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Variations in *Helicobacter pylori* Lipopolysaccharide To Evade the Innate Immune Component Surfactant Protein D

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***Helicobacter pylori* is a common and persistent human pathogen of the gastric mucosa. Surfactant protein D (SP-D), a component of innate immunity, is expressed in the human gastric mucosa and is capable of aggregating *H. pylori*. Wide variation in the SP-D binding affinity to *H. pylori* has been observed in clinical isolates and laboratory-adapted strains. The aim of this study was to reveal potential mechanisms responsible for evading SP-D binding and establishing persistent infection. An escape variant, J178V, was generated in vitro, and the lipopolysaccharide (LPS) structure of the variant was compared to that of the parental strain, J178. The genetic basis for structural variation was explored by sequencing LPS biosynthesis genes. SP-D binding to clinical isolates was demonstrated by fluorescence-activated cell sorter analyses. Here, we show that *H. pylori* evades SP-D binding through phase variation in lipopolysaccharide. This phenomenon is linked to changes in the fucosylation of the O chain, which was concomitant with slipped-strand mispairing in a poly(C) tract of the fucosyltransferase A (*fucT1*) gene. SP-D binding organisms are predominant in mucus in vivo ($P = 0.02$), suggesting that SP-D facilitates physical elimination. Phase variation to evade SP-D contributes to the persistence of this common gastric pathogen.**

The gram-negative motile bacterium *Helicobacter pylori* is a gastroduodenal pathogen that is a causative agent of chronic gastritis, associated with peptic ulcer development, and a cofactor in the development of adenocarcinoma and mucosa-associated lymphoma (13, 14, 29, 40). *H. pylori* colonizes more than one-half of the world's population. However, only 15% of infected individuals are clinically symptomatic (38). While it is clear that the organism induces a strong innate and adaptive immune response leading to active inflammation in the gastric mucosa, the ability of *H. pylori* to establish persistent infection so efficiently has not been adequately explained. *H. pylori* possesses a number of virulence factors, and some, such as urease and flagella, contribute to its persistence (12, 31), whereas others, such as the product of the cytotoxin-associated gene A (CagA, encoded in the *cag* pathogenicity island) and vacuolating cytotoxin (VacA), appear to confer increased virulence (7, 21). Nevertheless, the interplay between *H. pylori* and host defense mechanisms requires further dissection to establish the mechanisms of persistence.

H. pylori occupies a unique ecological niche in the gastric mucus and on the surfaces of epithelial cells. Within this environment, the organism minimizes recognition by the innate immune system and evades phagocytosis because neutrophils and macrophages do not appear to traverse the gastric epithelium. However, within the mucus layer, there is a further component of the innate immune system, surfactant protein D (SP-D) (37). SP-D, a member of the collectin family, is a multimeric protein with 12 carbohydrate recognition domains that displays an affinity preference for maltose, mannose, and glucose (9, 42). SP-D was originally identified as a component of surfactant in the lung, where it is mainly expressed in alveolar type II and Clara cells (8, 53). However, a growing body of evidence confirming the presence of SP-D at other mucosal sites in close contact with numerous pathogens suggests that SP-D plays a role in innate immunity at mucosal surfaces as a pathogen-associated molecular pattern (PAMP) recognition receptor (24). Specifically, SP-D has been shown to be involved in antibody-independent pathogen recognition and clearance (16, 28). Moreover, there is evidence for the interaction of SP-D with a wide range of microorganisms, including viruses (such as influenza A virus), fungi (such as *Aspergillus fumigatus* and *Saccharomyces cerevisiae*), and gram-negative bacteria (such as *Escherichia coli* and *Klebsiella pneumoniae*), inducing their aggregation in a calcium-dependent and lectin-specific manner (1, 15, 18, 19). Furthermore, SP-D aggregation of microorganisms can enhance phagocytosis by neutrophils and macrophages (10, 22, 39), and SP-D binding has been implicated in bactericidal activity (55).

Whereas most studies have focused on the interaction of SP-D with lung pathogens (30), we have recently demonstrated the expression of SP-D in gastric mucosa and the interaction between SP-D and *H. pylori* (37). In addition, experiments in SP-D-deficient ($SP\text{-}D^{-/-}$) mice have revealed that colonization with *Helicobacter* was more common in the absence of SP-D (W. Khamri, M. L. Worku, A. E. Anderson, M. M.

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Walker, S. Hawgood, K. B. M. Reid, H. W. Clark, and M. R. Thursz, submitted for publication). These data suggest that this component of innate immunity is likely involved in the immune response to *H. pylori* in the stomach. Although certain redundancy of innate immune mechanisms may occur, for successful persistent infection by *H. pylori* to occur implies that either SP-D-mediated immunity is ineffective or that the bacterium can evade SP-D binding, or both.

Lipopolysaccharide (LPS), the main component of gram-negative bacterial cell walls, is the putative target of SP-D binding (19), including that of *H. pylori* (37). The LPS of *H. pylori* strains expresses fucosylated oligosaccharides known as Lewis (Le) antigens that are structurally related to human blood group antigens. It has been shown that 80% of strains express the type 2 glycoforms Le^x and/or Le^y, whereas less than 5% contain the type 1 glycoforms Le^a and Le^b (34, 54). *H. pylori* LPSs display marked antigenic variation and may be used as the basis for differentiation between the strains (34). Phase variation is the reversible switching of surface epitopes, including those of LPS (36), and is genetically controlled by on-and-off switching of specific genes involved in LPS biosynthesis, particularly Lewis antigen mimicry (4, 5, 47). It has been suggested that phase variation contributes to the heterogeneity of *H. pylori* and might contribute to a better adaptation to the host (3, 36).

This study investigated the relationship between SP-D binding and LPS phase variation in vitro and in vivo, with the specific aim of revealing potential mechanisms of bacterial persistence due to avoidance of recognition by SP-D, a member of the collectin family of innate immunity molecules.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* reference strains ATCC 26695 and J178 were kindly donated by M. Anyim (London School of Hygiene and Tropical Medicine, London, United Kingdom) and J. C. Atherton (Division of Gastroenterology, University Hospital, Nottingham, United Kingdom), respectively. *H. pylori* J178 was originally isolated and typed at the Vanderbilt University School of Medicine (Nashville, TN) from a patient with nonulcer dyspepsia. This strain is motile and characterized by the expression of the virulence factors CagA and VacA. These *H. pylori* strains were grown on blood agar medium composed of Columbia agar base (Oxoid, Basingstoke, United Kingdom) supplemented with 10% (vol/vol) laked horse blood (Oxoid). The bacterial cells were then incubated at 37°C in a microaerobic atmosphere generated by Anaerocult C gas-generated envelopes (Oxoid) for 48 h.

