Analysis of the role of HP0208, a phase-variable open reading frame, and its homologues HP1416 and HP0159 in the biosynthesis of *Helicobacter pylori* lipopolysaccharide

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The roles of the three ORFs HP0208, HP0159 and HP1416 in the biosynthesis of *Helicobacter pylori* 26695 LPS were investigated in this study. These ORFs represent a paralogous family of genes with homology to the *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S. typhimurium*) *waaJ* gene, which encodes an α -1,2-glycosyltransferase required for core LPS biosynthesis. HP0208 contains multiple tandem repeats of the dimer 5'GA at its 5' end and its expression is predicted to be subject to phase variation. The number of 5'GA repeats present in this ORF was found to be non-permissive for the expression of HP0208 in the majority of *H. pylori* strains examined. To determine a role for this ORF in LPS biosynthesis a non-phase-variable, constitutively expressed variant of HP0208 was constructed and introduced into the genome of *H. pylori* 26695. Analysis of the LPS profile of this strain by Tricine-SDS-PAGE and immunoblotting with anti-Lewis Y antigen (Le^y) mAbs confirmed a role for HP0208 in the biosynthesis of core LPS. A role for HP0159 and HP1416 in the biosynthesis of core LPS was also established. Although homologous to *waaJ*, *H. pylori* HP0208, HP0159 and HP1416 failed to complement an *S. typhimurium waaJ* mutant, suggesting that these ORFs encode functionally different enzymes.

Received 27 July 2004
Accepted 17 March 2005

INTRODUCTION

Helicobacter pylori is a major cause of chronic gastritis and plays a pivotal role in the development of both gastric and duodenal ulcers (Blaser, 1998). Chronic infection with this organism is associated with an increased risk for the development of gastric adenocarcinoma and gastric lymphoma.

LPS has been implicated as a contributory factor in *H. pylori* pathogenesis (reviewed by Monteiro, 2001). Structurally, *H. pylori* LPS comprises lipid A, an inner core of one molecule of 2-keto-3-deoxy-octulosonic acid (KDO) and four molecules of heptose, and, attached to the inner core, an outer core composed of a heteropolymer of the neutral sugars glucose and galactose and an O-antigen side chain. A polymeric α -1,6-glucan chain is also variably present on full-length smooth LPS (S-LPS) in *H. pylori* 26695 (Monteiro *et al.*, 2000).

Structural analysis of the O-antigen moiety of *H. pylori* 26695 LPS has revealed that this molecule contains an elongated,

partially fucosylated *N*-acetylglucosamine (LacNAc) polysaccharide backbone (Aspinall *et al.*, 1996). The fucosylated O-antigen units within the O-antigen side chain have structural identity to the human Lewis X antigen (Le^x) while the terminal O-antigen unit comprises a single Lewis Y (Le^y) determinant (Monteiro *et al.*, 2000). Lewis X structures in the O-antigen have been implicated in the adhesion of *H. pylori* to gastric epithelial cells (Edwards *et al.*, 2000). In contrast, a role for Le^y in pathogenesis has yet to be defined. More recently it has been demonstrated that, in addition to Le^x and Le^y, *H. pylori* strains also have the genetic capacity to synthesize O-antigen determinants with structural identity to Le^a and Le^b (Appelmek *et al.*, 2000; Monteiro *et al.*, 2000). The expression of these Lewis antigens is subject to phase variation, which promotes intra-strain variation in O-antigen structure.

The molecular basis of O-antigen phase variation has been attributed to four genes, *futC*, which encodes an α -1,2-fucosyltransferase (Wang *et al.*, 1999), *galT*, which encodes a β -1,3-galactosyltransferase (Appelmek *et al.*, 2000), and *futA* and *futB*, which encode α -1,3-fucosyltransferases of differing fine specificity (Appelmek *et al.*, 1999; Ge *et al.*, 1997; Martin *et al.*, 1997). All four genes contain a homopolymeric tract of

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Abbreviations: Le^x, Lewis X antigen; Le^y, Lewis Y antigen.

dC within the 5' end of their ORFs. The number of dC residues in each tract can vary as a consequence of slipped-strand mispairing. This results in translational frame shifting, placing the initiation codon in or out of frame with the remainder of the ORF. As a consequence of this mechanism the carbohydrate structure of the O-antigen expressed by *H. pylori* can vary from generation to generation.

In contrast to O-antigen, little is known about the synthesis of core LPS structures, and the repertoire of genes implicated in this process has not been defined. Inspection of the *H. pylori* 26695 LPS structure indicates that there are three biosynthetic steps involved in synthesis of the core, which are predicted to be catalysed by family 8 glycosyl transferases (Coutinho & Henrissat, 1999). This family represents a group of structurally related retaining enzymes that includes the WaaJ family of LPS glycosyl transferases (Heinrichs *et al.*, 1998). Proteins within this group share four highly conserved regions of primary sequence that are implicated in the formation of an α -glycosidic linkage from an α -linked nucleotide diphospho-sugar donor (Shibayama *et al.*, 1998).

In the *H. pylori* 26695 genome sequence (Tomb *et al.*, 1997) three candidate ORFs that encode family 8 glycosyl transferases have been annotated. HP0208, HP0159 and HP1416 are homologues of the *S. typhimurium waaJ* gene that encodes an α -1,2-glycosyltransferase essential for core LPS synthesis. Expression of one of the *H. pylori waaJ* homologues, HP0208, is predicted to be phase variable since it contains multiple tandem repeats of the dimer 5'GA within the 5' end of its ORF. However, the number of 5'GA repeats present is not permissive for expression of the ORF. The same is true for the HP0208 homologue in the published genome sequence of *H. pylori* J99 (Alm *et al.*, 1999). The oligosaccharide structure synthesized by the product of HP0208 is therefore unlikely to be represented in the published LPS structure of 26695 or J99 (Monteiro *et al.*, 2000).

