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RESEARCH ARTICLE

Helicobacter pylori Gene Expression *in Vivo*: A Genomic Perspective

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ABSTRACT: Compared with many other bacteria whose natural environmental niche is the human gastrointestinal tract, *Helicobacter pylori* possesses a relatively limited genetic repertoire. This limitation is likely the result of a long and successful co-evolutionary process of adaptation to the demanding environment within the human gastric mucosa. It is more difficult to understand in terms of high infection rates worldwide, indicating a similar efficiency in transferring to and infecting new hosts. Insight into these and other aspects of *H. pylori* biology will likely be advanced by new studies that consider the bacterial genome as a functioning entity. Extending these genome-wide analyses to bacterial gene expression in the natural context of interactions with individual human hosts may be necessary to understand the complexities of *H. pylori* disease pathogenesis.

Keywords: Genome, *pylori*, Mucosa, Transcription, RNA, Regulation, *in Vivo*.

1. THE *H. pylori* 26695 GENOME

Helicobacter pylori is a motile gram-negative pleiomorphic spiral-shaped bacterium found almost exclusively in the human gastric mucosa. In terms of laboratory culture requirements, it is both nutritionally fastidious and sensitive to ambient oxygen levels. Isolates grow poorly in laboratory broth media without supplemental agents that potentially sequester toxic compounds present in cultures (charcoal, cyclodextrans, bovine serum albumin, starch, serum, etc.). Growth also does not initiate well from typically sized bacterial inocula, and therefore only a few doublings (μ = 6–8 h for freshly isolated strains) are typically seen before batch cultures become saturated. Viability counts on bacteria in saturated cultures can then decrease because of both autolysis [1] and conversion to nonculturable morphologically distinct forms that show reduced metabolic activity [2].

These exacting culture conditions are reflected in the limited metabolic capacity depicted in the *H. pylori* genome that was first described for the laboratory passaged strain 26695 in 1997. The single circular *H. pylori* chromosome contains approximately 1.65 million base pairs and is about half the size of that of *Escherichia coli* K12. The current annotation of the *H. pylori* 26695 genome (http://genolist.pasteur.fr/PyloriGene/) indicates 1637 predicted coding sequences (1594 for proteins), with 1281 shown to be conserved among all 15 strains examined by Salama et al. [3]. Although analyses of the genome sequences of strains 26695 and J99 have indicated that *H. pylori* lacks enzymes in known biosynthetic pathways for several constituents required in culture media, prior metabolic studies of other strains have demonstrated enzymatic activities for which genes were not identified [4]. Genes for glycolytic enzymes are present in these genomes, but studies of bacteria in typical laboratory culture media indicate that Entner-Doudoroff and pentose phosphate pathways are more active [5]. The *H. pylori* 26695 genome lacks genes for key NADH-generating enzymes, and NADPH is likely a preferred electron donor with a terminal cytochrome *bc* oxidase, cytochrome *c* peroxidase, or fumarate reductase used in respiration [6].

The *H. pylori* genome does contain a large number of genes (>2% of open reading frames (ORFs)) for different putative adhesins, lipoproteins, and other outer membrane proteins [7, 8], emphasizing the importance of adherence for life in the gastric mucosa (as discussed below). These coding sequences frequently show upstream homopolymeric tracts and dinucleotide repeats providing regions for recombination during DNA replication and translational frameshifting (mechanisms of phase and antigenic variation). A similarly large

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portion of the genome is devoted to restriction-modification systems. Estimates of the number in the genome of strain 26695 have been as high as 25 restriction enzyme homologues and 27 DNA methylases [9]. Within each strain, the majority of these systems are not functionally active [10]. The corresponding methylases therefore potentially have alternative roles, which may include regulation of bacterial gene expression [11], as a recent study suggests [12] (see below).

