Inactivation of a Helicobacter pylori DNA methyltransferase alters dnaK operon expression following host-cell adherence

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Abstract

The Helicobacter pylori hpyIM gene encodes a type II DNA methyltransferase that is highly conserved among strains. To investigate the potential role of M. hpyI methyltransferase activity in controlling gene expression in H. pylori, we analyzed gene transcription profiles in wild-type strain J166 and an isogenic hpyIM mutant strain using gene arrays. This analysis showed that the expression of a majority of genes was unaffected by hpyIM mutation, especially in exponential phase cultures. However, in stationary phase cultures and in cells adherent to AGS gastric epithelial cells in vitro, loss of hpyIM function altered the expression of the stress-responsive dnaK operon. Complementation of the hpyIM mutation using a shuttle plasmid encoding a wild-type copy of the gene re-established the wild-type pattern of dnaK operon expression. These data suggested that hpyIM, encoding a DNA methyltransferase, may have a role in H. pylori physiology that supersedes its original function in a type II restriction–modification system. ß 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Helicobacter pylori is a microaerophilic, Gram-negative bacterium that persistently colonizes the mucous layer overlying the gastric epithelium [1]. Colonization with H. pylori results in chronic superficial gastritis [2], which increases the risk for the development of peptic ulcer disease and distal gastric carcinoma [3–4]. However, the majority of persons colonized with H. pylori remain asymptomatic. Although several strain-specific factors have been identified that may be markers for the different clinical outcomes of H. pylori colonization [5–8], we presently have an incomplete understanding of the role these bacterial factors might have in progression to disease.

In studies designed to specifically identify virulence determinants in pathogenic versus non-pathogenic H. pylori strains, we identified a genetic locus that is transcriptionally activated following adherence of an ulcer-derived H. pylori strain to gastric epithelial cells in vitro [8]. The locus includes a highly conserved DNA adenine methyltransferase, encoded by hpyIM, that modifies the sequence CATG [9] and a 5'-flanking gene, named iceA, that shows extensive diversity between strains [8,10] and includes two mutually exclusive gene families, designated iceA1 and iceA2. In a minority of strains the iceA1 allele encodes a functional restriction enzyme (RE) that recognizes CATG, suggesting this genetic locus originated as a type II restriction–modification (R–M) system. However, mutations in the majority of iceA1 alleles prevent translation of a functional RE partner for the methyltransferase [10]. iceA2 genes are highly diverse and have low homology to iceA1 and other sequences in public databases [10], and it is not known whether iceA2 directs the synthesis of a functional protein in vivo.

Transcription of hpyIM initiates at promoters located within iceA1 or iceA2 [11,12] and Northern blot analysis of RNA from exponential phase broth cultures demonstrates that hpyIM transcript levels are higher in iceA1 strains than in iceA2 strains (J.P. Donahue, unpublished).

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Because it is unlikely that iceA1 and iceA2 encode functional proteins in the majority of strains, we hypothesized that an association of iceA1-containing strains with duodenal ulcer disease [8,13], might be related to differences in DNA methylation, catalyzed by M.HpyI, that affect transcription of genes involved in pathogenesis.

To test the aforementioned hypothesis, we used gene arrays to compare global gene transcription in H. pylori wild-type strain J166 and an isogenic hpyIM mutant strain in exponential and stationary phase broth cultures and following adherence to AGS gastric epithelial cells in vitro. This analysis showed that in stationary phase cultures and in cells adherent to host cells in vitro, loss of hpyIM function significantly altered the expression of the stress-responsive dnaK operon [14], suggesting a role for hpyIM in H. pylori physiology that is unrelated to type II R–M systems.

2. Materials and methods

2.1. Bacterial strains, plasmids and genomic DNA isolation

H. pylori strains J166 (wild-type) and J166M– (hpyIM::aphA) have been previously described [8,15]. Plasmid pVU1041 was constructed by inserting a 2000-bp DNA fragment encoding strain J166 iceA1-hpyIM (HP1209-HP1208) [16] into the Escherichia coli–H. pylori shuttle vector, pHel2 [17]. Plasmid pVU1041 was methylated in vitro using J166 cell-free extract prior to natural transformation of H. pylori strain J166M– [15]. For preparation of genomic DNA, H. pylori cells were harvested from 24-h cultures grown on agar plates and genomic DNA was isolated as described [18].