Isolation of clinical isolates from mucus versus tissue of biopsy samples. Antral biopsy specimens were collected from patients undergoing upper gastrointestinal endoscopy for dyspepsia after Ethical Committee approval and written consent. Patients were selected if they were over the age of 18 and were suffering with dyspepsia. Patients who had been on proton pump inhibitors or antibiotics within the previous 3 months were excluded. All patients had chronic active gastritis that was demonstrated histologically. The whole biopsy sample was transferred aseptically onto the surface of a selective agar plate consisting of blood agar supplemented with *H. pylori* selective supplement (DENT; Oxoid). The mucus layer was carefully scrubbed off the surface of the biopsy specimen using a 1-μl loop and streaked onto the plate. Subsequently, the intact tissue portion was lifted and transferred to a new selective agar plate and rubbed over the surface. After inoculation, the plates were incubated microaerobically for up to 5 days at 37°C. The relative proportion of isolated bacteria in mucus to tissue was 90:10. A sufficient number of colonies was tested to ensure they were representative of the overall bacterial population.

Bacterial standard curves. Bacterial strains were resuspended in Tris-buffered saline, pH 7.4 (TBS), and the optical density at 550 nm (OD_{550}) was determined using a Smart Spec 3000 spectrophotometer (Bio-Rad, Hemel Hempstead, United Kingdom). Subsequently, 10-fold serial dilutions (up to a 10^{-6} dilution) were made, the OD values were determined for each dilution, and then aliquots (10 μl) of each dilution were plated onto blood agar. After incubation, colonies were counted and a standard curve was produced that correlated OD with CFU.

***H. pylori* agglutination assay.** Native human SP-D (nhSP-D) was purified from the lung lavage fluid of patients with alveolar proteinosis according to the method of Strong et al. (48). The purity of SP-D preparations was greater than 95% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (48). SP-D binding to *H. pylori* strains was assessed using an agglutination assay as previously described (37). The bacterial cells were incubated with 2.5 μg/ml nhSP-D in the presence of 10 mmol/liter $CaCl_2$ for 1 h at 37°C, and agglutination was assessed as the difference in the OD_{770} of suspensions of bacterial cells after incubation for 45 min in the presence and the absence of nhSP-D.

Generation of SP-D escape variant. The escape variant of J178 (J178V) was generated following the agglutination of bacterial cultures derived from a single colony of the *H. pylori* parental strain, J178, in the presence of 2.5 μg/ml nhSP-D and 10 mmol/liter $CaCl_2$. The agglutinate was filtered through a 2-μm filter, and the free bacteria were collected and cultured on blood agar. This process was repeated for four cycles to generate J178V.

Restriction fragment length polymorphism (RFLP) analysis. Genomic DNA was extracted from *H. pylori* J178 and J178V using a standard technique involving proteinase K digestion and ethanol precipitation. Sequence data for the urease gene cluster (*ureA*, *ureB*, *ureC*, and *ureD*) (20) were used to design primers to amplify a portion of the *ureC* gene as described previously (32). The forward primer was 5′-TTTGGGACTGATGGCGTGAGGGGTAA-3′, the reverse primer was 5′-GGACATTCAAATTCACCAGGTTTTGAGG-3′, and the predicted amplification product was 1,132 nucleotide pairs in length. PCR amplification was performed in a 50-μl reaction volume with 1× PCR buffer; 0.125 mmol/liter $MgCl_2$, 200 μmol/liter (each) dATP, dCTP, dGTP, and dTTP (Roche Diagnostics, East Sussex, United Kingdom); 100 ng of each of the forward and reverse primers (Genosys, Haverhill, United Kingdom); 2.5 U of *Taq* polymerase (Gibco BRL, Paisley, United Kingdom); and 10 μl of template DNA sample (containing between 10 and 100 ng of DNA). Reactions were performed with a cycling program of 25 cycles of 94°C for 1.5 min, 48°C for 1 min, and 72°C for 2 min. The *ureC* amplicon was examined for homogeneity and yield by separation on a 0.8% agarose gel. A volume of 10 μl of the PCR product was digested using the appropriate restriction enzymes (NdeII and CfoI) (Roche Diagnostics), which were chosen because of their increased discriminatory ability in comparison to HindIII (data not shown). Digestion products were electrophoresed on a 2% agarose gel.

Competitive inhibitory SP-D enzyme-linked immunosorbent assay (ELISA) for determination of SP-D binding to LPS. LPS preparations were extracted from biomass of *H. pylori* J178 and J178V that had been grown on solid medium, using hot-phenol water treatment as described previously (35). SP-D binding to LPSs extracted from J178 and J178V was tested using the competitive inhibitory ELISA described elsewhere (37). Briefly, LPSs were preincubated with SP-D before addition to mannan-coated Maxisorb Immunoplates (Life Technologies, Paisley, United Kingdom). The plates were washed and sequentially incubated with biotin-labeled rabbit anti-SP-D polyclonal antibody (23), with ExtrAvidin-peroxidase conjugate (Sigma-Aldrich, Haverhill, United Kingdom), and with tetramethylbenzidine substrate (Bio-Rad, Hemel Hempstead, United Kingdom). Readings were carried out at 450 nm (Titertek Multiscan PLUS MKII) (37). The 50% inhibitory concentrations (IC_{50}) were calculated using GraphPad Prism software.

FACS analyses of SP-D binding to whole bacteria. Bacterial cell suspensions were adjusted to an OD_{550} of 0.24 (10^6 bacteria/ml) and washed three times in TBS by centrifugation at 1,700 × *g* for 8 min. The cells were then resuspended in TBS, and 100-μl aliquots were added to fluorescence-activated cell sorter (FACS) tubes and incubated in the presence of 2.5 μg/ml human recombinant SP-D, which consists of three carbohydrate recognition domains and the α-helical neck region (6), with 10 mmol/liter $CaCl_2$ for 1 h at 37°C. The cells were then washed twice in TBS and incubated in the presence of 1/10,000 biotin-labeled rabbit anti-recombinant SP-D antibody (23) for 1 h at 37°C. Subsequently, the cells were washed and finally incubated in the presence of fluorescein isothiocyanate-streptavidin (Serotec, Oxford, United Kingdom) at a 1/1,000 dilution for 30 min in the dark at room temperature. The cells were then washed three times and resuspended in 1% paraformaldehyde in phosphate-buffered saline (10 mmol/liter phosphate buffer, 2.7 mmol/liter KCl, and 137 mmol/liter NaCl, pH 7.4) solution. Incubation of the bacterial cells in the presence of 100 mmol/liter maltose and a goat immunoglobulin G (IgG) isotype control (R&D Systems, Oxon, United Kingdom) at 1/1,000 dilution were used as negative controls. The analysis was performed using a FACSCalibur flow cytometer, and data were analyzed using CELLQuest software (Becton Dickinson, Palo Alto, CA). The flow cytometer detectors were set as follows: forward scatter = E-01, side scatter = 413, and fluorescent channel 1 = 551. A total of 10,000 gated events were analyzed for each cell population. All experiments were done in duplicate. The estimation of positively labeled cells was determined by