The limited genetic repertoire of *H. pylori* is particularly striking in terms of the paucity of previously described bacterial transcriptional regulatory apparatus. Like other bacteria with small genome sizes, only a small percentage of *H. pylori* genes (estimated at 1.1%) are predicted to encode proteins that regulate gene expression based on identifiable regulatory protein motifs [13]. However, the similarly sized genome of *Haemophilus influenza* KW20, a species that also inhabits only a restricted human host niche, is predicted to encode a 3-fold greater number of these traditional regulatory proteins. While the *H. pylori* 26695 genome has only three recognizable sigma factor genes (*rpoD*, *rpoN*, and *fliA*), the *H. influenza* KW20 genome contains five. In terms of classical bacterial two-component systems, the genomes of these two species are similar. *H. pylori* has only four histidine kinases and six response regulators [8], while the *Haemophilus influenza* KW20 genome has four two-component gene pairs and one unpaired response regulator [14]. Compared with *Helicobacter pylori*, *Campylobacter jejuni* appears to have a somewhat broader repertoire of these regulatory systems within a genome of similar size. While the *C. jejuni* genome also encodes three predicted sigma factors (also designated *rpoD*, *rpoN*, and *fliA*), it contains seven predicted two-component systems and three orphan response regulators [15]. As *C. jejuni* is known to inhabit multiple hosts and ecological niches, this is perhaps a surprisingly modest corresponding increase in easily recognized regulatory capacity.

2. TRANSCRIPTIONAL REGULONS

Roles of alternative *H. pylori* sigma factors RpoN (σ^{54} and FliA (σ^{28}) have so far been described as centered on the production of flagella. Recently Josenhans et al. [16] described an anti-sigma factor (FlgM or HP1122) that modulates the activity of FliA. Global analysis of RNA expression patterns of relevant mutant *H. pylori* strains identified an outer membrane protein (HorE or HP0472), an enzyme in lipoprotein synthesis (EnvA or HP1052), and a conserved hypothetical peptide (HP1051) whose expression was modulated by the activities of FlgM and FliA. This study demonstrated regulation of *H. pylori* gene expression by an alternative sigma factor in metabolic activities other than flagellar biosynthesis, but also indicates a relatively limited role for σ^{28} in this capacity.

Three recent analyses have successfully identified small regulons controlled by *H. pylori* two-component signal transduction systems. Delany et al. [17] showed that transcription of the gene for chemotaxis protein TlpB was dependent on an intact gene encoding the unpaired response regulator HP1043. TlpB interacts with the products of the *cheW-cheA* two-component system that responds to environmental chemotactic signals. A second study showed that expression of RNAs corresponding to ORFs HP1408 and HP0119 was dependent on the HP0165-HP0166 two-component system [17]. HP1408 is part of a putative five-gene *H. pylori*-specific operon (HP1408-HP1412) in strain 26695, and HP0119 is also an *H. pylori*-specific ORF with no other sequence annotation.

Derepression of the same *H. pylori*-specific operon (HP1408-1412) upon inactivation of the gene for this histidine kinase (HP0164) in strain B128 was also noted by Forsyth et al. [18]. However, this was not observed when the same mutation was created in a different *H. pylori* strain (26695). These researchers also identified additional RNAs whose expression levels were altered by this mutation in strain 26695, including transcripts from two small *H. pylori*-specific operons (HP0681-HP0682 and HP1288-HP1289), as well as HP0725 (*hopF*), HP1399 (*rocF* arginase), and HP1432 (encoding an unusual short histidine- and glutamine-rich peptide). These results demonstrate variable regulation of gene expression among different strains and, like others described below, raise the possibility that adaptive responses in *H. pylori* may involve genes and metabolic pathways unique to this bacterium.

