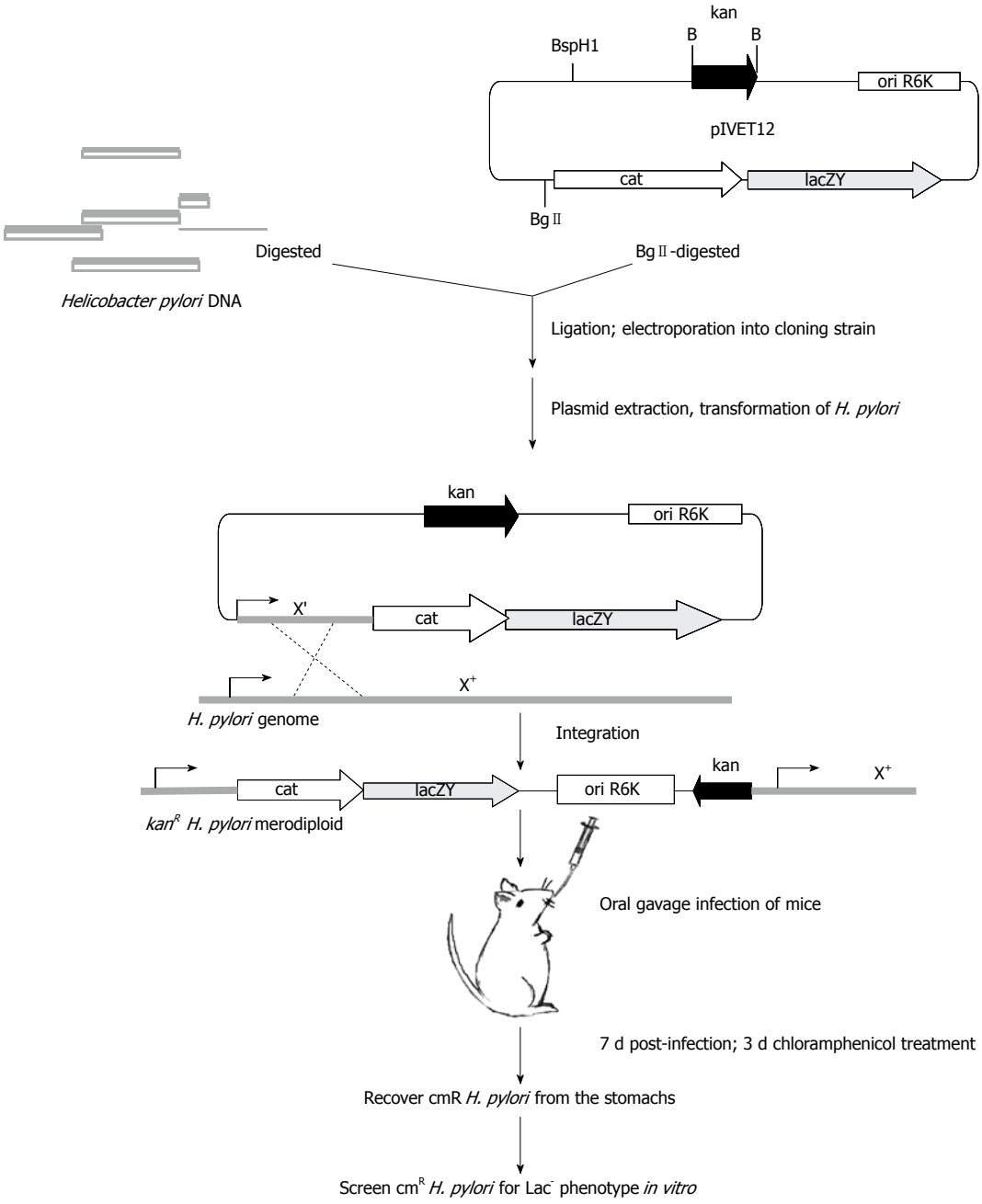

Figure 2 ***Helicobacter pylori*** **specific** ***in vivo*** **expression technology strategy.** *H. pylori*: *Helicobacter pylori*; B: *Bam*HI; Bg: *Bgl* II ; kan: Kanamycin gene.

to survive the systemic antibiotic treatment. However, the enzyme was expressed poorly when theses strains were grown *in vitro* on BLBB Kan X-gal medium. Operating on this premise, we focused our efforts on the characterization of these gene fusions because they may represent genes that are specifically induced *in vivo* (*ivi* genes).