TABLE 1. Primer sequences used in this study to amplify *H. pylori* candidate genes by PCR

Gene	Primer sequence (5′-3′)	PCR annealing temp (°C)	Product size (bp)
fuc T 2	Fuc1,2/F, ATGTTCCAACCCCTATTAGACGCC Fuc 2/R, CTTTTTAACCCATCTCCTTAT	52.9	1,428
lpxA	lpxA/F1, ATGAGTAAGATTGCAAAAACAG lpxA/R2, TTATTCCTCTTGTTTTTCTTCG	52.9	813
rfaF	rfaF/F1, ATGAGCGTAA ATGCACCCA rfaF/R1, TGGCCATCTTTTAGCACTCC	59	1,050
fucT 1	Fuc1,2/F, ATGTTCCAACCCCTATTAGACGCC Fuc1/RJ99, CAAACCCAATTTTTTAACCCATCT	55.5	1,278

setting an arbitrary cutoff gate at which discrimination between unstained (isotype control) and stained cell populations was detected. All experiments were compared using the same cutoff gate. The estimated proportions of SP-D binding cells were statistically compared between mucus and tissue isolates using Student's *t* test. Statistical significance was defined as a *P* value of <0.05.

LPS analysis with monoclonal antibodies. The expression of LPS epitopes, particularly Le antigen related, by J178 and J178V whole bacteria was measured in ELISA using specific monoclonal antibodies (MAbs) as described previously (3–5, 11). Polystyrene 96-well microtiter plates were coated at 7.5×10^6 CFU/ml with bacteria washed in 0.1 mol/liter phosphate-buffered saline, and the bacteria were tested for reactivity with MAbs (1 μg/ml). In addition, reactivity of antibodies with purified LPSs of *H. pylori* J178 and J178V with which microtiter plates were coated (50) was determined in ELISA as described previously (36) and by Western blotting after electroblotting of LPSs from sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (36). As outlined previously (27), the specificities of the antibodies in the assays were validated by their ability to bind the respective antigen from a panel of synthetic Le and blood group antigens (Isosep AB, Tullinge, Sweden, and Dextra Laboratories, Reading, United Kingdom).

LPS structural studies. Compositional analysis of *H. pylori* LPSs was performed using gas-liquid chromatography and gas-liquid chromatography–mass spectrometry as described elsewhere (20). Furthermore, detailed structural analysis was performed after the LPSs were subjected to standard mild acid hydrolysis, and the liberated saccharides were isolated using gel permeation chromatography (36). The isolated saccharides were subjected to physical analysis using nuclear magnetic resonance (NMR) spectroscopy (^{1}H-, ^{13}C-, and ^{31}P-NMR) and electrospray ionization mass spectrometry, as well as classical sugar and methylation analyses (36), and the structures of the core oligosaccharides and O chains of the respective LPSs were established. Details of the structural analysis will be reported in a separate paper (A. P. Moran, unpublished data).

DNA sequencing of LPS genes. Bacterial DNA was extracted using a standard technique involving proteinase K digestion and ethanol precipitation. PCRs were carried out in a 50-μl volume at the specific annealing temperatures listed in Table 1. The PCR products were immediately purified using a MinElute Extraction kit (QIAGEN, West Sussex, United Kingdom), and the products were subjected to cloning into a pGEM-T Easy vector system (Promega, Southhampton, United Kingdom) according to the manufacturer's instructions. The PCRs were repeated twice, and at least five clones from each PCR of each gene and each strain (J178, J178V, and J178V round 4) were sequenced. Of the cloned products, 1 μl was subjected to restriction digestion using EcoRI (New England Biolabs, Herts, United Kingdom) for 2 h at 37°C to verify the presence of the correct insert in each sample. The cloned products were sequenced using 3.2 pmol/liter of T7 and pUC/M13-Rev primers (5′-TAATACGACTCACTA TAG-3′ and 5′-CAGGAAACAGCTATGAC-3′, respectively). Sequencing was performed at the Sequencing Facility of the MRC-CSC Genetics Core Facility (Imperial College, Hammersmith Campus, London, United Kingdom).

Statistical analyses. Results were compared using the Student *t* test when normally distributed or using the Mann-Whitney U test for nonparametric data. Statistical significance was assumed for *P* values less than 0.05. All analyses were performed using Graph Pad Prism 3.0.

RESULTS

Generation of the J178 escape variant (J178V). Observations in our previous study characterizing SP-D binding and agglutination of *H. pylori* (37) revealed the presence of *H. pylori* cells that are not susceptible to binding by SP-D. This suggested that some bacterial cells lack a ligand for SP-D and are therefore capable of evading SP-D binding. We isolated an SP-D binding escape variant (J178V) in vitro after four cycles of agglutination and filtration of the parental strain J178 (a CagA-positive, VacA-positive laboratory-adapted strain) in the presence of 2.5 μg/ml nhSP-D and 10 mmol/liter $CaCl_2$ (Fig. 1A). J178V was resistant to binding and agglutination, even in the presence of excess concentrations (range, 2.5 to 12 μg/ml) of SP-D (Fig. 1B).

Agglutination of *H. pylori* in vitro was used as a measure of the relative binding of SP-D to J178 and J178V and to a well-characterized reference strain, ATCC 26695. Agglutination was inhibited in the presence of maltose and EDTA, demonstrating lectin-specific binding (Fig. 2A). In macroscopic observations and in agglutination assays, the reference strain ATCC 26695 had the highest binding to nhSP-D (δOD_{700} = 0.189), whereas J178V showed the lowest binding (δOD_{700} = 0.02). SP-D binding to J178 was fivefold higher than to J178V (P = 0.001) (Fig. 2A).

RFLP analysis of *H. pylori* J178 and J178V. In order to demonstrate that *H. pylori* J178V is derived from *H. pylori* J178 rather than arising through a nonclonal isolate, RFLP analyses were performed on DNAs extracted from both J178 and J178V using *ureC* primers for amplification. The PCR protocol successfully amplified a 1.1-kbp product which, when subjected to restriction analysis using NdeII and CfoI restriction endonucleases, yielded identical RFLP patterns for both strains (data not shown), thereby indicating a high degree of genetic relatedness.