H. pylori possesses a homologue of the bacterial ferric uptake regulator protein (Fur). Several operator sites and associated genes have been identified by a modified repressor titration assay screen (FURTA) developed for *Escherichia coli*. Fur was initially shown to bind to sites in *H. pylori fecA2* (HP0807) and *ribBA* (HP0804) promoter regions [19]. Iron-dependent expression of *H. pylori pfr* (HP0653) and *frpB* (HP0876) has also been shown to be Fur-dependent [20, 21]. In contrast to the role of *E. coli* Fur as a transcriptional repressor, *H. pylori* Fur can both directly activate or repress expression of genes in response to iron [21]. *fur* expression has also been shown to increase in response to reduced pH, establishing a link between iron availability and pH responses [22].

The unusually large number of restriction-modification systems identified in the *H. pylori* genome provided an immediate indication of the importance of bacterial DNA exchange in these naturally competent bacteria. Donahue et al. [12], in extending studies of Peek et al. [23], were among the first to provide evidence of potential alternative roles for these DNA methylases in the regulation of *H. pylori* gene expression. Studies by Heithoff et al. [24] on *Salmonella typhimurium* have recently brought DNA methylation into the mainstream as a mechanism for the global regulation of adaptive responses in bacterial pathogens. Donahue et al. [12] showed that inactivation of the *H. pylori hpyIM* gene (HP1208, immediately downstream of the *iceA* locus) altered

the expression of the *hrcA-dnaK-grpE* operon. Expression of the *dnaK* operon transcript in response to the onset of stationary phase in laboratory cultures or adherence to cultured AGS gastric epithelial cells was shown to be dependent on the presence of an intact *hpyIM* gene. The *H. pylori dnaK* operon contains a homologue of the bacterial *hrcA* regulatory gene, suggesting potential indirect effects of HpyIM activity on the expression of additional *H. pylori* genes beyond those identified by these studies.

Global regulation of bacterial gene expression is also mediated by ribonucleases that control RNA stability and mRNA turnover rates. In *E. coli*, a group of these enzymes form a multienzyme complex known as a degradosome. The *H. pylori* genome has homologues of numerous bacterial nucleases and degradosome components. Akada and colleagues [25] have shown that reduced environmental pH influences the relative decay rates of different transcripts of the urease operon (*ureABIEFGH*). Recent studies by the author [26] have identified RNAs encoding enzymes involved in bacterial transcript processing among those expressed by *H. pylori* in human gastric tissue. These studies indicated ribonuclease H (*rnhA* or HP0061) and an RNA DEAD helicase (HP0435) as candidates for mediating RNA stability in response to human host interactions (Table I).

3. GLOBAL ANALYSIS OF BACTERIAL GENE EXPRESSION *IN VITRO*

Several groups have now reported on the analysis of *H. pylori* gene expression associated with responses to reduced pH *in vitro*. Two studies examined total *H. pylori* RNA from bacteria grown on two different types of pH-adjusted plates [27, 28], and a third analyzed RNA in bacteria 30 min after resuspension in a reduced pH buffer [29]. It is not surprising perhaps that all three studies identified largely nonoverlapping sets of pH-responsive genes, given the different methods used to invoke bacterial adaptive responses. In addition, the study by Dong et al. [28] used differential display polymerase chain reaction (PCR) to identify bacterial RNAs, while the other two used genome array hybridization. Differential display identified 11 differentially expressed RNAs, including those for *grpE* and *ureB*, which were also identified by both array studies. The array studies by Ang et al. and Allan et al. [27, 29] describe 80 and 39 transcripts expressed in response to reduced pH, respectively; both studies identified differential expression of *H. pylori secA*, *secY*, and *secF*, and *envA*.

