Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin

Markus Gerhard*, Norbert Lehn†, Nina Neumayer*, Thomas Borén‡, Roland Rad*, Wolfgang Schepp§, Stephan Miehlke‖, Meinhard Classen*, and Christian Prinz*,**

*Department of Medicine II, Technical University of Munich, 81675 Munich, Germany; †Institute for Microbiology, University of Regensburg, 93053 Regensburg, Germany; ‡Department of Odontology, Umeå University, SE-90185 Umeå, Sweden; §Department of Medicine II, Bogenhausen Academic Teaching Hospital, 81925 Munich, Germany; and ‖Department of Medicine, University of Dresden, 01307 Dresden, Germany

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Infection with *Helicobacter pylori* is associated with different human gastric diseases. Biochemical studies, *in vitro* adherence assays, and *in vivo* animal models revealed that epithelial attachment of *H. pylori* can be mediated by the blood-group antigen-binding adhesin (BabA) targeting human Lewis^b surface epitopes. Studies with transgenic mice expressing the Lewis^b epitope have shown that such attachment can alter disease outcome. In the current study, the presence of the *babA2* gene encoding the adhesin was investigated in clinical isolates from a German population by using PCR and reverse transcription–PCR. A positive genotype was correlated to allelic variations in the genes encoding VacA and CagA and also to the prevalence of duodenal ulcer, distal gastric adenocarcinoma, mucosa-associated lymphoid tissue lymphoma, and antral gastritis. The presence of *babA2* was significantly associated with duodenal ulcer ($P = 0.0002$) and adenocarcinoma ($P = 0.033$). In contrast, type 1 strains (*vacAs1*- and *cagA*-positive) were associated with only duodenal ulcer ($P = 0.004$) but not adenocarcinoma ($P = 0.235$). Genotype presence of *babA2*, *vacAs1*, and *cagA* (''triple-positive'' strains) showed a highly significant correlation to the prevalence of ulcer ($P = 0.000002$) and adenocarcinoma ($P = 0.014$) and discriminated significantly better between disease outcome than did the current type 1 classification. These results indicate that the *babA2* gene is of high clinical relevance and would be a useful marker to identify patients who are at higher risk for specific *H. pylori*-related diseases.

VacA | CagA | ulcer | adenocarcinoma

Helicobacter pylori is a Gram-negative bacterium that colonizes the human stomach, mainly persisting within the gastric mucus layer (1). After acquisition in childhood, the organism persists for decades, and mixed infections are uncommon (2–5). Few bacteria can be detected in membrane-to-membrane contact with epithelial cells; contact enables them to interact directly with the human gastric epithelium (6). *H. pylori* infection regularly leads to active gastritis, which may progress to chronic gastritis with atrophy (7), duodenal ulcer (8), gastric adenocarcinoma (9, 10), or mucosa-associated lymphoid tissue (MALT) lymphoma (11). Environmental circumstances, host factors, as well as bacterial virulence factors may be important for the differential development of these diseases. The relevance of bacterial virulence factors was suggested by epidemiological studies in which a strong association between the virulence factors vacuolating cytotoxin (VacA) and cytotoxin-associated antigen (CagA) and the presence of duodenal ulcer was detected (12, 13).

VacA is a protein of 87 kDa that has been shown to vacuolate epithelial cells (14). CagA is a 120-kDa immunodominant antigen that elicits a strong immunological response to *H. pylori* strains (15, 16) and is used as a marker for the insertion of a large pathogenicity island encoding many proteins, several of which have been implicated in pathogenesis (17). Strains harboring the *VacA* phenotype or *vacAs1* genotype and the CagA protein or *cagA* gene have been detected at a higher frequency in patients with duodenal ulcer (18), atrophic gastritis, and gastric carcinoma (19–21). Because of the clinical importance of these virulence factors, *H. pylori* strains were classified as type 1 (VacA- and CagA-positive) and type 2 (both negative; ref. 22), but this classification alone did not allow for a proper clinical distinction between pathogenic and other strains because of the high prevalence of type 1 strains in western populations.

Recent studies provide evidence that bacterial adherence factors may contribute further to the specific tropism and pathogenicity of *H. pylori* in the human gastric epithelium. The blood-group antigen-binding adhesin, BabA, has been shown to mediate adherence of *H. pylori* to human Lewis^b (α-1,3/4-difucosylated) blood-group antigens on gastric epithelial cells (23, 24). *In vitro* adherence assays revealed that *H. pylori* bound in a lineage-specific manner to gastric surface mucous cells mediated by fucosylated blood-group antigens (25). Moreover, studies with transgenic mice expressing the human Lewis^b epitope in gastric epithelial cells indicated that Lewis^b functions as a receptor for a *H. pylori* adhesin and mediates its attachment to gastric pit and surface mucous cells (26). Attachment of *H. pylori* to gastric epithelial cells in such transgenic mice resulted in the development of chronic gastritis and gastric atrophy (27). Recently, the gene encoding BabA has been cloned (and termed *babA2*; ref. 28), which thus allows identification of *H. pylori* strains harboring the *babA2* genotype by PCR.

The clinical relevance of the *H. pylori* adherence factor BabA has not yet been determined in a larger series of clinical isolates obtained through routine endoscopy. Therefore, we investigated the presence and transcription of *babA2*, *cagA*, and *vacA* in clinical *H. pylori* isolates and correlated these data to the presence of gastritis, duodenal ulcer, gastric adenocarcinoma, or MALT lymphoma. Our data indicate that adherence of *H. pylori* to gastric epithelial cells may be crucial for the efficient delivery of bacterial toxins, resulting in specific *H. pylori*-related diseases.