Correlation between SP-D binding affinity of LPS and the corresponding live bacteria. We sought to determine whether disparity in affinities between different strains is related to components of the bacterial cell surface. A competitive inhibitory ELISA was therefore used to compare SP-D affinity to LPS molecules extracted from *H. pylori* J178 and J178V by measuring IC_{50} values in the competitive inhibition assay. SP-D had the highest affinity for J178 LPS (IC_{50} = 50.72 μg/ml), whereas affinity for J178V LPS was low (IC_{50} = 83.21 μg/ml) (Fig. 2B). These results

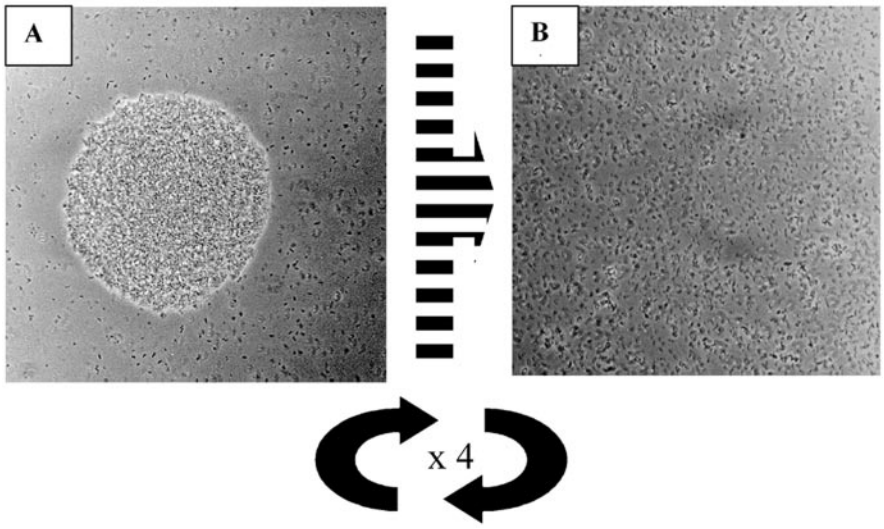

FIG. 1. Representation of the generation of the escape variant, *H. pylori* J178V. (A) Phase-contrast microscopy shows J178 after agglutination in the presence of 2.5 μg/ml nhSP-D and 10 mmol/liter $CaCl_2$. The agglutinated strain was filtered through 2-μm filters, and the collected filtrate was agglutinated and then filtered. The process was repeated for three additional cycles (×4) to yield the escape variant. (B) Phase-contrast microscopy of J178V showing no agglutination even in the presence of excess SP-D concentrations (range, 2.5 to 12 μg/ml).

were consistent with those obtained with the corresponding live bacterial strains (Fig. 2A). Binding was inhibited in the presence of maltose and EDTA (data not shown).

Variation in Le expression in LPS. Antigenic variations in the LPS structures of *H. pylori* J178 and J178V were explored using a panel of MAbs directed at specific Le antigens (terminal Le^x, polymeric Le^x, and terminal Le^y) (3–5, 11). The reactivities of these antibodies with whole bacterial cells of J178 and J178V in the ELISA revealed different reaction profiles. The MAb specific for polymeric Le^x bound strongly to both strains, whereas that specific for terminal (monomeric) Le^x bound to J178V, but to a much lesser extent than to J178. In

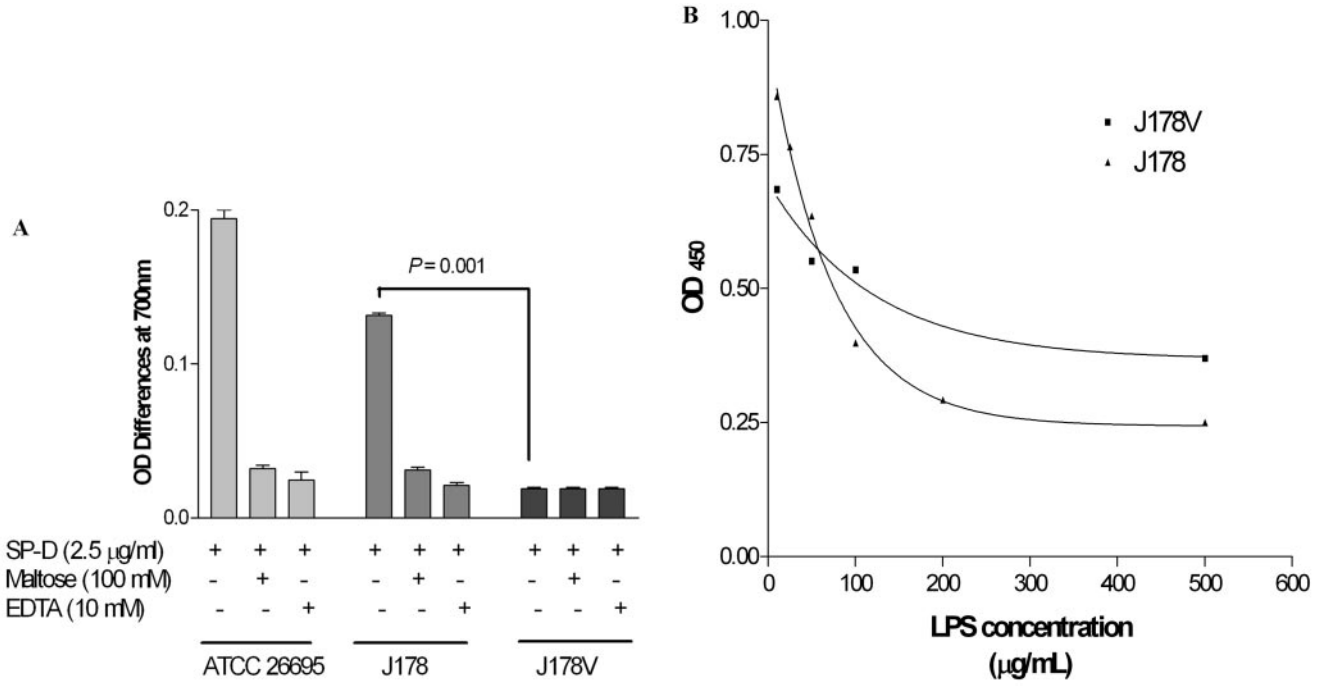

FIG. 2. Measurements of SP-D-mediated agglutination and SP-D binding of different *H. pylori* strains. (A) Agglutination was determined as the difference in OD_{700} between suspensions of bacterial cells after incubation for 45 min in the presence and the absence of SP-D. The error bars indicate standard deviations. (B) Inhibition of SP-D binding to mannan by *H. pylori* LPSs was demonstrated in dose-dependent competitive inhibition of SP-D-mannan binding by LPSs extracted from *H. pylori* J178 and J178V. Curve fitting was performed using GraphPad prism software (Prism 3.0).