To determine whether the *H. pylori* WaaJ homologues play a role in LPS biosynthesis a constitutively expressed non-phase-variable variant of HP0208 was constructed and the expression of HP0159 and HP1416 was disrupted by insertional mutagenesis. Based upon the LPS profile of the resultant mutants a role for each gene in core LPS biosynthesis is predicted.

METHODS

Bacterial strains, plasmids and culture conditions. *H. pylori* strains were routinely cultured on chocolate agar plates composed of Columbia blood agar base (Difco) (39 g l⁻¹) and horse blood (5% w/v) for 3 days at 37 °C in a microaerobic atmosphere. When appropriate, kanamycin (15 µg ml⁻¹) and chloramphenicol (35 µg ml⁻¹) were added to the media. *Escherichia coli* and *Salmonella enterica* serovar Typhimurium strains were cultivated in Luria broth supplemented with ampicillin (100 µg ml⁻¹), kanamycin (100 µg ml⁻¹) or chloramphenicol (35 µg ml⁻¹) where necessary. Solid medium was made by the addition of Bacto agar (1% w/v). All *Salmonella* strains used in this study were obtained from the Salmonella Genetic Stock Centre (<http://www.acs.ualgaly.ca/~kesander/about.html>).

Tricine-SDS-PAGE of LPS. LPS samples were prepared as previously described (Kimura & Hansen, 1986). To visualize core LPS profiles, purified LPS was resolved by Tricine (T)-SDS-PAGE according to the method described by Schagger & von Jagow (1987). To resolve O-antigen structures 16% SDS-PAGE gels were used. Following electrophoresis LPS was visualized by silver staining (Fomsgaard *et al.*, 1990).

Detection of LPS structures by immunoblotting. Bacterial colonies bound to nitrocellulose or Western transfers of fractionated LPS on nitrocellulose membranes were incubated at room temperature for a minimum of 2 h in PBS containing 3% (w/v) bovine serum albumin (PBS-B). This blocking solution was washed off with a solution of PBS containing 0.1% Tween 20 (PBS-Tween). Anti-Le^x mAbs (Calbiochem) were diluted appropriately in PBS-B, added to the filter and the incubation allowed to proceed at room temperature for 2 h. Unbound mAb was removed by five washes with PBS-Tween. The filters were then incubated for a further 2 h at room temperature with an alkaline phosphatase-conjugated sheep anti-mouse antibody diluted in PBS-B. After washing in PBS-Tween, the reaction was developed by the addition of alkaline phosphatase buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM IPTG, 5 mM MgCl₂) containing nitroterazolium blue (330 µg ml⁻¹) and bromochloroindolylphosphate (330 µg ml⁻¹).

DNA methodology. Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer Mannheim. Standard methods were used for restriction enzyme digestions, ligations, transformations and for the preparation of plasmid DNA from *E. coli*. Electroporation of *H. pylori* was carried out as described elsewhere (Ferrero *et al.*, 1992). *S. enterica* serovar Typhimurium (hereafter *S. typhimurium*) strains were transformed by electroporation using the method described by Sambrook & Russell (2001).

PCR. All of the oligonucleotide primers used for the PCR reactions described in this study are listed in Table 1. PCR reactions were carried out in a buffer containing 50 mM KCl, 10 mM Tris/HCl, pH 8, 2.5 mM MgCl₂ and 1% (w/v) gelatin. The reaction mixture was heated to 94 °C for 4 min prior to PCR amplification, which comprised 30 cycles of denaturation at 94 °C for 30 s, annealing at 2 °C below T_m of oligonucleotide pairs for 1 min and extension at 72 °C for 2 min.

Construction of a constitutively expressed HP0208 variant. A constitutively expressed variant of HP0208 was generated in a one-step PCR reaction. A 1700 bp fragment, comprising the 5' end of HP0208 and the adjacent ORF HP0209, was amplified from NCTC11637 chromosomal DNA by PCR using primers RL15 and RL17, which generated *Xba*I and *Xho*I restriction sites at the 5' ends of the PCR product, respectively (Fig. 1A). The required changes to the 5'GA tract that abolished phase variation and resulted in constitutive expression of HP0208 were incorporated into RL17 and introduced into the PCR product during amplification. The nucleotide sequence of RL17 and the modified 5'GA tract it generated is depicted in Fig. 1(B). The PCR product generated was digested with *Xho*I and *Xba*I and cloned into *Xba*I/*Xho*I-digested pBluescript SK+ (pBS). The resultant plasmid was then linearized at a unique *Bam*HI site within HP0209 and ligated to a 1.6 kb *Bam*HI fragment, derived from pUC4K, containing a kanamycin-resistance gene cassette. This plasmid was subsequently introduced into *H. pylori* 26695 by electroporation and the resultant strain termed *H. pylori* HP0208-ON.

Determination of the number of 5'GA repeats in HP0208. A 254 bp region encompassing the 5'GA repeats was amplified using primers MG3 and RL6. The resultant PCR product was then directly sequenced using the University of Manchester, School of Biological Sciences, in-house sequencing service.