Materials and Methods

Patient Population. *H. pylori* isolates were obtained from 114 patients with histologically confirmed distal gastric adenocarcinoma (total of $n = 27$; $n = 20$ intestinal type; $n = 7$ diffuse type), duodenal ulcer ($n = 23$), MALT lymphoma of the stomach ($n = 29$), or antral gastritis ($n = 35$). Cancers of the gastric cardia were excluded. Patients (55 male, 59 female) were age matched with median ages of 65 and 60.5 as well as 59 and 59 years, respectively. *H. pylori* isolates from patients with MALT lymphoma (23, 29) and distal gastric cancer (30) were recruited from

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Abbreviations: MALT, mucosa-associated lymphoid tissue; VacA, vacuolating cytotoxin; CagA, cytotoxin-associated antigen; BabA, blood-group antigen-binding adhesin.

**To whom reprint requests should be addressed. E-mail: christian.prinz@lrz.tum.de.

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previous studies, whereas isolates from patients with duodenal ulcer or gastritis were taken from routine diagnostics. All patients presented for routine endoscopy because of abdominal pain or dyspeptic complaints. Patients receiving antisecretory therapy (proton pump inhibitors or H2-receptor antagonists) or nonsteroidal antiinflammatory drugs were excluded. All biopsies were collected at the Technical University of Munich and one related teaching hospital.

Immunohistochemistry for Lewis^b. Immunostaining was performed according to standard protocols (31). Briefly, paraffin-embedded biopsies were deparaffinized and rehydrated. After blocking of endogenous peroxidase with 1% hydrogen peroxide, sections were incubated in normal serum for 30 min and then incubated with optimal diluted Lewis^b antibody (Signet Laboratories, Dedham, MA) for 2 h at room temperature. After washing, secondary antibodies were applied for 30 min and detected by peroxidase reaction.

***H. pylori* Culture.** Gastric biopsies were obtained from patients undergoing gastric endoscopy after informed consent. Each biopsy was homogenized in 0.5 ml of brucella broth with 10% (vol/vol) FCS without antibiotics. The suspension was inoculated subsequently onto three different media: Wilkins Chalgren agar (Oxoid, Basingstoke, U.K.) containing 10% (vol/vol) horse blood, Dent supplement (Oxoid), and 0.4 g/liter KNO_3 (Merck); Pylori agar (BioMérieux, Charbonnier les Bains, France); and nonselective Columbia chocolate agar (GCII, Becton Dickinson) without antibiotics. The media were incubated for up to 10 days at 36°C in an microaerobic atmosphere generated by gas insufflation: 0.6 bar of air (1 bar = 100 kPa) was evacuated from a jar and replaced by a mixture of 15% CO_2, 5% O_2, and 80% N_2 resulting in an atmosphere of 9% CO_2, 11% O_2, and 80% N_2. *H. pylori* was identified by colony morphology, Christensen urease (Bacto urea base, Difco), oxidase (Oxidase Dry Slide, Difco), catalase [3% (vol/vol) H_2O_2, Merck], and phase-contrast microscopy. Isolates were stored at −70°C in brucella broth plus 30% (vol/vol) FCS and 20% (vol/vol) glycerol. All frozen isolates were controlled for contamination.

***In Vitro* Adherence Assay.** Isolates were harvested from agar plates at stationary phase (during which BabA is mainly expressed in culture), resuspended in 0.2 M carbonate buffer, and digoxigenin labeled as described (32). Stocks were diluted to a density of 1 OD_{600} and frozen at −20°C in 100-μl aliquots. Universal Covalent 96-well microtiter-plates (Corning Costar, Cambridge, MA) were coated with 50 ng per well Lewis^b conjugated to human serum albumin (IsoSep AB, Stockholm) diluted in 0.2 M carbonate buffer (pH 9.6) to 1 ng/μl. A 50-μl aliquot of the solution (or 50 μl of buffer for controls) was added to each well, incubated for 1 h in the dark, and then removed with a pipette. Plates were exposed to UV light for 30 s in a Stratalinker (Stratagene) to immobilize the glycoproteins, and 100 μl of blocking buffer (0.5% nonfat dry milk/0.2% Tween 20) was added. After incubation for 1 h at room temperature, plates were decanted without washing, and 50 μl of bacterial suspension [diluted 1:2, including 10% (vol/vol) FCS] was added and incubated for 1 h at room temperature with gentle agitation (100 rpm) to reduce nonspecific binding. Each well was then washed three times with 100 μl of PBS, and detection was performed by using anti-DIG-HRP-antibody and ABTS-solution (both from Roche Diagnostics) according to the manufacturer's instructions. Extinction was quantified in a microplate reader (Bio-Rad) at 405 nm and normalized to controls (uncoated wells). In each single experiment, all strains were tested in two coated wells and two controls, and at least two independent assays were performed on each strain.

Preparation of DNA and RNA from *H. pylori* Cultures. For RNA preparation, agar plates were rinsed with 1 ml of lysis buffer [7 M urea/1.2% (vol/vol) SDS/35 mM NaCl/15 mM EDTA/10 mM Tris·HCl], and 800 μl of the initial volume was collected for phenol/chloroform extraction. Alternatively, bacteria grown in culture broth were pelleted by centrifugation for 5 min at 10,000 × *g*. The bacterial pellets were washed twice with PBS and then resuspended in 800 μl of lysis buffer (pH 5.2). RNA was extracted by phenol/chloroform and stored until use at −70°C. DNA was precipitated from the organic phase after removal of the aqueous phase by addition of 300 μl of 100% (vol/vol) ethanol, shaking, and centrifugation at 11,000 × *g* for 30 min. The pellet was washed twice with 70% (vol/vol) ethanol.