TABLE 2. Investigation of Le antigen expression by *H. pylori* J178 and J178V on whole bacterial cells

Strain	MAb specificity[a]			
	Monomeric LeX (6H3)	Polymeric LeX (54.1F6A)	LeY (Hp 151)	Core (6C11)
J178	+	+	−	+
J178V	−	+	+	+

[a] Lewis antigen expression on whole bacterial cells as detected in ELISA. Murine MAbs were 6H3 (IgM), 54.1F6A (IgM), Hp151 (IgG), and 6C11 for the detection of monomeric (or terminal) Le^x, polymeric Le^x, Le^y, and the core oligosaccharide region, respectively (3, 4, 11). −, OD_{492} <0.5; +, OD_{492} >2.

contrast, J178V expressed a terminal Le^y epitope that was present to a lesser extent on J178 (Table 2). Nevertheless, a MAb against a conserved epitope in the LPS core, which was used as a control, gave identical reactions with J178 and J178V whole cells (Table 2). Likewise, analysis of purified LPSs of J178 and J178V in immunoblotting and ELISA revealed the same spectrum of reactivities as those observed with the whole bacterial cells. In addition, however, these analyses showed that J178 LPS expressed predominantly an H-1 epitope terminally, but also to a lesser extent Le^x and Le^y units terminally, in contrast to J178V LPS, which expressed a terminal Le^y epitope alone (data not shown).

A detailed structural investigation of LPS from *H. pylori* J178 and J178V was undertaken using nuclear magnetic resonance spectroscopy (Moran, unpublished). Both strains were shown to contain an O-polysaccharide chain (smooth-form LPS) with polymeric Le^x. As shown in Fig. 3A and B, J178 LPS expressed terminal H-1 antigen, with lower expression of terminal Le^x and Le^y, consistent with the results of the antibody probing studies described above. In contrast, J178V was found to express terminal Le^y alone, but with less modification of the O chain by glucose and galactose and greater fucosylation than in J178 LPS. Specifically, analyses revealed a lower degree of fucosylation in J178 LPS than in the J178V LPS (10% versus 43%) and a higher degree of decoration with glucose (but also with galactose) in J178 LPS than in J178V LPS (60% versus 20%). Furthermore, structural studies revealed the occurrence of different core oligosaccharides in J178 and J178V LPSs (Fig. 3C and D). Compared with J178V LPS, a fourth heptose residue is absent from the core of J178, and the first residue of the O chain, *N*-acetylglucosamine, is in a β(1, 2) rather than β(1, 7) linkage to the terminal heptose of the core oligosaccharide.

J178V SP-D escape variation is an example of phase variation. Although RFLP confirmed that *H. pylori* J178 and J178V are derived from the same strain, there are clearly changes in their LPS structures, which allows J178V to evade binding by SP-D. SP-D binding to bacterial cells was demonstrated after labeling with fluorescein isothiocyanate-conjugated antibody using FACS analysis. This technique demonstrated that the proportion of the *H. pylori* ATCC 26695 cell population bound by SP-D was as high as 48% compared to 5.8% in the J178V population (Fig. 4). Moreover, a higher proportion of cells of the J178 population (44.9%) were capable of binding SP-D than in the J178V population. As shown in Fig. 4, serial rounds of culture (assigned rounds 1 to 4) of J178V in the absence of SP-D selection pressure revealed that this switch was reversible. A constant increase in the percentage of SP-D-bound cells was seen as the cycle numbers increased. The binding of the J178V cell population increased from 5.8% at round 1 to 24.5% at round 4, indicating reversion of a proportion of cells in the population to the J178 SP-D binding phenotype of the parental strain J178 population. Moreover, antibody probing and structural analyses of LPS from the J178V round 4 population showed reversion to the J178 phenotype (data not shown). Furthermore, comparison of the maximal growth rate of *H. pylori* J178 (8.58×10^4 CFU/hour) with that of J178V (7.6×10^4 CFU/hour) showed that the former had a growth rate advantage over the latter. Thus, in the absence of SP-D selective pressure, there was a spontaneous switchback from the J178V to the parental J178 phenotype, producing a replicative advantage but also loss of the escape variation.

SP-D escape variation in vivo. The proportion of SP-D binding cells in ex vivo clinical isolates from mucus and tissue was up to threefold lower than that with *H. pylori* ATCC 26695 (Fig. 4 and 5A), suggesting that SP-D exerts a significant selection pressure in vivo. The mean value (±standard deviation) for isolates from tissue samples was 16.30% ± 0.95%, whereas the mean value for isolates from mucus samples was 26.26% ± 2.71% (Fig. 5B). In all patients, the proportion of SP-D binding cells was lower than that seen with the reference strain in this study and with other collection strains (37). Nevertheless, *H. pylori* SP-D binding cells are more frequent in mucus isolates than in tissue isolates ($P = 0.02$), indicating that SP-D binding cells are concentrated in the mucus layer (Fig. 5B).

Genetic basis of escape variation. The genetic changes underlying phase variation between *H. pylori* J178 and J178V were sought by sequence analysis of genes involved in LPS biosynthesis. The LPS structural variation described above, particularly in the O chain, was used as a guide in selecting *H. pylori* genes. Three candidate genes were sequenced, which encode an α-1, 3-fucosyltransferase A (*fucT1*) and an α-1, 3-fucosyltransferase B (*fucT2*) and are involved in the synthesis of Le^x, Le^y, and Le^a, and an ADP-heptose-LPS heptosyltransferase II (*rfaF*) involved in synthesis of the LPS inner core (2, 33, 34, 49). A UDP–*N*-acetylglucosamine acyltransferase (*lpxA*) gene involved in lipid A synthesis (2, 33, 49) was used as a control gene and showed no amino acid changes (Table 3).