Table 1. Oligonucleotides used in this study

Oligonucleotide	ORF	Restriction site	Nucleotide sequence (5'–3')*
MG1	HP0208	<i>Xho</i> I	GAGATTATCCCTATTCTCGAGGC
MG2	HP0208	<i>Xba</i> I	CCCTTCTAGACAGTCGCTTCACT
MG3	5'GA repeats	–	GGCGTGGTTCTAATTCCTTATC
RL6	5'GA repeats	–	GAGATTATCCCTATTGTCGTGGC
RL2	HP1416	<i>Xho</i> I	GTATTTGATGAACCTCGAGCATGATG
RL3	HP1416	<i>Eco</i> RI	GGATACAGAATTCTTACCCCGATCAAATTATCC
RL10	HP0159	–	ATGAGGATCCTTATTCCTATTGTCATCGC
RL12	HP0159	–	AAAGGATTCTTTTAAGCGTTTGAAAATCC
RL15	HP0208-ON	<i>Xba</i> I	GATAACTCTAGAGCTCATCAAATAGCTTCTG
RL17	HP0208-ON	<i>Xho</i> I	CGCTTAAATTCTCGAGCAAACAATGGATTTGATAAAAGAGCTTTACACGTTTCGCGT TCTCGTTTGGCGTTTGCTAGCATGAAAAATAAGC
EXP1	HP0208 His tag	<i>Sac</i> I	GACTAGAGCTCCAAGAGATTATC
EXP2	HP0208 His tag	<i>Pst</i> I	AACCCCTGCAGGCTCGCATTAG
EXP3	HP1416 His tag	<i>Sac</i> I	CGCTTGAGCTCAGTATTATTAT
EXP4	HP1416 His tag	<i>Pst</i> I	CGCCTGCAGCGTGTTGCCAAAC
EXP5	HP0159 His tag	<i>Sac</i> I	GGATGAGCTCCTGGATACACAA
EXP6	HP0159 His tag	<i>Pst</i> I	AAATTCTGCAGCTAAAATGACGC

*Restriction sites are indicated by underlined base.

Insertional mutagenesis of the *H. pylori* family 8 glycosyltransferase genes. A 1200 bp fragment encompassing HP0208 was amplified from *H. pylori* MO19 (Falk *et al.*, 1993) chromosomal DNA by PCR using primers MG1 and MG2, which generated *Xba*I and *Xho*I restriction endonuclease recognition sites, respectively, at the 5' ends of the PCR product. The resultant PCR product was digested with *Xho*I and *Xba*I and ligated to *Xba*I/*Xho*I-digested pBS. HP0208 was then mutated by insertion of a chloramphenicol acetyltransferase (CAT) gene cassette isolated from plasmid pCAT (Wang & Taylor, 1990). pCAT was digested with *Hind*III and cloned into a unique *Eco*RI site within the HP0208 following Klenow treatment of all sticky ends.

HP1416 was amplified from *H. pylori* 26695 chromosomal DNA with primers RL2 and RL3, which generated *Xho*I and *Eco*RI, respectively, at the 5' ends of the PCR product. The resultant 1000 bp PCR product was then digested with *Eco*RI and *Xho*I and cloned into *Eco*RI/*Xho*I-digested pBS. Mutagenesis of HP1416 was achieved by insertion of an 800 bp *Hind*III fragment harbouring the CAT gene cassette into a unique *Hind*III restriction site in this plasmid. A 1100 bp PCR product harbouring HP0159 was amplified from *H. pylori* 26695 chromosomal DNA with primers RL10 and RL12. Following treatment with Klenow to generate blunt ends, the PCR product was cloned into *Eco*RV-digested pBS. The resultant plasmid was then linearized at a unique *Hind*III site within HP0159 and ligated to an 800 bp *Hind*III fragment harbouring the CAT gene cassette.

In all cases, ligation reactions were transformed into *E. coli* DH5 α and recombinant clones selected following growth on L-agar supplemented with ampicillin and chloramphenicol. Plasmids harbouring the mutated glycosyltransferase genes were subsequently introduced into *H. pylori* strains by electroporation.

Generation of N-terminal His-tagged recombinant HP0208, HP1416 and HP0159. The HP0208 ORF was amplified by PCR from NCTC11637 HP0208-ON chromosomal DNA with primers EXP1 and EXP2, which generated *Sac*I and *Pst*I restriction endonuclease sites, respectively, at the 5' ends of the PCR product. The 1250 bp PCR

product generated was digested with *Sac*I and *Pst*I and cloned into *Sac*I/*Pst*I-digested pQE30 (Qiagen), generating plasmid pQEHP0208-ON.

The HP1416 ORF was amplified from strain 26695 chromosomal DNA with primers EXP3 and EXP4, containing *Sac*I and *Pst*I restriction sites, respectively. The HP0159 ORF was amplified from strain 26695 using primers EXP5 and EXP6, which introduced *Sac*I and *Pst*I restriction sites into the PCR product, respectively. The 1200 bp PCR products generated were digested with *Sac*I and *Pst*I and cloned into *Sac*I/*Pst*I-digested pQE30, generating plasmids pQEHP1416 and pQEHP0159. All expression constructs were transformed into *E. coli* M15(pRep4) (Qiagen; Gottesman *et al.*, 1981) and recombinant clones were selected by growth on L-agar supplemented with ampicillin.

Generation of anti-HP0208 polyclonal antiserum. Plasmid pQEHP0208-ON was transformed into *E. coli* M15(pRep4). A 1 l culture was inoculated with 20 ml of an overnight culture of this strain and grown until an OD₆₀₀ of 0.6 was reached. Expression was then induced by the addition of IPTG to a final concentration of 1 mM. The culture was incubated for a further 5 h and bacteria harvested by centrifugation. The His-tagged HP0208 protein was then solubilized in 6 M guanidium hydrochloride and purified with Ni-NTA resin (Qiagen) according to the manufacturer's instructions. The Ni-NTA purified sample was then fractionated by SDS-PAGE and the over-expressed protein excised from the unstained polyacrylamide gel to purify the recombinant protein to homogeneity. To facilitate excision of the required peptide an equivalent gel was run simultaneously and stained with Coomassie blue to enable localization of the HP0208 protein on the preparative gel. Recombinant HP0208 protein was electroeluted from the polyacrylamide gel using a Bio-Rad model 422 electroeluter at 40 V overnight with constant stirring. The eluted protein was then concentrated and desalted in a 15 ml Centrplus YM-10 centrifugal filter device (Millipore). Rabbit polyclonal antiserum was then prepared from the purified protein by Harlan Seralab using a standard 77-day protocol.