IVET selection in cultured macrophages

RAW 264.7 macrophages were infected with *H. pylori* as described in the methods section. Chloramphenicol resistant bacteria were recovered from the lysates of RAW cells infected with merodiploid strains, but not from those infected with the wild type *H. pylori*. Chloramphenicol resistant colonies were pooled and the pools were used for the second round of infection of RAW 264.7 cells. Of a total of 231 merodiploid strains that survived chloramphenicol challenge, 15 (approximately 7%) were found to be inactive in β-galactosidase *in vitro* screens. In the pre-selection pool, 20% (41/206) of the *cat-lacZY* fusions were $LacZ^+$ *in vitro* (light blue) and 80% (165/206) were $LacZ^-$ (white). In contrast, after two rounds of chloramphenicol selection, 93% (216/231) were $LacZ^+$ and 7% (15/231) were $LacZ^-$. These strains likely contain fusions of *cat-lacZY* to *H. pylori* promoters which are active in macrophages but were not induced during *in vitro* growth.

Functional validation of IVET

To validate that the *in vivo* expressed pIVET11 and 12 proteins were under the control of *H. pylori* promoters,

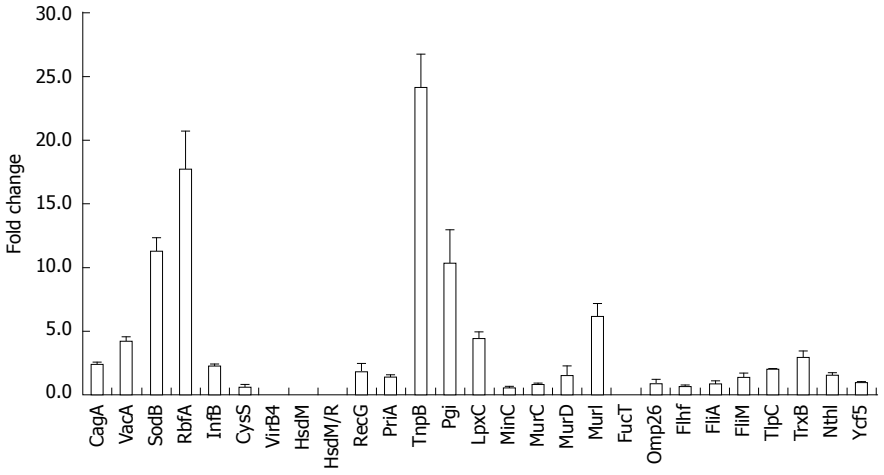

Figure 3 Gene expression of *Helicobacter pylori* induced by phagocytosis. Up-regulation and down-regulation of *Helicobacter pylori in vivo* induced genes expressed by macrophage engulfed bacteria.

we analyzed the nucleotide sequences and real time PCR results from infected mice (38 clones) and from infected RAW cells (15 clones). These clones contain the cat-lacZY reporter genes that are fused with genes in the *H. pylori* genome. The reporter genes should express in the host but not *in vitro*. Genomic DNA isolated from these clones was digested with *Eco*RI, *Bam*HI and *Hin*DⅢ and ligated to the compatible vectorette linkers. To sequence regions of genomic DNA flanking the inserted plasmid, we performed PCR using a primer homologous to the 5' end of *cat* gene in the IVET vectors and a primer unique to the vectorette linker. The resulting PCR products were sequenced directly or cloned and then sequenced.

Based on analysis of the nucleotide sequences of the individual inserts and comparison with the annotated genes of *H. pylori* in the GenBank database, we identified 31 genes. The list of the genes is shown in Table 3. The 31 genes included genes for virulence, cell envelope structures, motility, oxidative stress, nucleic acid and sugar metabolism, translation, protein synthesis and type Ⅳ secretion system. Four *ivi* conserved gene sequences did not show significant homology with any known genes in the Genbank database.

The real time PCR primers used to screen for *ivi* genes were tested using standard PCR conditions. Using *H. pylori* SS1 genomic DNA as the template, we found that only single PCR products resulted from each primer set. RNA was isolated from: (1) RAW 264.7 and *H. pylori* coculture; (2) *H. pylori* only; and (3) RAW 264.7 cells only. No product was obtained with the RNA of RAW 264.7 cells alone, confirming that there was no cross-reactivity that might have confounded the interpretation of data. Using 16s RNA as an internal control, we compared the expression profile of *H. pylori* and RAW 264.7 coculture with that of *H. pylori* only. The expression levels of genes differed from those observed in mice. We observed a 2-20 fold upregulation in *cag*A, *vac*A, *lpx*C, *mur*I, *tlp*C, *trx*B, *sod*B, *tnp*B, *pgi*, *rbf*A and *inf*B (Figure 3). In contrast, *hsd*M, *hsd*M/R, *fuc*T, *vir*B4, HP0426 and HP0427 were not upregulated. The expression levels of the remaining *ivi* genes remained the same.