Comparison of gene sequences between *H. pylori* J178 and J178V showed no changes in the *fucT2* gene. However, comparison of the full sequence of the cloned *rfaF* gene between J178 and J178V showed a transition from T to C at codon 120 in J178V, leading to an amino acid substitution: a phenylalanine-to-leucine change (Table 3). In addition, we identified a nucleotide deletion in the *fucT1* gene at position 13 in J178, leading to a frameshift and the induction of a termination codon (TAG) at codon 6 in J178 (Table 3). Hence, the predicted protein sequence for J178 from this nucleotide sequence is Met-Phe-Gln-Pro-Tyr-Stop, thereby prematurely terminating the translation process and, ultimately, resulting in the production of a truncated protein. This is likely to explain the lower degree of fucosylation in J178 than in J178V LPS revealed by the NMR structural analyses (Fig. 3A and B). Sequencing of J178V, which spontaneously switched back to the parental J178 phenotype in the absence of SP-D pressure (Fig. 4), showed that this variant has reverted to the J178 parental genotype (Table 3).

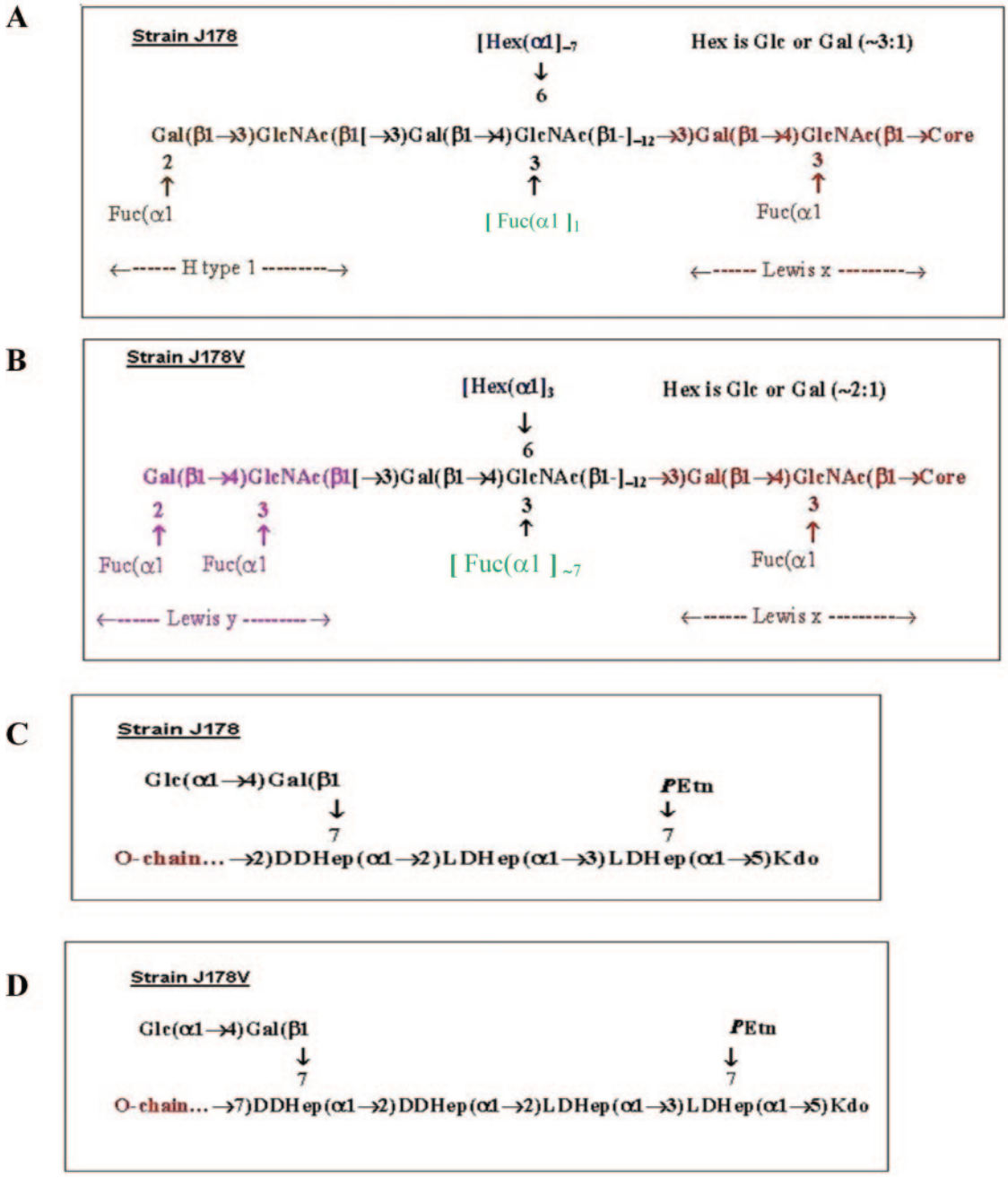

FIG. 3. Structural differences between LPSs from *H. pylori* J178 and J178V. (A and B) Proposed structures of the O chains of J178 and J178V LPSs, respectively. The O chain of *H. pylori* J178V had a higher degree of fucosylation (green) and a lower degree of glucose/galactose decoration (dark blue) than that of J178. In addition to the terminal H-1 units (dark brown) shown in J178, alternate terminal units of Le^x and Le^y also occurred to a lesser extent. (C and D) Proposed structures of the core oligosaccharides of J178 and J178V LPSs, respectively. Comparison of the cores shows that a fourth heptose residue is absent and that *N*-acetylglucosamine of the O chain occurs in β(1, 2) linkage to the terminal heptose of the core in J178 (C) rather than in β(1, 7) linkage, as in J178V (D). Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; Hex, hexose; DD-Hep, D-*glycero*-D-*manno*-heptose; LD-Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-2-octulosonic acid; *P*Etn, phosphorylethanolamine. The bracket around Fuc and Hex refers to an individual unit of the chain, and the numbers indicate how many of this unit are in the chain. The arrows indicate the linkage.

DISCUSSION

SP-D is a PAMP recognition receptor providing immediate defense against microbial invasion as part of the innate immune system at mucosal surfaces (10). Moreover, the importance of SP-D in the immune response against *Helicobacter* infection has been revealed in experiments in the SP-D-deficient ($SP\text{-}D^{-/-}$) mouse, where colonization with *Helicobacter* was more common in the absence of SP-D (Khamri et al., submitted). Although a certain redundancy of innate immune