A new study by Thompson et al. [30] reports RNA expression profiling by genomic array for the Sydney strain 1 (SS1) during growth in broth cultures. Of particular interest is the increased reliability achieved by preparing total RNA at eight consecutive time points to follow progressive changes in *H. pylori* gene expression in batch cultures (Brucella Broth with 10% fetal calf serum). Among those RNAs described as increasing in steady-state levels with the age of cultures are those for *flaA*, *napA* (neutrophil activating protein), and *pfr* (a putative iron storage protein). RNA expression levels for two genes associated with iron uptake, *fecA* and *frpB*, were also reduced over the time course. Only approximately 20% (325) of array features showed more than 2-fold changes in signal intensity over the time course used in these studies, with about half of these increasing and half decreasing. The authors note that a large number of *H. pylori* genes were not expressed at all in broth culture, given the detection threshold chosen in identifying constitutively expressed genes. The percentage of genes detected at any single time point is reported to be about 40% [30]. Interestingly, our studies [26] using the same PCR products arrayed on nylon with radiolabeled cDNA probes showed approximately 70% of array features hybridizing in analyses of RNAs from mid-logarithmic phase *H. pylori* cultures grown in the same media. This difference may originate in the use of different array hybridization methods [31], as well as differences in criteria used as transcript detection thresholds. These are important questions to resolve in terms of *H. pylori* functional genomics, as we try to determine whether there are important metabolic capacities or virulence determinants that are not elaborated at any stage of growth in laboratory cultures.

Table I. ORFs with informative annotations corresponding to differentially expressed *H. pylori* RNAs obtained from human gastric biopsies.[a]

ORF	Annotation[b]
Strain B128	
HP0228	Probable sulfate permease
HP0347	*rluD1*, pseudouridine synthase
HP0358	Predicted outer membrane protein
HP0441	*vir*B4-like Type IV system
HP0645	*slt* murein transglycosylase
HP0661	*rnhA* ribonucleaseH
HP0667	Res.-Mod.-like peptide
HP0718	Predicted integral membrane protein
HP1349	Predicted periplasmic (*C. jejuni* homologue)
HP1421	*trbB* Type IV system
HP1545	*folC* folylpolyglutamate synthase
Strain B213	
HP0038	*com*B8 Type IV system
HP0040	*com*B9 Type IV system
HP0045	*wbcJ*, GDP fucose synthase
HP0048	*hypF* hydrogenase transcriptional regulator
HP0228	Probable sulfate permease
HP0263	*hpy99*VIIIM cytosine methytransferase
HP0321	*gmk* guanylate kinase
HP0435	DEAD-box RNA helicase
HP0463	*hsdM1* restriction enzyme
HP0667	Res.-Mod.-like
HP1492	*nifU*-like, Fe-S assembly protein
HP0981	*xseA*, DNA repair exonuclease
HP0696	Urease-like *N*-methylhydantoinase
JHP1132	*iceA2* cell adherence-associated RNA

[a] As previously described by Graham et al. [26].
[b] Updated annotations are from http://genolist.pasteur.fr/PyloriGene.

4. STUDIES OF *H. pylori* GENE EXPRESSION IN HUMAN GASTRIC MUCOSA

Experimental models of human infections have provided enormous amounts of valuable information about how bacteria interact with their hosts. However, even the very best of these models cannot reproduce all of the important parameters that signal responses from infecting microbes. Although specific human host cell interactions can be modeled in cell cultures, human primary cells are removed from the normal contact with immune cells and cytokines that typically modulate their activities. In practice, experimental cell culture infections also often employ unnaturally high multiplicities of infection to obtain measurable responses from both the host and microbe. The use of transformed cell lines can contribute increased reproducibility in these types of studies, as well as allow an increase in scale to increase detection. Unfortunately, almost all immortalized cells are known to either lack or lose important relevant characteristics on laboratory passage. Animal models can replace valuable parameters missing from studies of microbial interactions with cell monocultures in laboratory media, particularly for bacteria that remain attached to cell surfaces. However, bacterial interactions with surrogate host tissues are only expected to reproduce common subsets of interactions and environments normally encountered by human pathogens during natural infections.