DISCUSSION

Characterization of microbial genes that are specifically induced during infection is important to the understanding of the mechanisms by which microbial pathogens cause disease. Intracellular pathogens have to evolve strategies to overcome the unfavorable environment met inside the host, which is very different in a culture broth outside the host. *H. pylori* colonizes the gastric mucosa during infection and synthesizes defense molecules to survive in the acidic gastric environment. Therefore, it is important to identify the genes of *H. pylori* that are upregulated in the intracellular environment of the host.

IVET has previously been attempted in *Salmonella typhimurium, Vibrio cholerae* and *Pseudomonas aeruginosa*[5,10,24-27]. In the present investigation, an antibiotic-based IVET has been applied in *H. pylori* for the first time. Novel *H. pylori* specific plasmids, pIVET11and pIVET12 (Figure 1), were constructed by modifying the plasmid pIVET8[10] and then used to construct the *H. pylori* library. Although this library does not contain the entire *H. pylori* genome, it will likely give insight into the type of genes upregulated and hence necessary for the bacterium to evade host immune defenses. On the basis of chloramphenicol selection, 31 genes were identified (Table 3). These include genes responsible for a broad and varied group of cellular structures and functions: virulence, cell envelope structures, motility, oxidative stress, nucleic acid and sugar metabolism, translation, protein synthesis, type Ⅳ secretion system and few conserved and hypothetical proteins. Virulence genes such as *cagA* and *vacA* were induced and upregulated *in vivo*. *CagA* is translocated into gastric epithelial cells and induces numerous alterations in cellular signaling[28-30]. Several *H. pylori* factors are known to interact directly with immune cells and modulate immune responses to *H. pylori*. One of these factors is *vacA* which

alters the function of T lymphocytes, B cells, macrophages and mast cells[31,32].

H. pylori null mutant strains defective in the production of flagella are unable to colonize animal models[33]. Flagella facilitate bacterial motility resulting in bacterial penetration of the mucus layer. Hence, upregulation of *flhf*, a global regulator of flagella biosyntheis and *fli*A, *fli*M, flagella motor switch proteins and *tlp*C, a methyl accepting chemotaxis protein is significant.

During host infection, animal pathogens are exposed to reactive oxygen species, such as superoxides, hydrogen peroxides, or organic peroxides, as a result of the release of lysosomal contents within inflammatory cells[34]. In our IVET screen, proteins involved in oxidative stress protection, *trx*B, thioredoxin reductase, *sod*B, iron-dependent superoxide dismutase, nth1, endonuclease Ⅲ and *ycf*5, cytochrome c biogenesis were upregulated. Thioredoxins have been implicated in a variety of physiological processes and biological pathways. In addition, they play a role in defense against oxidative stress, either by reducing protein disulfide bonds produced by various oxidants or by scavenging reactive oxygen species[35]. Superoxide dismutase has been demonstrated to play an important role in oxidative stress defense mechanisms to counter iron-promoted DNA damage in *H. pylori*[27].

Bacterial surface structures (adhesins, pilins, lipopolysaccharide, capsules) are often involved in direct contact with host cells, signaling molecules, and or immune defenses (e.g., antibody). Hence the production and/or modification of many of these surface structures *in vivo* is often hypervariable in order to facilitate dissemination and to avoid immune defense mechanisms[36]. In our system, several cell envelope structure-related proteins were identified. These included *fuc*T, *lpx*C, *min*C, *mur*D, *mur*C, *mur*I, and *omp*26.

Our IVET screening revealed the host-induced expression of several genes involved in nucleic acid metabolism, including *hsd*M/R, *hsd*M, *rec*G, *pri*A and *tnp*B (encodes the *H. pylori* IS606 transposase). This class of host-induced genes is involved in DNA synthesis and modification. Bacterial type Ⅱ restriction-modification systems involve a restriction endonuclease and, a methyltransferase[37,38]. The coordinated action of these enzymes mimics primitive immune defense mechanisms and protects bacterial cells from foreign DNA invasion[39,40]. In addition, DNA methylation may play a role in gene regulation by inhibiting the interaction between regulatory proteins and their target DNA sequences[41]. It may also be involved in the regulation of chromosomal DNA replication and gene expression[42], transposon movement[43], or DNA mismatch repair[44]. A potential role for *rec*G in recombination and in the rescue of stalled replication forks has been suggested[45-47]. Additionally, recent studies suggest that *rec*G provides a more general defense against pathological DNA replication[19]. Cells lacking *pri*A show a reduced viability and an increased sensitivity to DNA damage, phenotypes that are generally attributed to the deficiency in rescuing stalled or damaged forks[48]. Genes involved in transposition have been upregulated in microorganisms during interaction with a eukaryotic host. *IS*600 and *tnp*F genes were upregulated during interaction of *S. flexneri* with epithelial cells and HeLa monolayer respectively[49,50].