2.2. H. pylori growth and adherence to human AGS cells

H. pylori strains were grown in Brucella broth containing 10% fetal calf serum at 37°C and 5% CO2 with shaking (100 rpm). For exponential phase cultures, 25 ml of broth in a 250-ml culture flask was inoculated with a fresh overnight broth culture to a final optical density at 600 nm of (100 rpm). For exponential phase cultures, 25 ml of broth in 250-ml culture flasks was inoculated as described for exponential and stationary phase bacteria or 75 ml of broth in 250-ml culture flasks was inoculated as described for exponential and stationary phase bacteria. Stationary phase cells were collected by centrifugation and immediately processed for RNA isolation. For stationary phase cultures, and to obtain cells used for in vitro adherence to AGS gastric epithelial cells, 75 ml of broth in a 250-ml culture flask was inoculated as described for exponential and stationary phase cultures and incubated for 42 h. Stationary phase cells were collected by centrifugation and immediately processed for RNA isolation or were suspended in RPMI 1640 to an estimated final concentration of 1 × 10^10 CFU ml^{-1}. Human AGS gastric epithelial cells were grown in RPMI 1640 containing 10% fetal calf serum. Confluent cultures of AGS cells in T75 culture flasks (approximately 1 × 10^6 cells) were washed once with RPMI 1640 and infected with 1 × 10^10 H. pylori cells in a total volume of 10 ml of RPMI 1640. Infected AGS cell monolayers were incubated at 37°C (5% CO2). After 1 h, non-adherent bacteria were removed and AGS cell monolayers with adherent bacteria were washed with ice-cold PBS prior to isolation of total RNA.

2.3. RNA isolation

RNA was isolated from H. pylori exponential and stationary phase cells and from H. pylori-infected AGS gastric epithelial cell monolayers essentially as described [19]. Exponential and stationary phase bacterial cell pellets in 15-ml polypropylene centrifuge tubes or AGS cells with adherent H. pylori in T75 culture flasks were each lysed with 3 ml of lysis solution [0.5% SDS, 10 mM Na2EDTA (pH 8.0)]. Then, 3 ml of ice-cold wash solution [0.1 M sodium acetate (pH 5.2), 10 mM Na2EDTA (pH 8)] was added to the cell lysates in 15-ml polypropylene tubes. Cell lysates in culture flasks were transferred to 15-ml polypropylene tubes and flasks were rinsed with 3 ml of ice-cold wash solution which was then combined with cell lysates. Each cell lysate was then extracted with 6 ml of H2O-saturated phenol and centrifuged at 2600 × g for 10 min at 4°C. The aqueous phase (5.5 ml) was removed and added to 0.66 ml 1 M Tris-HCl (pH 8) and 0.27 ml 5 M NaCl and the RNA precipitated by the addition of 12 ml 100% ethanol and incubation at −20°C for a minimum of 30 min. The RNA was collected by centrifugation, washed with 70% ethanol, air dried and dissolved in 50 μl of DEPC-treated H2O. RNA was incubated with amplification grade DNase I (0.5 U μg^{-1} RNA; Life Technologies) as described by the manufacturer. The integrity of the RNA was assessed by agarose gel electrophoresis prior to labeling for hybridization to H. pylori gene arrays.