Reverse Transcription and PCR. PCR amplification of *H. pylori* gene loci was performed for *ureB* (as a positive control), *cagA*, *vacA* mosaics *vacAs1/2* and *vacAm1/2*, and *babA2*. Because allelic variations of the *cagA* gene locus have been described (33, 34), we used two different primer sets to amplify the middle region and 3′ end of the *cagA* gene (not shown). We obtained identical results in our German population and therefore used primers located in the middle region of the *cagA* gene to determine the *cagA* genotype status.

Primer sequences were *ureB* sense (5′-TTCACCCCAACA-AATCCCTACAG-3′), *ureB* antisense (5′-ACGGCCCATCGC-TTGAGAGT-3′), *cagA* sense (5′-GTATGGGGGCAATGGT-GGTC-3′), and *cagA* antisense (5′-GATTCTTGGAGGCGTT-GGTGTAT-3′; *vacA* primers were synthesized as described (13). The *babA2* primers were designed on the basis of the recently published signal sequence of the *babA2* gene (28) and were *babA2* sense (5′-AAT CCA AAA AGG AGA AAA AGT ATG AAA-3′) and *babA2* antisense (5′-TGT TAG TGA TTT CGG TGT AGG ACA-3′); identical primers for DNA and RNA amplification of *cagA* and *babA2* were used. For reverse transcription, 1 μg of total RNA (in a volume of 11 μl) and 1 μl of random hexamer primers (Amersham Pharmacia) were incubated at 70°C for 10 min and placed on ice for 5 min. cDNA synthesis was performed in a total volume of 20 μl with Superscript II (GIBCO/BRL) according to the manufacturer's instructions. cDNA (2 μl) or genomic DNA (20 ng) was used for amplification. Amplification was carried out in a total volume of 50 μl containing PCR buffer [50 mM KCl/10 mM Tris·HCl, pH 8.7/200 μM of each deoxynucleotide/2 units of *AmpliTaq* polymerase (Perkin–Elmer/Cetus)/1 μl of each primer (20 μM)]. $MgCl_2$ concentrations were adjusted for each primer pair. Reaction mixtures were overlaid with 25 μl of mineral oil (Serva) and amplified as follows: an initial denaturation for 5 min at 94°C; 30 cycles of 94° for 1 min, 55–62°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were analyzed on 1–2% agarose gels stained with ethidium bromide. Sequences were confirmed by restriction analysis and direct sequencing.

Statistical Analysis. The χ^2 test and the Fisher's exact test (because the numbers of isolates in the *vacAs1*, *cagA*, or *babA2* negative ulcer groups were determined to be zero) were used to compare the differences among the groups. *P* values are indicated in the text and in the figure legends. *P* values <0.05 were considered to be significant.

Results

PCR Analysis of the Different *H. pylori* Genotypes. *H. pylori* was successfully isolated and cultured from 114 patients. Integrity of DNA was confirmed by urease PCR, yielding clear signals from all isolates (not shown). Initially, sequence variations of the *babA* gene were investigated. The *babA2* genotype was detected by mismatch PCR. The upstream primer was located from −20 to +3 in the initial signal sequence, containing three mismatches to

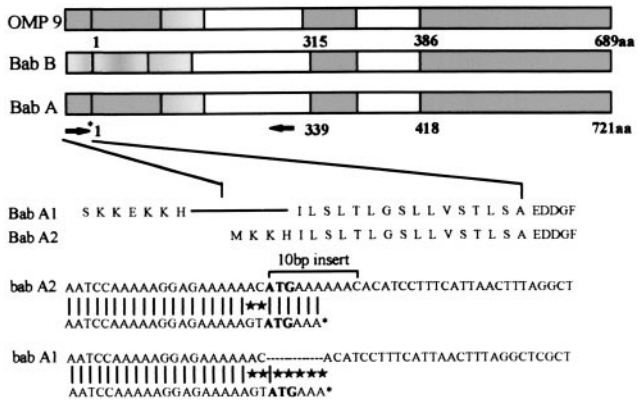

Fig. 1. *BabA* gene organization, nucleotide sequence (adapted from ref. 28), and primer positions for mismatch PCR. The binding of the sense primer to *babA1* and *babA2* genes is shown in relation to repeat motif. Matches are indicated by vertical bars; mismatches are indicated by asterisks. Regions of high homology to other genes (OMP9) are indicated by gray shading of open bars.

the *babA1* gene and two additional mismatches at −2/−3 (shown in Fig. 1). All PCR products were analyzed by sequencing, which confirmed the presence of *babA2* genotype. The results of DNA amplification for the *babA2* were confirmed by reverse transcription–PCR after RNA isolation, indicating that this gene was efficiently transcribed. The *babA2* genotype was detected in 82 of 114 isolates (71.9%).

Correlation of *babA2* Genotyping and *in Vitro* Adhesion Properties. A subset of 54 H. pylori strains was investigated for adhesion to Lewis^b in a microtiter assay. Of these, 23 strains were *babA2* negative, and 31 strains were *babA2* positive. Strains were considered positive if the ratio of $\text{extinction}_{\text{lewis}}/\text{extinction}_{\text{control}}$ was >1.5. *BabA2*-positive strains showed values between 1.8 and 2.0; negative strains showed values around 1.0. No binding was observed to Lewis^a. None of the *babA2*-negative strains showed any binding in the adherence assay. Of 31 *babA2*-positive strains, 28 showed a significant increase in extinction, indicating efficient binding, whereas 3 *babA2*-positive strains did not bind Lewis^b (Table 1).