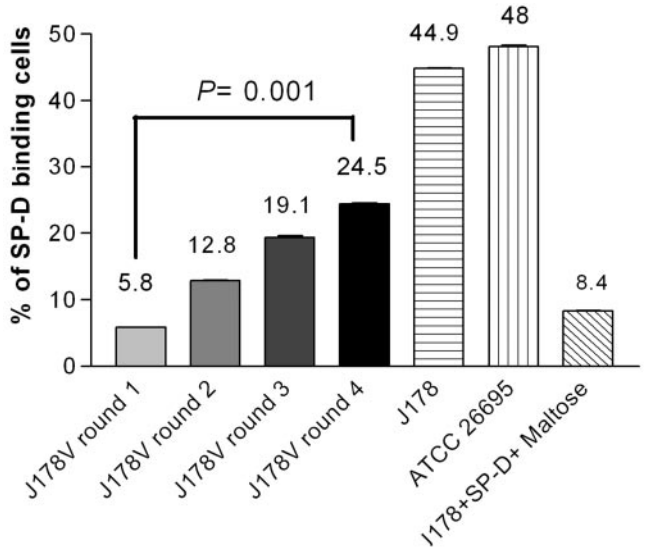

FIG. 4. Switchback from *H. pylori* J178V to the phenotype of J178 as demonstrated by FACS analyses during serial rounds of culture (assigned rounds 1 to 4) in the absence of SP-D. The proportions of SP-D binding cells in the J178V cell population at different subculture rounds were estimated starting from the original J178V population to the fourth subculture round, compared to *H. pylori* J178 and ATCC 26695 populations. A treatment with 100 mmol/liter maltose was included as a negative control in order to confirm the lectin-binding specificity of SP-D.

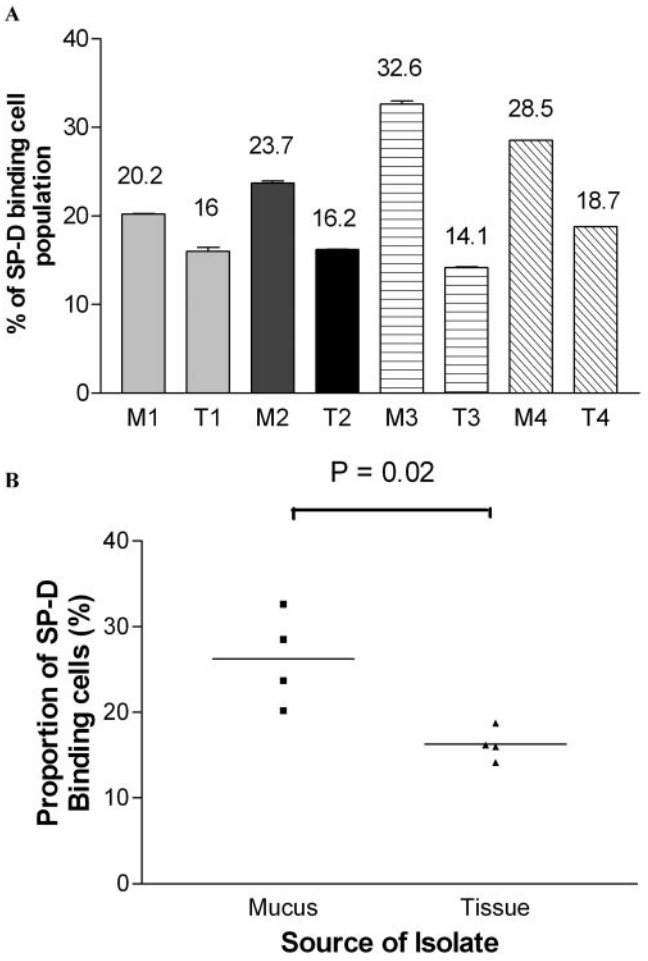

FIG. 5. Determination of the presence of low and high SP-D binding affinity *H. pylori* clinical isolates and the presence of escape variants in vivo. (A) Estimation of the proportions of binding cells in the individual *H. pylori* cell populations of clinical isolates 1, 2, 3, and 4 from mucus (M1, M2, M3, and M4) and tissue (T1, T2, T3, and T4). The bacterial populations from mucus samples displayed a higher fluorescence than those from matched tissues. (B) The proportion of SP-D binding cells in cell populations of individual *H. pylori* isolates from mucus was significantly higher than those from tissue. $P = 0.02$ (Student t test). Horizontal lines represent mean values.

mechanisms may occur, to establish persistent infection, *H. pylori* may develop mechanisms to evade SP-D binding and hence avoid this important innate immune response. This study identified and characterized the mechanism used by *H. pylori* to avoid SP-D binding.

The outer leaflet of the cell wall of *H. pylori*, like those of other gram-negative bacteria, is predominately composed of LPS consisting of the hydrophilic O chain, the core oligosaccharide region, and the hydrophobic lipid A moiety (34); the O chain may express Lewis antigens (33). Serological probing of whole bacterial cells in ELISA indicated that both the J178 and J178V strains had high expression of polymeric Le^x. However, compared to *H. pylori* J178, which expressed a terminal (monomeric) Le^x epitope, the variant strain J178V displayed a higher expression of terminal Le^y epitope. Likewise, analysis of purified LPSs of J178 and J178V in immunoblotting and ELISA revealed the same spectrum of reactivities as observed with the whole bacterial cells but also showed that J178 LPS expressed predominantly an H-1 epitope terminally and also to a lesser extent Le^x and Le^y units terminally, in contrast to J178V LPS, which expressed a terminal Le^y epitope alone. As well as confirming these results, structural studies found that the O chain from J178V possessed a higher degree of fucosylation and a lower degree of decoration with glucose (but also with galactose) than J178. These results are consistent with reports on the selectivity of SP-D binding to different monosaccharides in vitro. Inhibition of SP-D binding to solid-phase mannan by specific saccharides has demonstrated that SP-D has a low affinity for fucose relative to its high affinity for glucose or galactose (42). Hence, the changes in LPS structure replacing glucose and galactose with fucose residues on the O chain likely explain the changes in SP-D affinity for *H. pylori* J178 and J178V.

Although serological probing indicated the occurrence of some conservation of the inner core in LPSs of J178 and J178V, structural analyses revealed a new type of core oligosaccharide in J178 LPS, where an *N*-acetylglucosamine is β(1, 2) rather than β(1, 7) linked in the J178 LPS. It is therefore conceivable that changes in the core oligosaccharide region also influence the binding of SP-D by *H. pylori*. From the structural investigations, it would appear that changes in the core oligosaccharide induce complementary changes in the O chain, or vice versa. Therefore, at present, it is difficult to determine whether the differences seen in the genes responsible for the synthesis of the O chain are primary or compensatory, following the change in the core oligosaccharide region.