2.4. Gene array hybridization

33P-labeled cDNA was synthesized from 15 μg RNA (exponential and stationary phase bacteria) or 75 μg RNA (adherent bacterial and AGS cell total RNA) using random hexamer primers and Superscript II reverse transcriptase (Life Technologies). Reactions were incubated at 42°C for 2 h and then 70°C for 15 min. After this incubation, RNA was hydrolyzed by incubation in 0.3 N NaOH, 17 mM Na2EDTA, 0.3% SDS at 68°C for 30 min. Hydrolysis reactions were neutralized by adding 2 N HCl and the labeled cDNA was separated from unincorporated [α-33P]dATP using a Gel-Spin S50 column as described by the manufacturer (Worthington). H. pylori gene arrays (Eurogentec) were pre-hybridized in 5 × SSC (pH 7.5), 5 × Denhardt’s solution, 0.5% SDS [19] for a minimum of 2 h at 65°C. The 33P-labeled cDNA was denatured in 0.3 N NaOH at 37°C for 10 min and hybridized to the gene array at 65°C for 20 h in the same solution used for pre-hybridization. Hybridized gene arrays
were washed with 2×SSC, 0.1% SDS at 65°C for 20 min and then with 0.2×SSC, 0.1% SDS at 65°C for 1 h. Gene arrays were autoradiographed using Kodak Bio-Max MR X-ray film.

2.5. RNA slot-blot analysis

DNase I-treated *H. pylori* RNA was applied to Hybond-N (Amersham Life Science) membranes using a slot-blot apparatus and crosslinked to the membrane by exposure to ultraviolet light as described [11]. DNA fragments used as probes were synthesized by PCR using gene-specific oligonucleotide primers and *H. pylori* J166 genomic DNA as template. DNA fragments were labeled with [α-32P]dCTP by random priming using Prime-It RmT (Stratagene) as described by the manufacturer. Unincorporated [α-32P]dCTP was removed from labeled DNA fragments using 1-ml Sephadex G-50 spin columns essentially as described [19]. RNA slot blots were pre-hybridized, hybridized to 32P-labeled DNA probes, and then washed as described by us for Northern blots [11]. Blots were autoradiographed using Kodak Bio-Max MR X-ray film.
film and imaged using a Fuji phosphorimaging screen (BAS-MP2040).

3. Results

3.1. Gene array analysis of transcription in H. pylori wild-type and hpyIM mutant bacteria

To assess whether hpyIM, encoding a DNA adenine methyltransferase, may be involved in the control of expression of specific genes in H. pylori, we used gene arrays to compare global transcription in H. pylori wild-type strain J166 and isogenic hpyIM mutant strain J166M− in exponential and stationary phase broth cultures and following 1 h of adherence to AGS gastric epithelial cells in vitro. Steady-state mRNA levels were analyzed using the Eurogentec H. pylori gene array which contains 1578 PCR products representing 97% of the ORFs from strain 26695.

Fig. 4. Complementation of the hpyIM mutation re-establishes the wild-type levels of dnaK operon transcripts in stationary phase and adherent cells. A: RNA slot-blot analysis of transcript levels in H. pylori wild-type strain J166, hpyIM mutant strain J166M−, and J166M− ΔpVU1041(hpyIM−). RNA isolated from stationary phase cells and from cells adherent to AGS gastric epithelial cells in vitro was applied to Hybond-N membranes and hybridized to 32P-labeled DNA probes encoding hrcA, grpE, dnaK or 16S rRNA as indicated. B: Quantitation of dnaK operon transcript levels by phosphorimager analysis of three independent RNA slot blots including the blot shown in (A). Photo-stimulated luminescence (PSL) values represent the mean ± S.E.M.

WT, strain J166; M−, strain J166M−; and M+/M+, strain J166M− ΔpVU1041(hpyIM−). Stripped columns, RNA from stationary phase cells; solid columns, RNA from adherent cells.