Simultaneous Expression of *babA2*, *cagA*, and *vacA* Genotype. The clinical isolates were analyzed further with regard to the simultaneous presence of the *cagA*, *vacA*, and *babA* genotype. CagA gene expression was also verified by amplification of RNA with reverse transcription–PCR in identical isolates. These PCR experiments yielded identical products, indicating efficient transcription of this gene in *cagA*-positive strains. *vacA* mosaics were determined with regard to *s1/s2* and *m1/m2* sequences according to previously published gene variations (13). The *vacAs1* genotype has been correlated to the presence of the VacA toxin *in vivo* (13). Thus, the *vacAs1* genotype served as an indicator for toxin-positive strains and was detected in a total of 99 isolates (Table 2), whereas 15 had the *vacAs2* genotype. A statistically significant correlation was observed between *vacAs1* and *cagA* genotypes ($P < 0.001$; $\chi^2 = 34.5$; not shown), between the *babA2* and *cagA* genotypes ($P < 0.01$; $\chi^2 = 15.3$), and between the *babA2* and *vacAs1* genotypes ($P < 0.01$; $\chi^2 = 17.5$), as shown in Table 2. No correlation, however, was observed between the presence of the *vacAm1* or *vacAm2* genotype and *cagA* status (not shown). When the simultaneous presence of *vacAs1*, *cagA*, and *babA2* genes was investigated, we found that 78 of 99 isolates with *vacAs1* genotype were *babA2* positive, as were 70 of 86 isolates with *cagA* genotype. Of 82 isolates with *babA2* genotype, 68 were triple positive and thus contained all three genes (Table 2). Thus, the presence of the *babA2* genotype was significantly associated with the simultaneous presence of type 1 strain genotypes ($P < 0.01$; $\chi^2 = 12.87$).

Table 1. Correlation between the *babA2* genotype and adherence to Lewis^b epitopes in an *in vitro* adherence assay in a subset of 54 *H. pylori* strains

Adhesion to Lewis^b epitopes	*babA2* genotype-negative (n = 23 strains)	*babA2* genotype-positive (n = 31 strains)
Adhesion to Lewis^b	0	28
No adhesion to Lewis^b	23	3
Correlation	100%	91%

Table 2. Frequency and correlation of the *vacAs1*, *cagA*, and *babA2* genotypes in a total of 114 *H. pylori* isolates

Genotype (n = 114)	*babA2*-positive (n = 82)	babA2-negative (n = 32)	χ^2 value	P value
vacs1-positive (n = 99)	78	21	17.5	<0.01
cagA-positive (n = 86)	70	16	15.3	<0.01
Type 1 (n = 84)	68	16	12.8	<0.01

Genotype Distribution in Different Gastric Diseases. Table 3 shows the distribution of various genotypes in different gastric diseases. The overall *babA2* genotype prevalence ranged at 71.9% in the current study. The *babA2* genotype was detected in all patients with duodenal ulcer (23 of 23 ulcer isolates, 100%) and in 77.8% of patients with distal gastric adenocarcinomas (21 of 27 isolates) but only in 69% of isolates from patients with MALT lymphoma (20 of 29 isolates). *BabA2* was detected at a significantly lower frequency in isolates from patients with chronic gastritis (18 of 35, 51.4%). A highly statistically significant correlation was obtained between the *babA2* genotype and the presence of duodenal ulcer (Fisher's exact two-sided $P = 0.00024$). In addition, *babA2* genotype showed a significant correlation with the presence of gastric adenocarcinoma ($\chi^2 = 4.535$; $P = 0.033$).

The *vacAs1* genotype prevalence ranged at 86.8% in the current study (Table 3) and was detected at high frequency in isolates from patients with gastritis (82.9%). The presence of the *VacAs1* genotype correlated with the presence of duodenal ulcer when a two-sided χ^2 test was applied ($\chi^2 = 4.3$; $P = 0.036$), whereas Fisher's exact test did not reveal statistical significance (two-sided P value of 0.072). No correlation was found between *vacAs1* and adenocarcinoma ($\chi^2 = 2.7$; $P = 0.126$). Interestingly, the *vacAm1* genotype was detected at a higher frequency in isolates from patients with gastric adenocarcinoma (74.1% vs. 34–44% in other gastric diseases) and thereby showed a significant association to only this disease ($\chi^2 = 4.65$; $P = 0.025$).

The *cagA* genotype (Table 3) was significantly associated with duodenal ulcer (100%; Fisher's exact two-sided $P = 0.008$) but not with gastric adenocarcinoma (total of 92.6%; $\chi^2 = 3.5$; $P = 0.094$). Type 1 strains (*vacAs1*- and *cagA*-positive) were found in 23 of 23 cases with ulcer and in 23 of 27 cases with adenocarcinoma. Type 1 strains were significantly associated with duodenal ulcer (Fisher's exact two-sided $P = 0.0041$) but not with gastric adenocarcinoma ($\chi^2 = 1.65$; $P = 0.235$). A two-group χ^2 test with a one-sided significance level indicated a 91% power to detect the difference between the ulcer and gastritis group in type 1 strains, whereas the identical test yielded only a 28%

Table 3. Correlation between virulence factor genotypes and disease prevalence

Gene	Total, n = 114 (%)	Gastric adenocarcinoma, n = 27 (%)	Duodenal ulcer, n = 23 (%)	MALT, n = 29 (%)	Gastritis, n = 35 (%)	*P* value gastric adenocarcinoma vs. antral gastritis	*P* value duodenal ulcer vs. antral gastritis
vacAs1	99 (86.8)	26 (96.3)	23 (100)	21 (72.4)	29 (82.9)	0.126	0.036
vacAs2	15 (13.2)	1 (3.7)	0 (0)	8 (27.6)	6 (17.1)	n.s.	n.s.
vacAm1	54 (47.4)	20 (74.1)	8 (34.7)	10 (34.5)	16 (45.7)	0.025	n.s.
vacAm2	60 (52.6)	7 (25.9)	15 (65.2)	19 (65.5)	19 (54.3)	n.s.	n.s.
cagA	89 (78.9)	25 (92.6)	23 (100)	14 (48.3)	27 (77.1)	0.094	0.008
babA2	82 (71.9)	21 (77.8)	23 (100)	20 (69.0)	18 (51.4)	0.033	0.0002
Type 1	84 (73.7)	23 (85.1)	23 (100)	12 (41.4)	25 (71.4)	0.235	0.0041
Triple-positive	68 (59.6)	20 (74.1)	23 (100)	20 (34.5)	15 (42.9)	0.014	2×10^{-6}

The *P* value of gastric adencarcinoma vs. antral gastritis was calculated with the χ^2 test. The *P* value of duodenal ulcer vs. antral gastritis was calculated with Fisher's exact two-sided *P* test. n.s., not significant.

power to discriminate gastric adenocarcinoma and gastritis, which was not statistically significant.