SDS-PAGE analysis of recombinant protein expression. Whole-cell extracts were resolved on a 12 % SDS-PAGE gel. The presence of

cially producing a 'phase on' mutant the phenotypic impact that the expression of HP0208 would have on the profile of NCTC11637 LPS could be determined.

To generate a non-phase-variable, constitutively expressed HP0208 variant, the 5'GA repeat region within the ORF was modified to eliminate slipped-strand mispairing and the reading frame altered to ensure expression of the full-length HP0208 ORF. This was achieved by replacing the guanine bases at 99 and 105 bp from the translational start of HP0208 with adenine, the adenine bases at positions 100, 102 and 106 with cytosine, and the adenine base at position 108 with thymidine (Fig. 1B). To ensure constitutive expression, two copies of 5'GA were deleted, placing the ORF in-frame. This mutation was generated using a one-step PCR strategy, which resulted in the generation of a 1700 bp PCR product consisting of the 5' end of HP0208, containing the desired mutation, and the adjacent ORF HP0209 (Fig. 1A). This procedure is described in detail in Methods.

The resultant PCR product was cloned into pBS SK+. Selection for *H. pylori* recombinants containing the 5'GA mutation was facilitated by insertion of a kanamycin-resistance gene cassette into HP0209, an ORF adjacent to the 5' end of HP0208 (Fig. 1A). This ORF encodes a hypothetical protein with no role in LPS biosynthesis and does not influence the LPS phenotype of the *H. pylori* 5'GA mutant (N. J. High, unpublished observation). Confirmation that co-transformation of the 5'GA mutation had occurred in kanamycin-resistant transformants was obtained by PCR amplification of the region surrounding the 5'GA mutation and direct sequencing of the PCR product. The detailed methodology for the construction of this mutant, referred to as *H. pylori* HP0208-ON to reflect the fact that the HP0208 ORF is constitutively expressed, is outlined in Methods.

Attempts to introduce the HP0208-ON mutation into *H. pylori* 26695 were unsuccessful and so NCTC11637 was used as the recipient for this experiment. Whilst the LPS expressed by these two strains may display structural differences, the enzymes encoded by HP0208 are predicted to have the same function in both backgrounds.

T-SDS-PAGE analysis of the LPS expressed by *H. pylori* HP0208-ON

To determine the impact of the *H. pylori* HP0208-ON mutation on LPS biosynthesis, samples of purified LPS were separated by T-SDS-PAGE and visualized by silver staining (Fig. 2A). Comparison of the LPS profile of the *H. pylori* HP0208-ON mutant with that of wild-type *H. pylori* LPS revealed that constitutive expression of HP0208 had altered the LPS profile. In the *H. pylori* HP0208-ON LPS sample the second-fastest migrating LPS species (indicated by an arrow in Fig. 2A) was much more intensely stained than the equivalent species in wild-type *H. pylori* LPS. In contrast, the relative intensity of all the other LPS species in both samples was comparable. This observation suggested that in *H. pylori* HP0208-ON there was increased expression of an LPS species that was either absent or in low abundance in the

Fig. 2. Characterization of the phenotype of the LPS NCTC11637 HP0208-ON mutant. (A) Analysis of LPS samples by T-SDS-PAGE and silver staining. The region of the gel depicting the core LPS structures is presented. The location of the LPS species with increased abundance in NCTC11637 HP0208-ON is indicated by an arrow. (B) A Western transfer of purified LPS fractionated by SDS-PAGE and probed with anti-Le^y mAbs. The region of the Western transfer depicting the O-antigen side chain is presented.

wild-type strain. Alterations in band intensities could also be due to different substitutions on the glycoforms of the wild-type and mutant that may alter reactivity to the silver stain rather than simply reflecting changes in quantity. That the increased intensity of this band is caused by expression of a novel, co-migrating LPS species also remains a possibility. In the absence of a chemical structure for this mutant, however, the precise nature of this LPS species remains open to speculation.

To determine whether constitutive expression of HP0208 had affected biosynthesis of the O-antigen, purified LPS was separated by SDS-PAGE and transferred onto a nitrocellulose membrane by Western blotting. The blot was then probed with anti-Le^y mAbs (Fig. 2B). No apparent difference in the reactivity of the *H. pylori* HP0208-ON O-antigen and wild-type *H. pylori* O-antigen was observed.

HP0208 ORF is expressed in nature

To determine whether expression of the HP0208 ORF could be detected in wild-type NCTC11637 and strains of *H. pylori* in which the predominant number of 5'GA repeats is permissive for the expression of the HP0208 ORF, such as MO19, an antiserum was generated against the HP0208-ON polypeptide. To achieve this, the HP0208-ON ORF was

amplified from NCTC11637 HP0208-ON by PCR and cloned into the expression vector pQE30 generating plasmid pQEHP0208-ON. This plasmid was then transformed into *E. coli* M15(pRep4) and expressed as a recombinant protein with an N-terminal His tag. pRep carries *laqI*, which encodes the Lac repressor and allows controlled expression of recombinant proteins. The his-tagged protein was then purified to homogeneity and used as an immunogen to generate polyclonal rabbit antiserum. These procedures are outlined in detail in Methods.