Fig. 5. Decrease in viability and RNA integrity in stationary phase cultures of the H. pylori hpyIM mutant strain. A: Shown are growth curves of strains J166, J166M−, and J166M− ΔpVU1041(hpyIM−) grown in 75 ml brucella broth plus 10% fetal calf serum as described in Section 2. Growth was monitored by measuring the change in optical density at 600 nm (A600) with time in hours. B: Shown is a 1% TAE-agarose gel stained with ethidium bromide. Total RNA (2 μg), isolated from 48-h stationary phase cultures of strains J166, J166M−, and J166M− ΔpVU1041(hpyIM−), was loaded on the gel as indicated above the lanes. The locations of 23S, 16S and 4S/5S RNA are indicated.
spotted in duplicate on a nylon membrane. This analysis showed that transcript levels of genes involved in bacterial growth and other normal cellular metabolic processes were similar in exponential phase cultures of the wild-type and hpyIM mutant strains (data not shown). However, analysis of the transcriptome in stationary phase cultures of the wild-type and hpyIM mutant strains revealed several potential differences. In particular, the transcript levels of the stress-responsive dnaK operon, comprised of hrcA, grpE and dnaK [14], were increased significantly in stationary phase cultures of the wild-type and hpyIM mutant strains, with higher levels evident in the hpyIM mutant (Fig. 1). Adherence of stationary phase wild-type and hpyIM mutant bacteria to AGS gastric epithelial cells in vitro for 1 h resulted in the rapid decrease in the levels of dnaK operon transcripts in the wild-type strain to pre-stationary phase levels, whereas dnaK operon transcript levels remained high in the hpyIM mutant strain. The data suggested that this was a specific effect on dnaK operon expression, because the transcript levels of many other genes, including catalase (kat, 3D14) and vacuolating cytoxin (vacA, 3E11) shown in Fig. 1, remained similar in the wild-type and hpyIM mutant. Also, independent of the hpyIM mutation, we found that bacterial adherence to gastric epithelial cells resulted in an increase in the transcript levels of many genes encoding proteins predicted to be involved in bacterial growth. For example, as seen in Fig. 1, transcript levels of peptide chain release factor (prfA, 1D5) were clearly visible in exponential phase cells, declined to low levels in stationary phase cells, and then increased in both the wild-type and hpyIM mutant strains following 1 h of host-cell contact.

The apparent altered control of dnaK operon transcription in the hpyIM mutant strain that was shown in gene-array hybridizations was confirmed by RNA slot-blot analysis. Total RNA from the same preparations used in the gene-array analysis shown in Fig. 1 was applied to Hybond-N membranes and probed with 32P-labeled DNA fragments encoding hrcA, grpE, dnaK or 16S rRNA. RNA slot-blot results are shown in Fig. 2 and transcript levels were quantitated by phosphorimager. This analysis showed that dnaK operon transcript levels in exponential phase cultures of both strains were approximately equal. In stationary phase cultures hrcA, grpE, and dnaK transcript levels were increased in the hpyIM mutant strain by three-fold, 1.6-fold and two-fold, respectively, relative to the wild-type strain. Following epithelial cell adherence, dnaK operon transcripts declined to pre-stationary phase levels in wild-type cells while remaining high in the hpyIM mutant cells. This resulted in hrcA, grpE and dnaK transcript levels that were 36-fold, 6.5-fold, and 10.3-fold, respectively, higher in the hpyIM mutant than in the wild-type strain. For all conditions tested, 16S rRNA levels were similar, indicating that equal amounts of total bacterial RNA were present in all slots on the membrane.

### 3.2. Complementation of the hpyIM mutation re-establishes wild-type dnaK operon transcript levels

To demonstrate that mutation of hpyIM was specifically responsible for the observed changes in control of dnaK operon transcript levels, we sought to complement the hpyIM mutation by introducing a plasmid encoding a wild-type copy of hpyIM into J166M3. Plasmid pVU1041 was constructed by inserting a DNA fragment encoding H. pylori strain J166 icaAl–hpyIM into the shuttle vector pHel2 [17]. This DNA fragment contains the native transcriptional and translational regulatory signals present in wild-type strain J166 upstream of hpyIM. Following transformation of strain J166M+, complementation of the hpyIM mutation was demonstrated by the restoration of resistance of genomic DNA to digestion by the RE NlaIII which recognizes the sequence CATG modified by M.HpyI (Fig. 3). Genomic DNA from J166M+ transformed with the parental shuttle vector, pHel2, remained susceptible to NlaIII digestion (Fig. 3).