The availability of the complete genome sequences of two

TABLE 3. Comparison of sequencing results of the *lpxA*, *rfaF*, and *fucT1* genes from J178, J178V, and the J178V-reverted strain (J178V round 4)

Gene	Codon position	Nucleotide sequence in *Helicobacter* strains[a]			Transition and amino acid change
		J178	J178V	J178V reverted[b]	
lpxA	10	A**TT**	A**TC**	A**TT**	T → C/Ile → Ile
rfaF	120	**T**TC	**C**TC	**T**TC	T → C/Phen → Leu
fucT 1	6	CCCTATTAG	CCC**C**TATTA	CCCTATTAG	C base deletion at position 13 resulting in a premature termination codon

[a] Boldface bases indicate the nucleotide change in the sequence. Underlining marks the position of the termination codon TAG in the *fucT1* gene.
[b] The reverted J178V strain shows the same genotype as the parental strain, *H. pylori* J178.

H. pylori strains (26695 and J99) (2, 49) permitted the determination of genes involved in LPS biosynthesis and thus facilitated the exploration of the possible genetic mechanisms underlying the observed SP-D escape mechanism. At least 27 genes likely to be involved in LPS biosynthesis have been identified in *H. pylori* (33). These genes are scattered throughout the genome and not clustered at one locus. Based on the specific changes seen in the LPS structure between J178 and J178V, we selected genes involved in the O chain and the core oligosaccharide region for sequence analysis. The *rfaF* gene encodes an ADP-heptose-LPS heptosyltransferase II involved in the synthesis of the heptose region of the inner core oligosaccharide. The *fucT1* (also referred to as *futA*) gene encodes an α-1,3-fucosyltransferase enzyme involved in Le^x and Le^y biosynthesis (2, 33, 49). This enzyme is responsible for catalyzing the transfer of L-fucose from donor GDP-β-L-fucose to the *N*-acetylglucosamine residues in the O chain of *H. pylori* LPS at an α-1, 3-glycosidic linkage (33). Recent reports indicate that this FucT enzyme acts as an α3- and α4-fucosyltransferase, capable of transferring fucose to C-3 and C-4 positions of the O-chain backbone, respectively (4, 43).

Comparison of the sequenced candidate genes of the two strains, J178 and J178V, revealed a nonsynonymous missense mutation in the *rfaF* gene sequence of J178V. The more important genetic change was found in the *fucT1* gene, where the tetranucleotide C tract in J178 lost a C base through phase variation, probably due to a slipped-strand mispairing during DNA replication (3). This deletion results in the formation of an early stop codon at position 6, leading to a nonfunctional gene product. Glucose and galactose that are found in the O chain of J178 are more avidly bound by SP-D than fucose (42). Hence, the structural and the genetic studies correlate with each other and with the changes in SP-D binding affinity. The changes in the *fucT1* and *rfaF* genes identified in this study are likely to explain the observed escape variation. Nevertheless, our sequencing analyses do not rule out the presence of variations in other genes or in regulation at the posttranscriptional level. Furthermore, alternative mechanisms of avoiding SP-D binding may exist in other strains.

Phenotypical variation in the surface epitopes expressed by bacteria has been well documented in *Haemophilus influenzae* (45) and *Neisseria* spp. (52) as an adaptive strategy for microbial evasion of the host immune response. The variation is characterized by the reversible switch between "on" and "off" statuses of a particular epitope, as well as different levels of its expression, resulting in potential flexibility in LPS structure. Consistent with the results of the present study, the LPS structure of *H. pylori* is known to be complex and phase variable (3–5, 36, 47).

Lack of SP-D binding in clinical isolates was found to be much more prevalent than in laboratory-adapted strains that have been subcultured for numerous generations in the absence of SP-D. This is consistent with the possibility of SP-D exerting selection pressure in vivo. Furthermore, SP-D binding cells were significantly more frequent in mucus isolates than in isolates taken from the tissue portion of samples. It appears likely that SP-D facilitates clearance of *H. pylori* by trapping the organism in the mucus layer, where it can be physically eliminated. On the other hand, nonbinding *H. pylori* cells would have a selective advantage for colonizing tissue.

Although SP-D binding can cause bacterial agglutination and inhibition of motility (37), SP-D has been reported to be capable of inhibiting the growth of gram-negative bacteria by increasing membrane permeability (55). Also, SP-D aggregation of microorganisms can enhance phagocytosis by neutrophils and macrophages (10, 22, 39). Therefore, evasion of SP-D binding by *H. pylori* may contribute to the persistence of the infection, and thus, SP-D nonbinding variants would appear to have an advantage over binding variants. However, SP-D binding cells were seen to persist in vivo, suggesting that there must be a counterbalancing advantage. We found that J178 had a growth rate advantage over J178V (maximal growth rate, 8.58×10^4 versus 7.6×10^4 CFU/hour). Thus, although J178V rapidly switches to the J178 phenotype in the absence of SP-D selection pressure and therefore loses the ability to evade SP-D binding, the bacterium can gain a greater growth rate. The reason for the differential growth rate remains unclear.

H. pylori is not the only bacterium to have developed a strategy for avoiding SP-D-mediated elimination. A recent study by Mariencheck and colleagues showed that *Pseudomonas aeruginosa*-produced elastase is capable of degrading SP-D, as well as SP-A, thereby allowing the organism to evade the SP-D-mediated immune response (26). Moreover, gram-positive bacteria, such as *Streptococcus pneumoniae*, fail to bind SP-D after serotype switching (17). *Cryptococcus neoformans* evades SP-D through the secreted capsular component glucuronoxylomannan (51), and encapsulation of *Klebsiella pneumoniae* also facilitates SP-D evasion (46). Hence, it could be deduced that development of strategies by pathogens to escape SP-D binding reflects the important role of SP-D in the innate immune response.

The prevalence of *H. pylori* infection in the human popula-

tion attests to the success of the organism in avoiding host defenses. There is considerable redundancy in the innate immune system, and thus, the ability of *H. pylori* to establish persistent infection implies that the organism has strategies to avoid many components. *H. pylori* is predominantly a noninvasive bacterium, and although the gastritis associated with infection is characterized by infiltration of the lamina propria by neutrophils and macrophages, these phagocytic cells are not seen in the gastric lumen or mucus at the site of colonization. Residence in the mucus layer also minimizes the interaction of *H. pylori* with other PAMP receptors, such as the Toll-like receptors. Furthermore, a lower ability of LPSs of *Helicobacter* spp. to interact with such receptors may also contribute to persistence (25). In general, *H. pylori* LPS displays considerably less immunoactivity than those of other gram-negative bacteria, and it is therefore less likely to trigger an effective response (41). Moreover, *H. pylori* has acquired a system for evading complement-mediated lysis (44), and we have now demonstrated *H. pylori* evasion of the collectin SP-D. These insights into the mechanisms by which *H. pylori* circumvents host defense could provide novel strategies for development of future therapeutic approaches. If the organism is dependent on phase variation in the fucosyltransferase gene for survival, then targeting that enzyme may inhibit persistent infection.

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