Western transfers of whole-cell extracts of *H. pylori* NCTC11637, 26695, J99, SS1, P446 and MO19 were probed with anti-HP0208-ON polyclonal antiserum (Fig. 3). An *E. coli* M15(pRep4)(pQEHP0208-ON) cell extract was used as a positive control for reactivity of the polyclonal antiserum. In this experiment anti-HP0208 reactive bands of approximately 49 kDa and 47 kDa were identified in the MO19 sample and the NCTC11637 HP0208-ON sample, respectively. This confirmed that expression of HP0208 can occur naturally in *in vitro* grown *H. pylori*. In contrast, no reactive bands were detected in the cell extracts from any of the other strains tested, confirming that HP0208 is not expressed, as predicted by the number of 5'GA repeats present in the ORF. The absence of detectable HP0208 expression suggests that, in populations of these strains, organisms expressing this ORF are either in low abundance, or completely absent. In support of this hypothesis, when immunoblots of approximately 3000 NCTC11637 colonies were probed with anti-HP0208-ON antiserum, no reactive colonies were observed (data not shown). This suggests that off-to-on phase-switching of HP0208 must occur at a frequency of 10^{-3} per cell per generation or less.

Generation of an HP0208 insertion mutation in *H. pylori* strain MO19

To provide further evidence that HP0208 plays a role in the biosynthesis of *H. pylori* LPS the ORF was disrupted by insertional mutagenesis in MO19 as outlined in Methods. That expression of HP0208 had been abolished was determined by probing Western blots of MO19HP0208 with anti-HP0208-ON polyclonal antiserum (Fig. 4A). The LPS profile of the resultant mutant was then compared to that of wild-type MO19 by T-SDS-PAGE analysis of purified LPS samples

(Fig. 4B). In the MO19HP0208 mutant an additional LPS species, of higher molecular mass than the core LPS species present in wild-type MO19 LPS, was observed (indicated by i in Fig. 4B). In addition, a ladder-like pattern of LPS species, reminiscent of the synthesis of a polymeric carbohydrate structure was also apparent in the mutant. Based upon this observation it appears that in MO19 expression of HP0208 may modify the structure of an LPS acceptor either by adding a different terminal sugar or by generating an alternative sugar linkage such that the activity of at least one of the glycosyl transferases expressed by this strain is inhibited.

Western blots of equivalent gels probed with an anti-Le^x mAb revealed no detectable difference in the O-antigen structures expressed by wild-type MO19 and the MO19-HP0208 mutant, confirming that mutagenesis of HP0208 affects only the core oligosaccharide structure (Fig. 4C).

Characterization of the roles of HP1416 and HP0159 in LPS biosynthesis

HP1416 and HP0159 encode proteins that are 42 % and 30 % identical to HP0208, respectively. To determine whether these ORFs also play a role in LPS biosynthesis, insertion mutations were introduced into each ORF as outlined in Methods. PCR amplification and insertional mutagenesis were only successfully achieved for HP1416 when using NCTC11637 chromosomal DNA as a template, suggesting that HP0159 is either not present in this strain or its nucleotide sequence differs from that of strain 26695 in the region of the gene from which PCR primers were designed. As a consequence all mutagenesis experiments were subsequently carried out in *H. pylori* 26695.

To determine the phenotype of each mutant, purified LPS was analysed by T-SDS-PAGE and compared to that of wild-type *H. pylori* 26695 (Fig. 5A). As depicted in Fig. 5(A) the HP1416 mutant expresses the same repertoire of core LPS species as wild-type *H. pylori*. However, one LPS species that is barely detectable in wild-type *H. pylori* is present in greater abundance in the HP1416 mutant. In the HP0159 mutant, the two predominant LPS species apparent in the wild-type LPS sample are absent and replaced by a strongly stained lower-molecular-mass LPS species, indicating that truncation of the core had occurred.

Fig. 3. HP0208 expression in *H. pylori* strains was analysed by probing a Western transfer of whole-cell lysates, separated by SDS-PAGE, with anti-HP0208 polyclonal antiserum. Cell lysate from *E. coli* M15(pRep4)(pQEHP0208-ON) was used as a positive control for reactivity of the anti-HP0208 antiserum. Molecular mass markers are indicated on the left of the Western blot.

Fig. 4. SDS-PAGE analyses showing the effect of insertional mutagenesis of HP0208 on the LPS profile of *H. pylori* MO19. (A) Western transfers of cell lysates from NCTC11637 HP0208-ON, MO19 and MO19HP0208 probed with anti-HP0208 antiserum. (B) Purified LPS samples from MO19 and MO19HP0208 were separated by T-SDS-PAGE and silver stained. A novel LPS species present in MO19HP0208 is indicated by i. The relative positions of O-antigen and core LPS species are indicated by braces. (C) Western transfers of LPS separated by T-SDS-PAGE probed with an anti-Le^y mAb. The Le^y-reactive O-antigen LPS species is indicated.

To investigate the impact of these mutations on O-antigen biosynthesis Western transfers of equivalent gels were probed with an anti-Le^y Mab (Fig. 5B). From these experiments it was revealed that insertion mutations in HP0159 had no effect on O-antigen biosynthesis. In contrast, no Le^y-reactive structures were expressed by the 26695 HP1416 mutant and no O-antigen was synthesized, as confirmed by silver-stained SDS-PAGE gels (Fig. 5C).