Direct analysis of bacteria RNA in microbes present in infected tissues is clearly the most feasible way of examining microbial gene expression during actual human infections [26]. This approach may be particularly valuable in understanding *H. pylori* biology and pathogenesis. Well-recognized strain variability, together with variation in individual hosts and host responses, makes it likely that there are important interactions occurring in some infections and not others. *H. pylori* is also known to interact with human fucosylated and sialylated carbohydrate epitopes that are absent in nonprimate tissues or expressed at only very low levels by human cell lines. These include recently identified human tissue antigens that are expressed in response to *H. pylori* infection [32]. Unlike other bacterial infections, biopsies from human gastric tissues are quite often obtained from *H. pylori*-infected patients for histological examination. These specimens usually contain reasonable numbers of bacteria, on the order of 10^3 to 10^4 cultivatable [33] microbes per gastric tissue specimen.

Despite the existence of appropriate specimens, analyses of *H. pylori in vivo* gene expression have been limited. Rick Peek and colleagues initiated studies designed to examine *in vivo* expression of an *H. pylori* gene that was initially identified by differential display PCR as induced on contact with cultured AGS gastric epithelial cells [23]. The specific RNA region identified by these early studies corresponded to *H. pylori iceA* (HP1209 or JHP1132), an ORF predicted to encode part of a degenerate restriction-modification system. Donahue et al. [34] subsequently showed that in all of four *H. pylori* strains examined, a dicistronic transcript of the *iceA* region was synthesized that encoded a highly conserved DNA methylase (HpyIM). Peek et al. [35] used reverse transcription–PCR (RT-PCR) on total RNA prepared from 41 gastric biopsy specimens to compare detection of *H. pylori* 16S rRNA and transcripts for each of two different *iceA* alleles. These studies noted that approximately half of strains for which *H. pylori* 16S rRNA was detected expressed detectable levels of *iceA1* allele transcripts in the mucosa, and those patients showed more mucosal inflammation. Subsequent studies by Takeuchi et al. [34] showed that detection of transcripts from the highly conserved downstream *hpyIM* region varied among different strains *in vitro* and *in vivo* and was not related to the degree of inflammation in different patient infections. Expression levels for *hpyIM* RNA were shown to vary with growth phase *in vitro*, suggesting that methylase expression in the mucosa may also be modulated during infection.

Other studies of *H. pylori* gene expression in its natural environmental niche also used targeted RT-PCR to assess the expression of bacterial *ureA* (urease subunit), *katA* (catalase), and *alpA* (adhesin) in both mouse and human mucosa [36]. Fluorescence-monitored quantitative PCR showed that these genes are expressed during both mouse and human gastric infections, and that relative steady-state transcript levels for these genes (rRNA > *ureA* > *katA* > *alpA*) are consistent for *H. pylori* present in both mouse and human mucosa.

5. GLOBAL ANALYSIS OF *H. pylori* GENE EXPRESSION IN NATURAL HUMAN INFECTIONS

Analysis of global patterns of microbial gene expression in bacteria present in naturally infected human tissues was recently described for the first time [26]. These studies were made possible by the combination of a novel PCR-mediated cDNA capture method (SCOTS) with bacterial genome array hybridization. Genome-wide *H. pylori* RNA expression was examined for bacteria in two patient gastric biopsy specimens and for the isolated strains (B128 and B213) during growth in laboratory broth cultures. Many *H. pylori* genes were identified as potentially expressed in response to environmental factors and interactions with human gastric mucosa. These included several for which some idea of the function of the encoded product can be inferred by comparison with gene sequences in other bacterial species (Table I). However, the majority of genes identified were largely without known homologues in other species. A subsequent reannotation of the *H. pylori* 26695 genome as described by Boneca et al. [37] reduced the total number of hypothetical *H. pylori*-specific coding regions to 334 (21.5% of all ORFs), largely based on the publication of the *Campylobacter jejuni* genome sequence. Almost half (45%) of the 65 genes identi-

Table II. Putative species-specific *H. pylori* operons corresponding to RNAs identified as expressed in response to human gastric mucosa.[a]