Distribution of Triple-Positive Strains. *H. pylori* strains that harbored the *vacAs1* gene (toxin-positive strains) and the *cagA* genotype and that were also positive in regard to *babA2* expression showed a highly significant correlation to the presence of duodenal ulcer (total of 100%; Fisher's exact two-sided P = 0.000002) and a significant correlation to gastric adenocarcinoma (total of 74.1%; χ^2 = 6.04; P = 0.014). Triple-positive strains were detected only in 34.5% of isolates from patients with MALT or 42.9% of isolates from patients with gastritis. The distribution of *vacAs1*-, *cagA*-, and *babA2*-positive strains in the gastritis group is illustrated in Fig. 2. A two-group χ^2 test with a one-sided significance level indicated a 99% power to detect the difference between the ulcer and gastritis group and a 77% power to detect the difference between adenocarcinomas and gastritis. The statistical significance levels and the discriminating power exceeded the results from type 1 strains by far. Hence, a different strain-typing definition is suggested (Fig. 3), based on the close correlation of the triple-positive strains to ulcer and gastric adenocarcinoma.

Lewis^b Epitope Expression in Patients with Gastritis. Lewis^b epitope expression was determined in all 35 patients with gastritis. Immunohistochemistry was performed as described in *Materials and Methods.* By using a monoclonal antibody against Lewis^b, 33 patients showed positive staining for Lewis^b in the gastric mucosa; only 2 were Lewis^b negative. All 15 patients harboring triple-positive strains showed expression of Lewis^b antigens in the gastric mucosa. Of the 17 triple-negative patients, 2 were Lewis^b negative.

Follow-Up of the Gastritis Group. Finally, a follow-up study was performed with the patients included in the gastritis group (n = 35) for a median of 5 years (4.0–5.5 years). Three patients were excluded, because no data were available. Of the remaining 32 isolates from the gastritis group, 15 were initially triple positive, harboring *babA2*, *vacAs1*, and *cagA* genes. From these patients, one patient developed gastric adenocarcinoma; one had a bleeding ulcer 2 years after initial endoscopy; and two developed intestinal metaplasia as determined in routine gastric biopsies. Furthermore, two patients were treated by eradication triple therapy because of a positive family history for gastric cancer; two others were subjected to eradication triple therapy, because they complained about chronic abdominal pain and felt improvement after therapy (total of eight patients with complaints). In contrast, 1 of the triple-negative patients (n = 17) developed intestinal metaplasia, but none had any history of ulcer or gastric malignancy. One patient in this group was treated by eradication triple therapy because of dyspeptic complaints.

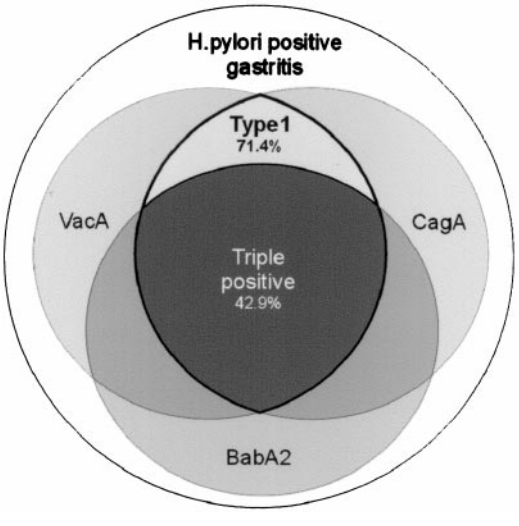

Fig. 2. Schematic illustration of the distribution of *vacAs1*, *cagA*, and *babA2* strains in a total of 35 *H. pylori* isolates from patients with gastritis.

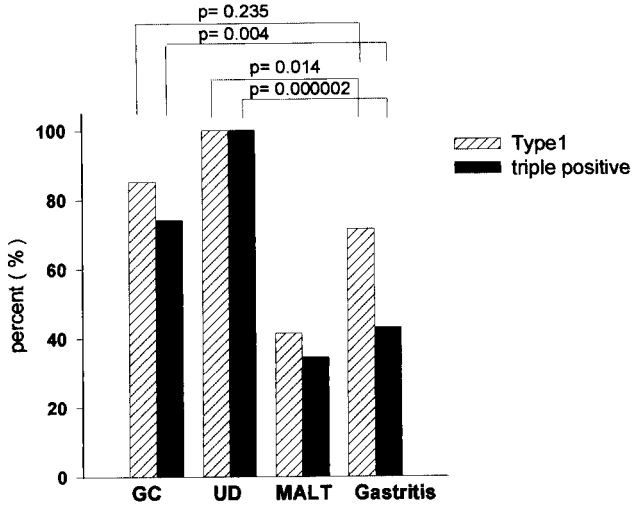

Fig. 3. *babA2*, type 1, and triple-positive genotypes in *H. pylori* isolates from patients with different diseases. GC, gastric adenocarcinoma; UD, duodenal ulcer.