Our IVET screens showed that genes involved in sugar metabolism (*pgi*), translation and regulation (*rbf*A and *inf*B), as well as, protein and peptide synthesis (*cys*S) were also upregulated. We also detected upregulation of four hypothetical proteins: HP0426, HO0427, HP0423 and HP0424. These genes encode putative proteins with unknown functions and do not show significant homology to known proteins. This finding has been observed in most genome-wide analyses, including IVET studies[7]. In a recent IVET study of *V. cholerae*, the largest class of *ivi* genes was found to encode hypothetical proteins[51]. These results indicate that the function of many genes required for growth and survival in complex niches remain uncharacterized and additional functional analyses of these genes are needed.

Transcription profiles of all *ivi* genes were confirmed by real time PCR of *H. pylori* RNA isolated from *H. pylori* infected RAW 264.7 macrophages. These experiments were conducted to determine how well *in vitro ivi* genes in macrophages mirror *in vivo ivi* genes inside the host. The expression levels of several *ivi* genes in macrophages varied from the levels observed in mice. For example, *cag*A, *vac*A, *lpx*C, *mur*I, *tlp*C, *trx*B, *sod*B, *tnp*B, *pgi*, *rbf*A and *inf*B showed a 2-20 fold upregulation (Figure 3). However, *hsd*M, *hsd*M/R, *fuc*T, *vir*B4, HP0426 and HP0427 were not upregulated in the macrophage cell line, and there was no change in the expression of the remaining *ivi* genes. These data suggest a strong correlation between results obtained *in vitro* in the macrophage cell line and in the intact animal. Thus, the macrophages are suitable for the study of initial stages of host cell and bacterium interaction. However, the *in vivo* animal IVET screenings provide a broader and more comprehensive picture of *ivi* genes necessary for infection and colonization. In this study, we identified novel *H. pylori in vivo* induced genes that belonged to several functional gene families, including several well known virulence factors that are expressed by bacterium in infected mouse stomachs. The positive identification of these genes demonstrates that our IVET systems are powerful tools for studying *H. pylori* gene expression in the host environment, and points to potential *H. pylori* specific targets that allow *H. pylori* to circumvent host immune defenses.

ACKNOWLEDGMENTS

We would like to acknowledge Stephanie Marshall for her contribution in the construction of pIVET9 and pIVET10 plasmids.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) chronically infect 50% to 90% of the world's population. Gastritis and ulcers are seen in 15% to 20 % of the infected popula-

tion and gastric cancers occur in 1% to 2% of the same group. Identification of bacterial genes (virulence factors) accounting for *H. pylori* survival in the host is fundamental to understanding the mechanisms of pathogenesis.

Research frontiers

Several methods such as signature-tagged mutagenesis, selective capture of transcribed sequences, differential fluorescence induction and microarray analyses have been used to study bacterial genes that are expressed during infection of animal hosts. These strategies are often limited by their inability to reproduce the complex environments encountered by pathogens in their hosts. To overcome these limitations, *in vivo* expression technology (IVET) has been developed. IVET has resulted in the identification of bacterial genes involved in infection, survival and pathogenesis.

Innovations and breakthroughs

IVET has been utilized extensively in *Salmonella typhimurium, Vibrio cholerae* and *Pseudomonas aeruginosa* to identify potential virulence factors. This technology has not been exhaustively utilized in *H. pylori* because of limitations imposed by the genetic intractability of this bacterium. Recombination-based *in vivo* expression technology (RIVET) approach has been used with *Vibrio cholerae*, *Lactobacillus plantarum*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Bordetella pertussis*. RIVET is a variant of the original IVET in which a promoter transcriptional event is captured permanently as a conversion of the infecting strain from antibiotic resistant to antibiotic sensitive. Recently, RIVET has been utilized to identify *H. pylori* genes important for host colonization. In this study, authors have developed IVET approach for screening *H. pylori* genes that are specifically expressed *in vivo*.

Applications

The study results suggest that this IVET approach may provide powerful tools for studying *H. pylori* gene expression in the host environment. Identification of *H. pylori in vivo* induced genes will provide an improved understanding of metabolic, physiological, and genetic factors that contribute to survival and virulence of this pathogen. It may also lead to the identification of possible vaccine targets.

Terminology

IVET is a genetic method used to determine which bacterial genes are upregulated when bacteria invade the stomach of a host.

Peer review

This study demonstrated the efficacy of *in vivo* expression technology for screening *H. pylori* genes that are expressed *in vivo* in mice and macrophage hosts. In this study, genes responsible for a broad group of functions were identified. Although no screen of this type can provide an exhaustive account of all genes induced *in vivo*, it will likely give insight into the type of genes upregulated and hence necessary for the survival of *H. pylori* in gastric mucosa.

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