To further characterize the roles played by HP1416 and HP0159 in LPS biosynthesis, each mutation was introduced into an *H. pylori* 26695 *galE*-null mutant (Edwards *et al.*, 2000) and its impact on LPS biosynthesis determined by T-SDS-PAGE analysis of purified LPS samples (Fig. 6). Insertional mutagenesis of *galE* results in the expression of a galactose-deficient LPS that lacks O-antigen and is composed of a highly truncated core with an abundant glucan chain α -Glc-(1-[-6]- α -Glc-(1-)]_n-2)-. When visualized on T-SDS-PAGE the glucan chain appears as a diffuse band in *H. pylori* 26695 *galE* (Fig. 6) and a ladder of low-molecular-mass LPS species in *H. pylori* NCTC11637 *galE* (Edwards *et al.*, 2000). Insertional mutagenesis of HP1416 resulted in loss of the diffuse band, representing the glucan chain, and an increase in abundance of a high-molecular-mass core-LPS species, which was present at negligible levels the 26695 *galE* LPS

sample. Synthesis of the glucan chain was also abolished following insertional mutagenesis of HP0159; however, the rest of the core appeared unaltered.

Complementation of a *S. typhimurium waaJ* mutant with HP0208, HP1416 and HP0159

To obtain further information about the functional role played by the *H. pylori* WaaJ homologues HP0208, HP1416 and HP0159, their ability to *trans* complement an *S. typhimurium waaJ* mutant was determined. To achieve this, each ORF was cloned into pQE30 and expressed as a recombinant protein with an N-terminal His tag in the *S. typhimurium* wild-type strain SL3770 *waa+* and SL375 *waaJ417*, which lacks a functional copy of *waaJ* (Roantree *et al.*, 1977), as outlined in Methods. In all three cases no complementation of the *S. typhimurium waaJ* mutation was observed. The catalytic activity of the *H. pylori* WaaJ homologues is therefore concluded to be distinct from that of the *S. typhimurium* WaaJ (MacLachlan *et al.*, 1991).

DISCUSSION

The *H. pylori* 26695 genome contains three genes HP0208, HP1416 and HP0159 that encode family 8 glycosyl trans-

Fig. 5. Characterization of the effect of insertional mutagenesis of HP1416 and HP0159 on the LPS profile of *H. pylori* 26695. (A) Silver-stained T-SDS-PAGE gel of purified LPS samples. The novel high-molecular-mass core-LPS species expressed by 26695HP1416 is indicated by i and the truncated LPS species expressed by 26695HP0159 is indicated by ii. (B) Western transfers of LPS fractionated by SDS-PAGE and probed with anti-Le^Y mAbs. (C) Silver-stained SDS-PAGE gel of LPS samples. The relative positions of the O-antigen side chain and core LPS are indicated on the right of the gel.

ferases. One of the genes in this family, HP0208, contains multiple tandem repeats of 5'GA within the 5' end of its ORF, suggesting that its expression is subject to phase variation. The number of repeats present in this gene varies and, of the strains surveyed in this study, is only consistent with expression in MO19, a strain isolated from an asymptomatic infection. Similar observations have also been made by Salaun *et al.* (2004), who reported that of 20 *H. pylori* isolates from diverse geographical locations only one strain was capable of expressing HP0208, as predicted by the number of 5'GA repeats. Moreover, in this study no evidence of phase switching in an off-to-on direction was obtained for HP0208 by immunoblotting NCTC11637 colonies using an anti-HP0208 polyclonal antiserum, suggesting that this event occurs rarely during *in vitro* growth. These observations indicate that the necessary selective pressure to enrich for *H. pylori* populations that predominately express HP0208 is likely to be encountered exclusively within a human host, at a specific stage in pathogenesis.

In support of this hypothesis the phase-variable expression of pili in *Haemophilus influenzae* has also been shown to be strongly influenced by *in vitro* growth. Following subculture, clinical isolates of *Haemophilus influenzae* isolated from the nasopharynx rapidly lose the capacity to express pili (Apicella *et al.*, 1984). This phenotypic change correlates with a shift in the number of 5'TA repeats present in the promoter region situated between *hifA*, which encodes the pilin-subunit

protein, and *hifB*, which encodes a chaperonin (van Ham *et al.*, 1993). When combined with buccal epithelial cells, a phase switch occurs and non-piliated micro-organisms become piliated within 30 h, enabling adhesion of *Haemophilus influenzae* to occur (Patrick *et al.*, 1989). This example indicates that selection pressure within the host can be critical in maintaining the expression of certain phase-variable phenotypes. Accordingly, *H. pylori* strains isolated directly from the human stomach may contain a number of 5'GA repeats permissive for the expression of HP0208, but rapidly lose this ability following subculture. Why MO19 retains the ability to express HP0208 is unclear, but may reflect the fact that this strain, unlike the others, was isolated from an asymptomatic infection.

To mimic the phenotypic impact of switching-on the expression of HP0208 through phase variation a constitutively expressed variant of HP0208 was generated and introduced into the genome of NCTC11637. This mutant had an altered core-LPS profile relative to the wild-type strain. A role for HP0208 in core-LPS biosynthesis was further confirmed by insertional mutagenesis of the equivalent ORF in MO19, which also resulted in the synthesis of a modified core-LPS structure. So, although naturally occurring 'phase on' and 'phase off' HP0208 variants have yet to be identified, the phenotypes of the two mutants generated in this study

Fig. 6. SDS-PAGE analysis of the effect of insertional mutagenesis of HP0208, HP1416 and HP0159 on the LPS profile of 26695galE. The region of the gel depicting the core LPS structures is presented. The position of the glucan chain expressed by 26695galE and the novel core-LPS species expressed by 26695gal/EHP1416 are indicated by i and ii, respectively.

suggest that phase-variable LPS structures do occur in the core oligosaccharide and are not restricted to the O-antigen side chain.