ORF	Annotation[b]	Putative operon	Coding regions (no.)
Strain B128			
HP0061	Hypothetical	HP0057-HP0066	10
HP0274	Hypothetical	HP0268-HP0277	10
HP0343	Hypothetical	HP0334-HP0346	13
HP0441	*vir*B4-like, Type IV	HP0441-HP0446	6
JHP0945	Hypothetical	JHP0944-JHP0950	7
Strain B213			
HP0038/40/45	Type IV system	HP0037-HP0042	6
HP0061	Hypothetical	HP0057-HP0066	10
HP0338/HP0343	Hypothetical	HP0334-HP0346	13
HP0435	Hypothetical	HP0433-HP0436	4
HP0893	Hypothetical	HP0893-HP0895	3
JHP0945	Hypothetical	JHP0944-JHP0950	7
JHP0958	Hypothetical	JHP0954-JHP0962	9

[a] As previously described by Graham et al. [26].
[b] Updated annotations are from http://genolist.pasteur.fr/PyloriGene.

fied by our studies remained without identified homologues in any other bacterial species [26]. Many of these *H. pylori*-specific genes are found in putative operons that are almost entirely composed of genes unique to *H. pylori*. Table II lists these putative operons for those containing three or more coding sequences. As *H. pylori* only inhabits a single environmental niche that is only transiently shared by a few other bacterial species, a significant amount of additional effort directed specifically at understanding the natural biology of this bacteria in its native habitat will be necessary to obtain functional information about these genes.

Our RNA analyses also directly identified *H. pylori* genes expressed by bacteria in human mucosa by sequencing cDNA clones obtained by SCOTS [26]. This approach allowed the unambiguous determination of the expression of specific paralogous gene family members, including those encoding members of the large outer membrane protein family that includes the *babA2* and *Alp* adhesins. We were therefore able to describe *in vivo* expression of *hopE* (HP0706), *alpB* (HP0913), *hopI* (HP1156), *hopL* (HP1157), *babA2* (HP1243), *hopN* (HP1343), and *horJ* (HP1469) by *H. pylori* B128 with this approach. Potential cross-hybridization complicated similar identification of *in vivo* expression of these genes by the array hybridization methods used. As sequences for these genes were obtained from a limited sampling of clones from a bacterial cDNA library obtained by SCOTS from gastric tissue, RNAs for these genes were potentially expressed by *H. pylori* B128 at high levels during infection.

6. CONCLUSIONS

The human gastric mucosa is a dynamic environment with regard to bacterial colonization. It is hypothesized that daughter cells derived from minor adherent subpopulations of *H. pylori* replace those that reside in the mucus layer as they are removed by mechanical action [38]. The mucus layer itself, in which the vast majority of *H. pylori* reside (e.g., 99% [39]), is constantly moving material away from the sites of *H. pylori* colonization and overlies an epithelium where cell turnover is rapid (typically replacing individual cells every 3 to 4 days). These environmental demands are a driving evolutionary force and, when combined with a microbial propensity to both exchange DNA and allow genetic change, appear to have resulted in a relatively simple *H. pylori* genome with significant investment in adherence mechanisms. Although few regulatory genes have been identified by similarity to other known bacterial regulatory genes, the need for *H. pylori* to at least survive transfer to new hosts at reasonable rates suggests that less well-recognized regulatory mechanisms, including DNA methylation, are important. However, the coordinate regulation of *H. pylori* gene expression resulting from incompletely described regulatory mechanisms may not be apparent under laboratory conditions currently capable of supporting growth in laboratory media. If there are important *H. pylori* adaptive responses exhibited only *in vivo*, *these* may include those about which we have no previous information from studies of other bacteria. It will be interesting to determine global changes in *H. pylori* gene expression associated with now well-described transitions to potentially more environmentally tolerant "dormant" morphological variants *in vitro*, and to compare expression of relevant genes in *H. pylori* present in different human clinical specimens (e.g., biopsies, dental plaque, feces).

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