Discussion

Key factors in the specific tropism and pathogenicity of *H. pylori* in the stomach include mechanisms of adherence and secretion of bacterial toxins. Several lines of evidence suggest a key role of Lewis^b antigens as receptors for adherence of *H. pylori* to gastric surface mucosal cells. Biochemical studies have identified a 78-kDa protein from *H. pylori* strains (termed BabA) that allows binding to the blood-group antigen Lewis^b present on the surface of gastric epithelial cells (24). Two corresponding genes encoding BabA have been cloned: *babA1* and *babA2* (28). Only the *babA2* gene is functionally active. These two genes have almost complete sequence homology, with the exception of a 10-bp insert, found only in *babA2*, which creates a translational initiation codon in the signal peptide sequence (28). We used this sequence difference to amplify the *babA2* gene selectively by mismatch PCR. In all *H. pylori* strains investigated, results obtained from DNA and RNA amplification of the *babA2* gene were concordant, indicating that when the *babA2* gene is present, it is efficiently transcribed. Sequencing confirmed the specificity of the assay in all cases.

Our current results further substantiate the observation that bacteria harboring the *babA2* gene express the adhesin and can thereby bind to Lewis^b antigens on gastric epithelial cells. The correlation between *babA2* genotype and *in vitro* binding to Lewis^b antigens was determined in a subset of strains ($n = 54$) by using an adherence assay performed in Lewis^b-coated microtiter plates. In these experiments, we determined that *babA2*-negative strains did not bind Lewis^b epitopes, indicating the specificity of our PCR assay. Also, no binding to Lewis^a was observed. Moreover, the correlation between *babA2* gene presence and adhesin expression was greater than 90%, indicating that, in most *babA2*-positive strains, the adhesin is expressed and functionally active. Some strains may lack the protein because of differential gene regulation, as suggested previously (35). These strains may be able to adapt their outer membrane protein expression to conditions in the environment by switching gene expression on and off. The presence of highly homologous genes could allow allelic replacement between genomic areas of different transcriptional activity. Thus, the *in vitro* binding activity may not necessarily reflect the *in vivo* situation. Furthermore, the expression of the protein may depend further on bacterial growth and may differ at various time points of culture or *in vivo* conditions.

The presence of the *babA2* genotype can therefore be regarded as a good indicator for the ability of strains to express the Lewis^b-binding adhesin. The presence of the corresponding Lewis antigens was verified in the current study in a subset of patients with gastritis. As shown by immunohistochemistry, the majority of patients (>90%) expressed Lewis^b in gastric biopsies. This result is in concordance with histopathological studies that found Lewis^b expression in up to 95% of gastric tissue (36). Absence of Lewis^b expression in the gastric mucosa seems to be rare, and the predictive value therefore may not be increased substantially by determination of Lewis status.

babA2 genotype distribution was determined in different gastric diseases and was a good marker for the presence of duodenal ulcer (100% vs. 51.4% in gastritis) and adenocarcinoma (77.8%). The close correlation between *babA2* and the presence of duodenal ulcer was not unexpected, because it had been anticipated in previous biochemical studies (23) and *in vitro* assays (25). Thus, our current clinical and experimental data confirm previous experimental studies indicating a central role of *H. pylori*'s adhesin and Lewis antigens in the pathogenesis of ulcer disease.

Our finding regarding the correlation between *babA2* gene presence and gastric cancer suggests that the presence of *H. pylori*'s adhesin and simultaneous Lewis^b epitope expression in the gastric mucosa may play an important role in the pathogenesis of gastric cancer as well. Previous studies have shown that *H. pylori* can attach directly to areas of intestinal metaplasia in the stomach (37). This finding may be explained the fact that Lewis^b expression is also detected at a high percentage in areas of intestinal metaplasia, potential precursor lesions of gastric cancer (36). Therefore, adherence of *babA2*-positive strains to areas of intestinal metaplasia and the direct interaction of bacteria with these cells may be a critical factor for the development of distal gastric cancer.

Another study could not find any effect of Lewis antigen expression on the adherence of *H. pylori* to human gastric epithelial cells (38). This study, however, investigated only a small number of individuals ($n = 19$) and used isolated gastric cells, which might not reflect *in vivo* conditions in different gastric diseases. Additional adherence factors independent of Lewis^b have also been shown to mediate adherence of *H. pylori*, but the clinical relevance of these has not been determined yet. Thus, *BabA* is not the only adherence factor of *H. pylori*. Our current data, however, give evidence for the clinical relevance of *BabA* and further suggest that this factor may have special importance for the induction of pathogenicity when additional factors, such as *vacA* and *cagA*, are present.

Therefore, we investigated the simultaneous presence of the bacterial virulence factor genes *cagA*, *vacA*, and *babA2* among clinical isolates of different patient groups. We initially found that the *babA2* status was significantly associated with *cagA*-genotype-positive strains. This finding is in accordance with observations made by Ilver *et al.* (28), who found expression of both *cagA* and *babA* in 70% of their isolates investigated. The *vacAs1* genotype was also significantly associated with the presence of the *babA2* genotype (Fig. 2), indicating that, in some (triple-positive) strains, the presence of all three factors might be important for mediating pathogenicity. On the other hand, this observation might reflect only a coincidence because of the high prevalence of *cagA*-, *vacAs1*-, and *babA2*-positive strains (>70%). Our current data, however, clearly indicate that the simultaneous presence of these three genes represents a specific intersection within several groups of strains with defined virulence, as illustrated in Fig. 2.

Classification of *H. pylori* strains by the simultaneous expression of *vacAs1*, *cagA*, and *babA2* allowed identification of a subgroup of type 1 strains that are associated with specific gastric diseases in human adults. The statistical correlation of these triple-positive strains showed a remarkable difference in regard to disease distribution and significance levels compared with type 1 strains alone.