Based upon cumulative information derived from phase-variable LPS genes in organisms such as *Haemophilus influenzae* (Weiser & Pan, 1998) and *Neisseria gonorrhoeae* (van Putten, 1993) it is reasonable to assume that phase variation of HP0208 contributes to the niche adaptation of *H. pylori*. Its precise role, however, awaits determination. In MO19, the loss of a functional copy of HP0208 results in abundant synthesis of a polymeric oligosaccharide species that, in the absence of structural data, is predicted to be the α -Glc-(1-[-6]- α -Glc-(1-)]_n-2)- glucan chain. Based upon studies carried out using the non-typable *H. pylori* strain PJ2 this structure has been implicated in colonization of the murine stomach (Altman *et al.*, 2003). If phase variation of HP0208 dictates whether or not the glucan chain is expressed, then it might be anticipated that expression of this ORF would need to be switched off to facilitate colonization of the murine stomach.

Two homologues of the phase-variable HP0208 ORF, HP1416 and HP0159, are also present in the genome of *H. pylori* 26695, both of which are implicated in core LPS biosynthesis. Insertional mutagenesis of HP1416 abolished O-antigen biosynthesis in strain 26695 and biosynthesis of the glucan chain in 26695galE. The apparent loss of both

structures from a mutation in a single ORF suggests that the polymerization of O-antigen may be dependent upon the prior synthesis of the glucan chain, or vice versa. The phenotype of the HP0159 LPS mutants indicates that this ORF is required for the synthesis of both the outer core and the glucan chain, suggesting that ordered synthesis of these structures is likely to occur. An interesting feature of the 26695HP0159 mutant is that although it has a more severely truncated outer-core LPS than the 26695HP1416 mutant it is still able to synthesize O-antigen. This distinction may be reflected in structural differences in the residual glucan chain expressed by these two mutants. Comparative LPS structural analysis is therefore essential to conclusively determine the biosynthetic steps that trigger the initiation of O-antigen synthesis/polymerization.

In summary, it has been demonstrated that the phase-variable ORF HP0208 plays a role in core oligosaccharide biosynthesis. This suggests that, in addition to the O-antigen side chain, a structure within the core oligosaccharide moiety of *H. pylori* LPS is also subject to phase variation. HP1416 and HP0159, which are homologues of HP0208, are also implicated in core-LPS biosynthesis. Analysis of the LPS phenotypes of strains carrying mutations in these ORFs suggests that core oligosaccharide and O-antigen biosynthesis in *H. pylori* is a complex process and that ordered addition of sugar units is crucial for the formation of the branched LPS structure characteristic of this organism.

ACKNOWLEDGEMENTS

Rebecca Langdon was funded by a BBSRC studentship. Work in N. J. H.'s laboratory is funded by the Wellcome Trust.

REFERENCES

- Alm R.A., Ling L.S., Moir D.T. and 20 other authors (1999). Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* **397**, 176–180.
- Altman, E., Smirnova, N., Li, J., Aubry, A. & Logan, S. M. (2003). Occurrence of a nontypable *Helicobacter pylori* strain lacking Lewis blood group O antigens and DD-heptoglycan: evidence for the role of the core 1,6-glucan chain in colonization. *Glycobiology* **13**, 777–783.
- Apicella, M. A., Shero, M., Dudas, K. C., Stack, R. R., LaScolea, L. J., Murphy, T. F. & Mylotte, J. M. (1984). Fimbriation of *Haemophilus influenzae* species isolated from the respiratory tract of adults. *J Infect Dis* **150**, 40–43.
- Appelmek, B. J., Martin, S. L., Monteiro, M. A. & 10 other authors (1999). Phase variation in *Helicobacter pylori* lipopolysaccharide due to changes in the lengths of poly(C) tracts in 3-fucosyltransferase genes. *Infect Immun* **67**, 5361–5366.
- Appelmek, B. J., Martino, M. C., Veenhof, E. & 7 other authors (2000). Phase variation in H type I and Lewis a epitopes of *Helicobacter pylori* lipopolysaccharide. *Infect Immun* **68**, 5928–5932.
- Aspinall, G. O., Monteiro, M. A., Pang, H., Walsh, E. & Moran, A. P. (1996). Lipopolysaccharides of *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504): Structures of the O-antigen and core oligosaccharide regions. *Biochemistry* **35**, 2489–2497.
- Blaser, M. J. (1998). *Helicobacter pylori* and gastric diseases. *Br Med J* **316**, 1507–1510.