RNA was isolated from strain J166, J166M−, and J166M+/pVU1041 stationary phase cultures and from cells adherent to AGS gastric epithelial cells in vitro and dnaK operon transcript levels were assessed by RNA slot-blot analysis. As shown in Fig. 4, dnaK operon transcripts were more strongly induced in both stationary phase and adherent cells of hpyIM mutant strain, J166M−, than in the wild-type strain, J166. This result was consistent with the results of gene-array and RNA slot-blot analyses shown in Figs. 1 and 2. Complementation of the hpyIM mutation in strain J166M−/pVU1041 resulted in the re-establishment of wild-type dnaK operon transcript levels in both stationary phase and adherent cells. Quantitation of transcript levels by phosphorimager analysis of the RNA slot blot shown in Fig. 4A and two other RNA slot blots from independent experiments demonstrated the reproducibility of these effects on dnaK operon expression (Fig. 4B). These data indicate that M.HpyI methyltransferase function, encoded by hpyIM, was responsible for the observed alterations in control of dnaK operon transcript levels in J166M−. The complementation observed argues against a role in this phenomenon for undetermined secondary mutations or polarity effects on gene expression as a consequence of hpyIM insertional mutagenesis.

### 3.3. The hpyIM mutant exhibits decreased survival in stationary phase compared to the wild-type

Under the growth conditions we used for the analysis of dnaK operon transcript levels in stationary phase and adherent H. pylori, the hpyIM mutant strain consistently lost viability sooner in stationary phase than the wild-type strain. This is illustrated in Fig. 5A which shows typical growth curves for strains J166, J166M− and the complemented hpyIM mutant J166M+/pVU1041. The growth rates of the hpyIM mutant, wild-type and complemented
hpvIM mutant strains were similar in the exponential phase of growth, and all cultures entered stationary phase after approximately 24 h of incubation. After an additional 24 h of incubation, the density of the hpvIM mutant culture declined relative to cultures of the wild-type and complemented hpvIM mutant. This decline in the culture density of the hpvIM mutant corresponded to an approximately three-fold reduction in the number of CFU ml⁻¹, as determined by viable cell plate counts. Consistent with a loss in viability, RNA isolated from 48-h cultures of the hpvIM mutant strain appeared degraded, whereas RNA from the wild-type and complemented hpvIM mutant appeared intact (Fig. 5B). It should be noted that for the studies reported in this communication, RNA was isolated from cultures of the hpvIM mutant strain prior to the decline in viability and consequent RNA degradation.

4. Discussion

DNA methylation in prokaryotes occurs primarily within the context of R–M systems. However, the Dam methyltransferase of enteric bacteria has been shown to exert a regulatory role in a number of important cellular processes, including the timing of DNA replication, methyl-directed DNA mismatch repair, and the regulation of virulence gene expression [20,21]. In this communication we showed that inactivation of the H. pylori hpvIM gene, encoding a type II DNA methyltransferase, altered the control of dnaK operon transcript levels in stationary phase cultures and following host-cell contact in vitro. These data suggest that DNA methylation, mediated by M.HpyI methyltransferase, may have an important role in H. pylori physiology that is independent of R–M systems.

It is presently unknown how the loss of M.HpyI methyltransferase activity caused the alteration in dnaK operon expression that we observed in these studies. Gene-array experiments showed that hpvIM transcript levels decreased in stationary phase cultures and then increased following host-cell contact. This pattern of expression is consistent with a repressive effect of M.HpyI activity on dnaK operon transcription. However, a direct effect of DNA methylation on dnaK operon transcription, similar to the regulation of transcriptional initiation of the pap operon of E. coli by Dam methyltransferase [22], is unlikely for two reasons. First, the dnaK operon promoter region does not contain the sequence CATG that is recognized by the M.HpyI methyltransferase [16,23]. Second, dnaK operon transcript levels are not induced in exponential phase cultures of the hpvIM mutant strain where M.HpyI methyltransferase activity is completely absent. Other possible mechanisms to account for our observations include alterations in dnaK operon mRNA stability and/or an abnormal increase in the formation of products that induce dnaK operon transcription, e.g., unfolded or partially folded proteins. The decreased viability of the hpvIM mutant strain in stationary phase suggests that M.HpyI methyltransferase activity may function in survival mechanisms that normally operate in stationary phase, and the loss of this activity may result in intracellular conditions that lead to substantial increases in expression of the stress-responsive dnaK operon expression as a consequence of hpvIM inactivation and to elucidate the potential role of DNA methylation and M.HpyI methyltransferase function in H. pylori physiology.

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