The association of type 1 strains with duodenal ulcer or gastric carcinoma has been reported in several studies before (7, 12, 18, 21, 39, 40). We also confirmed the association between type 1 strains and duodenal ulcer in our study, although the absolute difference between these groups was relatively small. In contrast, we found that type 1 strains were not associated with adenocarcinoma, a finding that might be explained by the high prevalence of type 1 strains in our population. However, because only 10–20% of *H. pylori*-infected patients with chronic gastritis will develop duodenal ulcer and less then 1% may develop gastric adenocarcinoma, our current data confirm that genotyping of *H. pylori* strains by the presence *vacA and* cagA only in patients with gastritis (>70% type 1) cannot identify a small subgroup of patients with chronic gastritis who might benefit from *H. pylori* eradication strategies.

Genotyping of *H. pylori* strains by the additional presence of the adherence factor gene *babA2* in type 1 strains, however, yielded a highly significant association with ulcer ($P = 2 \times 10^{-6}$) and distal gastric adenocarcinoma ($P = 0.014$). The association between triple-positive strains and duodenal ulcer is in accordance with the single correlation of type 1 and *babA2* strains with

this disease, whereas the significance level and the discriminating power exceed the previously reported values for type 1 strains by far. Our observation that triple-positive (in contrast to type 1) strains are associated with gastric adenocarcinoma indicates even more clearly that *babA2* is able to discriminate between type 1 subgroups with different prognoses. In this context, definition of a triple-positive subgroup yielded a 77% power in a two-group χ^2 test, whereas type 1 strains showed only a 28% power to detect carcinoma and thus were not significantly associated. Because the absolute percentage of triple-positive strains in patients with chronic gastritis was smaller than that of type 1 strains, *H. pylori* eradication of patients in this group seems to be more reasonable. Indeed, a follow-up analysis of the patients harboring triple-positive strains in the gastritis group showed that these patients developed gastric abnormalities more frequently. This observation may further support the view that triple-positive strains in patients with chronic gastritis should be eradicated, because the combined presence of these genes may lead to duodenal ulcer or, after years of infection, to the generation of adenocarcinoma.

Previously, the presence of triple-positive strains has been determined in a small population of children (41). In this study, no correlation between ulcer development or gastric inflammation and the presence of cag-pathogenicity island, cytotoxin production, and binding to Lewis^b oligosaccharide was found. However, this lack of correlation may be a specific finding in children and may be explained by the fact that Lewis^b expression seems to be less frequent in children than in adults (41).

In contrast to the correlation of *babA2*, *cagA*, and *vacAs1* genotype with ulcer or adenocarcinoma, we found an inverse relation of triple-positive strains to the presence of MALT lymphoma. These three genotypes were significantly lower in patients with MALT lymphoma. In this group, *vacAs2* strains were detected more frequently. This finding stands in contrast to previously reported associations (42); however, it may be explained by the fact that the pathomechanism for the development of MALT lymphoma is initiated by *H. pylori* infection but differs from the mechanism by which *H. pylori* induces ulcers or adenocarcinoma, such as clonal expansion of B cells induced by T cell stimulation (43, 44).

Our current data support the assumption that the virulence factors *VacA* and *CagA* may have special importance when bacteria are able to adhere tightly to gastric surface mucous cells via their adhesin, and subsequently, infection with these strains leads to epithelial damage, caused either by the direct delivery of bacterial toxins or by the indirect induction of an immune response. We further suggest that *H. pylori* can be classified as a triple-positive strain with simultaneous presence of *vacAs1*, *cagA*, and *babA2* and that the combined expression of these genes is related to ulcer or adenocarcinoma development.