- Coutinho, P. M. & Henrissat, B. (1999).** Carbohydrate-active enzymes: an integrated database approach. In *Recent Advances in Carbohydrate Bioengineering*, pp. 3–12. Edited by H. J. Gilbert, G. Davies, B. Henrissat & B. Svensson. Cambridge: The Royal Society of Chemistry.
- Edwards, N. J., Monteiro, M. A., Faller, G., Walsh, E. J., Moran, A. P., Roberts, I. S. & High, N. J. (2000).** Lewis X structures in the O-antigen side chain promote adhesion of *Helicobacter pylori* to the gastric epithelium. *Mol Microbiol* **35**, 1530–1539.
- Falk, P., Roth, K. A., Boren, T., Westblom, T. U., Gordon, J. I. & Normark, S. (1993).** An *in vitro* adherence assay reveals that *Helicobacter pylori* exhibits lineage-specific tropism in the human gastric epithelium. *Proc Natl Acad Sci U S A* **90**, 2035–2039.
- Ferrero, R. L., Cussac, V., Courcoux, P. & Labigne, A. (1992).** Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. *J Bacteriol* **174**, 4212–4217.
- Fomsgaard, A., Freudenberg, M. A. & Galanos, C. (1990).** Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. *J Clin Microbiol* **28**, 2627–2631.
- Ge, Z., Chan, N. W. C., Palcic, M. M. & Taylor, D. E. (1997).** Cloning and heterologous expression of an 1,3 fucosyltransferase gene from the gastric pathogen *Helicobacter pylori*. *J Biol Chem* **272**, 21357–21363.
- Gottesman, C. S., Halpern, E. & Trisler, P. (1981).** Role of *sulA* and *sulB* in filamentation by Lon mutants of *Escherichia coli* K-12. *J Bacteriol* **148**, 265–273.
- Heinrichs, D. E., Montiero, M. A., Perry, M. B. and Whitfield, C. (1998).** The assembly system for the lipopolysaccharide R2 core-type of *Escherichia coli* K-12 and *Salmonella enterica*. Structure and function of WaaK and WaaL homologues. *J Biol Chem* **273**, 8849–8859.
- Kimura, A. & Hansen, E. J. (1986).** Antigenic and phenotypic variants of *Haemophilus influenzae* type b lipopolysaccharide and their relationship to virulence. *Infect Immun* **51**, 69–79.
- Lee, A., O'Rourke, J., De Ungria, M. C., Robertson, B., Daskalopoulos, G. & Dixon, M. F. (1997).** A standardised mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* **112**, 1386–1397.
- MacLachlan, P. R., Kadam, S. K. & Sanderson, K. E. (1991).** Cloning, characterisation, and DNA sequence of the *rfa* region for lipopolysaccharide synthesis in *Salmonella typhimurium* LT2. *J Bacteriol* **173**, 7151–7163.
- Marshall, B. J. & Warren, J. R. (1984).** Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **i**, 1311–1315.
- Martin, S. L., Edbrooke, M. R., Hodgman, T. C., van den Eijnden, D. H. & Bird, M. I. (1997).** Lewis X biosynthesis in *Helicobacter pylori*. Molecular cloning of an alpha(1,3)-fucosyltransferase gene. *J Biol Chem* **272**, 21349–21356.
- Monteiro, M. A. (2001).** *Helicobacter pylori*: a wolf in sheep's clothing: the glyco-type families of *Helicobacter pylori* lipopolysaccharides expressing histo-blood groups: structure, biosynthesis, and role in pathogenesis. *Adv Carbohydr Chem Biochem* **57**, 99–158.
- Monteiro, M. A., Appelmek, B. J., Rasko, D. A. & 10 other authors (2000).** Lipopolysaccharide structures of *Helicobacter pylori* genomic strains 26695 and J99, mouse model *H. pylori* Sydney strain, *H. pylori* P466 carrying sialyl Lewis X, and *H. pylori* UA915 expressing Lewis B classification of *H. pylori* lipopolysaccharides into glyco-type families. *Eur J Biochem* **267**, 305–320.
- Patrick, C. C., Patrick, G. S., Kaplan, S. L., Barrish, J. & Mason, E. O. (1989).** Adherence kinetics of *Haemophilus influenzae* type b to eucaryotic cells. *Pediatr Res* **26**, 500–503.
- Roantree, R. J., Kuo, T. T. & MacPhee, D. G. (1977).** The effect of defined lipopolysaccharide core defects upon antibiotic resistances of *Salmonella typhimurium*. *J Gen Microbiol* **103**, 223–234.
- Salaun, L., Linz, B., Suerbaum, S. & Saunders, N. J. (2004).** The diversity within an expanded and redefined repertoire of phase-variable genes in *Helicobacter pylori*. *Microbiology* **150**, 817–830.
- Sambrook, J. & Russell, D. W. (2001).** Transformation of *E. coli* by electroporation. In *Molecular Cloning: a Laboratory Manual*, pp. 1–119. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schagger, H. & von Jagow, G. (1987).** Tricine-sodium dodecylsulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. *Anal Biochem* **166**, 368–379.
- Shibayama, K., Ohsuka, S., Tanaka, T., Arakawa, Y. & Ohta, M. (1998).** Conserved structural regions involved in the catalytic mechanism of *Escherichia coli* K-12 WaaO (RfaI). *J Bacteriol* **180**, 5313–5318.
- Tomb, J. F., White, O., Kerlavage, A. R. & 39 other authors (1997).** The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**, 539–547.
- van Ham, S. M., van Alphen, L., Mooi, F. R. & van Putten, J. P. (1993).** Phase variation of *H. influenzae* fimbriae: transcriptional control of two divergent genes through a variable combined promoter region. *Cell* **73**, 1187–1196.
- van Putten, J. P. (1993).** Phase variation of lipopolysaccharide directs the interconversion of invasive and immuno-resistant phenotypes of *Neisseria gonorrhoeae*. *EMBO J* **12**, 4043–4051.
- Wang, Y. & Taylor, D. E. (1990).** Chloramphenicol resistance in *Campylobacter coli*: nucleotide sequence, expression and cloning vector construction. *Gene* **94**, 23–28.
- Wang, G., Boulton, P. G., Chan, N. W., Palcic, M. M. & Taylor, D. E. (1999).** Novel *Helicobacter pylori* alpha1,2-fucosyltransferase, a key enzyme in the synthesis of Lewis antigens. *Microbiology* **145**, 3245–3253.
- Weiser, J. N. & Pan, N. (1998).** Adaptation of *Haemophilus influenzae* to acquired and innate humoral immunity based on phase variation of lipopolysaccharide. *Mol Microbiol* **30**, 767–775.