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1. Blaser, M. J. (1997) *J. Clin. Invest.* **100,** 759–762.
2. Covacci, A., Telford, J. L., Del, G. G., Parsonnet, J. & Rappuoli, R. (1999) *Science* **284,** 1328–1333.
3. Miehlke, S., Thomas, R., Guiterrez, O., Graham, D. Y. & Go, M. F. (1999) *J. Clin. Microbiol.* **37,** 245–247.
4. Suerbaum, S., Smith, J. M., Bapumia, K., Morelli, G., Smith, N. H., Kunstmann, E., Dyrek, I. & Achtman, M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 12619–12624.
5. Kersulyte, D., Chalkauskas, H. & Berg, D. E. (1999) *Mol. Microbiol.* **31,** 31–43.
6. Thomsen, L. L., Gavin, J. B. & Tasman, J. C. (1990) *Gut* **31,** 1230–1236.
7. Kuipers, E. J., Uyterlinde, A. M., Pena, A. S., Roosendaal, R., Pals, G., Nelis, G. F., Festen, H. P. & Meuwissen, S. G. (1995) *Lancet* **345,** 1525–1528.
8. Anonymous (1994) *J. Am. Med. Assoc.* **272,** 65–69 (abstr.).
9. Forman, D. (1996) *Scand. J. Gastroenterol. Suppl.* **214,** 31–33.
10. Parsonnet, J., Friedman, G. D., Vandersteen, D. P., Chang, Y., Vogelman, J. H., Orentreich, N. & Sibley, R. K. (1991) *N. Engl. J Med.* **325,** 1127–1131.
11. Wotherspoon, A. C., Ortiz, H. C., Falzon, M. R. & Isaacson, P. G. (1991) *Lancet* **338,** 1175–1176.
12. Blaser, M. J. (1996) *Aliment. Pharmacol. Ther.* **10,** Suppl. 1, 73–77.
13. Atherton, J. C., Cao, P., Peek, R. M., Jr., Tummuru, M. K., Blaser, M. J. & Cover, T. L. (1995) *J. Biol. Chem.* **270,** 17771–17777.
14. Papini, E., de, B. M., Milia, E., Bugnoli, M., Zerial, M., Rappuoli, R. & Montecucco, C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9720–9724.
15. Peek, R. M., Jr., Miller, G. G., Tham, K. T., Perez, P. G., Cover, T. L., Atherton, J. C., Dunn, G. D. & Blaser, M. J. (1995) *J. Clin. Microbiol.* **33,** 28–32.
16. Crabtree, J. E., Taylor, J. D., Wyatt, J. I., Heatley, R. V., Shallcross, T. M., Tompkins, D. S. & Rathbone, B. J. (1991) *Lancet* **338,** 332–335.
17. Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R. & Covacci, A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14648–14653.
18. Atherton, J. C., Peek, R. M., Jr., Tham, K. T., Cover, T. L. & Blaser, M. J. (1997) *Gastroenterology* **112,** 92–99.
19. Crabtree, J. E., Farmery, S. M., Lindley, I. J., Figura, N., Peichl, P. & Tompkins, D. S. (1994) *J. Clin. Pathol.* **47,** 945–950.
20. Kuipers, E. J., Perez, P. G., Meuwissen, S. G. & Blaser, M. J. (1995) *J. Natl. Cancer Inst.* **87,** 1777–1780.
21. Blaser, M. J., Perez, P. G., Kleanthous, H., Cover, T. L., Peek, R. M., Chyou, P. H., Stemmermann, G. N. & Nomura, A. (1995) *Cancer Res.* **55,** 2111–2115.
22. Xiang, Z., Censini, S., Bayeli, P. F., Telford, J. L., Figura, N., Rappuoli, R. & Covacci, A. (1995) *Infect. Immun.* **63,** 94–98.
23. Boren, T., Falk, P., Roth, K. A., Larson, G. & Normark, S. (1993) *Science* **262,** 1892–1895.
24. Boren, T., Normark, S. & Falk, P. (1994) *Trends Microbiol.* **2,** 221–228.
25. Falk, P., Roth, K. A., Boren, T., Westblom, T. U., Gordon, J. I. & Normark, S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2035–2039.
26. Falk, P. G., Bry, L., Holgersson, J. & Gordon, J. I. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1515–1519.
27. Guruge, J. L., Falk, P. G., Lorenz, R. G., Dans, M., Wirth, H. P., Blaser, M. J., Berg, D. E. & Gordon, J. I. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3925–3930.
28. Ilver, D., Arnquist, A., Ögren, J., Frick, I., Kersulyte, D., Incecik, E. T., Berg, D., Govacci, A., Engstrand, L. & Boren, T. (1998) *Science* **279,** 373–377.
29. Neubauer, A., Thiede, C., Morgner, A., Alpen, B., Ritter, M., Neubauer, B., Wundisch, T., Ehninger, G., Stolte, M. & Bayerdorffer, E. (1997) *J. Natl. Cancer Inst.* **89,** 1350–1355.
30. Miehlke, S., Hackelsberger, A., Meining, A., Hatz, R., Lehn, N., Malfertheiner, P., Stolte, M. & Bayerdorffer, E. (1998) *Br. J. Cancer* **78,** 263–266.
31. Mason, D. Y. & Sammons, R. (1978) *J. Clin. Pathol.* **31,** 454–460.
32. Falk, P., Boren, T., Haslam, D. & Caparon, M. (1994) *Methods Cell Biol.* **45,** 166–188.
33. Covacci, A., Censini, S., Bugnoli, M., Petracca, R., Burroni, D., Macchia, G., Massone, A., Papini, E., Xiang, Z., Figura, N., *et al*. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5791–5795.
34. Miehlke, S., Kibler, K., Kim, J. G., Figura, N., Small, S. M., Graham, D. Y. & Go, M. F. (1996) *Am. J. Gastroenterol.* **91,** 1322–1325.
35. Alm, R. A., Ling, L. S., Moir, D. T., King, B. L., Brown, E. D., Doig, P. C., Smith, D. R., Noonan, B., Guild, B. C., deJonge, B. L., *et al*. (1999) *Nature (London)* **397,** 176–180.
36. Kobayashi, K., Sakamoto, J., Kito, T., Yamamura, Y., Koshikawa, T., Fujita, M., Watanabe, T. & Nakazato, H. (1993) *Am. J. Gastroenterol.* **88,** 919–924.
37. Genta, R. M., Gurer, I. E., Graham, D. Y., Krishnan, B., Segura, A. M., Gutierrez, O., Kim, J. G. & Burchette, J. L., Jr. (1996) *Gastroenterology* **111,** 1206–1211.
38. Clyne, M. & Drumm, B. (1997) *Gastroenterology* **113,** 72–80.
39. Blaser, M. J. & Crabtree, J. E. (1996) *Am. J. Clin. Pathol.* **106,** 565–567.
40. Blaser, M. J. (1994) *Scand. J Gastroenterol. Suppl.* **205,** 1–5.
41. Celik, J., Su, B., Tiren, U., Finkel, Y., Thoresson, A. C., Engstrand, L., Sandstedt, B., Bernander, S. & Normark, S. (1998) *J. Infect. Dis.* **177,** 247–252.
42. Eck, M., Schmausser, B., Haas, R., Greiner, A., Czub, S. & Muller, H. H. (1997) *Gastroenterology* **112,** 1482–1486.
43. Zucca, E., Bertoni, F., Roggero, E., Bosshard, G., Cazzaniga, G., Pedrinis, E., Biondi, A. & Cavalli, F. (1998) *N. Engl. J. Med.* **338,** 804–810.
44. Zucca, E., Bertoni, F., Roggero, E., Cazzaniga, G., Bosshard, G., Biondi, A. & Cavalli, F. (1998) *Leukemia* **12,** 247–249.