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Cloning and Genetic Characterization of the *Helicobacter pylori* and *Helicobacter mustelae flaB* Flagellin Genes and Construction of *H. pylori flaA*- and *flaB*-Negative Mutants by Electroporation-Mediated Allelic Exchange

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Helicobacter pylori* is one of the most common human pathogens. It causes chronic gastritis and is involved in the pathogenesis of gastroduodenal ulcer disease and possibly gastric carcinoma. *Helicobacter mustelae* is a bacterium closely related to *H. pylori* that causes gastritis and ulcer disease in ferrets and is therefore considered an important animal model of gastric *Helicobacter* infections. Motility, even in a viscous environment, is conferred to the bacteria by several sheathed flagella and is regarded as one of their principal virulence factors. The flagellar filament of *H. pylori* consists of two different flagellin species expressed in different amounts. The gene (*flaA*) encoding the major flagellin has recently been cloned and sequenced. Here we report the cloning and sequencing of two highly homologous new flagellin genes from *H. pylori* 85P and *H. mustelae* NCTC 12032. The nucleotide sequence of the *H. pylori* gene proved that it encoded the second flagellin molecule found in *H. pylori* flagellar filaments. The genes were named *flaB*. The *H. mustelae* and *H. pylori flaB* genes both coded for proteins with 514 amino acids and molecular masses of 54.0 and 53.9 kDa, respectively. The proteins shared 81.7% identical amino acids. The degree of conservation between *H. pylori* FlaB and the *H. pylori* FlaA major flagellin was much lower (58%). Both *flaB* genes were preceded by σ^{54}-like promoter sequences. Mapping of the transcription start site for the *H. pylori flaB* gene by a primer extension experiment confirmed the functional activity of the σ^{54} promoter. To evaluate the importance of both genes for motility, *flaA*- and *flaB*-disrupted mutants of *H. pylori* N6 were constructed by electroporation-mediated allelic exchange and characterized by Western blot (immunoblot) analysis and motility testing. Both mutations selectively abolished the expression of the targeted gene without affecting the synthesis of the other flagellin molecule. Whereas *flaA* mutants were completely nonmotile, *flaB* mutants retained motility.

Helicobacter pylori (20) is a gram-negative, spiral-shaped bacterium that is now generally accepted to be the causative agent of chronic, active type B gastritis in humans. *H. pylori* infection is also regarded as a prerequisite for the occurrence of duodenal ulcers and a cofactor in the pathogenesis of gastric ulcer and gastric carcinoma (38, 41; for reviews see references 1 and 42).

Several potential virulence factors have been suggested to play a role in *Helicobacter* pathogenesis, those studied in most detail being the production of abundant amounts of urease, motility, the expression of cytotoxins and other extracellular enzymes, and adhesion to tissue-specific receptors (for reviews see references 1, 2, and 6). In regards to urease expression and motility, experiments using the gnotobiotic piglet model of *H. pylori* infection have supported the importance of these properties for the onset of colonization (7–9). A high degree of motility, even in a viscous environment (24), is conferred to *H. pylori* by a bundle of three to six flagella that extends from one pole of the bacterium. The flagella consist of a basal part that contains the flagellar motor and the hook structure, the central filament, and a membranous sheath that envelops each filament (16, 21). No details are known about the basal parts of *Helicobacter* flagella. As for the sheath, there is only relatively basic biochemical information available. It is a bilayered membrane that contains proteins, lipids, and lipopolysaccharides and thus closely resembles the outer membrane, although differences, especially in fatty acid composition and protein pattern, have been noted (18). The sheath has been suggested to play a role in protecting the filaments from the acidic environment of the gastric lumen. The filaments have been the subject of several detailed investigations. The major constituent of the *H. pylori* flagellar filament is a 53-kDa flagellin molecule. The gene encoding this major flagellin has recently been cloned and sequenced by Leying et al. (32) and is called *flaA*.

Recently, Kostrzynska et al. (26) have demonstrated the presence of a second flagellin molecule of slightly higher molecular mass in *H. pylori* flagellar filaments. It is expressed in much smaller amounts than the major flagellin and has been shown to be located primarily in the proximal part of the filament. The function of this protein and its importance for motility are as yet unknown. Research in the field of *Helicobacter* pathogenesis has been hampered by the difficulties encountered in the establishment of animal models. The three most promising animal models are the infection of gnotobiotic piglets with *H. pylori* (7–9), the colonization of the murine or rat stomach with *Helicobacter felis* (31), and the *Helicobacter mustelae* infection of the ferret. *H. mustelae* is a very common pathogen of ferrets, in which, like *H. pylori* in humans, it colonizes the gastric mucosa and

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TABLE 1. Vectors and hybrid plasmids used in this study

Plasmid	Vector	Size (kb)	Characteristics[a]	Reference
	pIC20R2	2.7	Rep_{Ec}, Ap^r	36
	pILL570	5.3	Rep_{Ec}, Mob, Sp^r	29
	pILL575	10	Rep_{Ec}, Rep_{Cj}, Mob, Cos, Km^r	29
pILL600	pBR322	5.7	Ap^r Km^r; source of kanamycin cassette	30
pILL683	pILL575	45	Km^r; cosmid containing *H. pylori flaB*	This study
pHL319-2-4	pIC20R2	6.3	Ap^r; plasmid containing *H. pylori flaA*	32
pSUS10	pIC20R2	7.7	Ap^r Km^r; *H. pylori flaA*ΩKm	This study
pSUS19	pILL570	8.8	Sp^r; subclone of pILL683, contains *H. pylori flaB*	This study
pSUS22	pILL570	10.2	Sp^r Km^r; *H. pylori flaB*ΩKm	This study
pSUS100	pILL570	12.3	Sp^r; plasmid containing *H. mustelae flaB*	This study
pSUS101	pILL570	8.3	Sp^r; subclone of pSUS100, contains *H. mustelae flaB*	This study

[a] Rep_{Ec} and Rep_{Cj}, plasmids capable of replicating in *E. coli* and *C. jejuni*, respectively; Mob, conjugative plasmid due to the presence of OriT; Ap^r, Km^r, and Sp^r, resistance to ampicillin, kanamycin, and spectinomycin, respectively; Cos, presence of lambda cos site.

causes chronic gastritis and ulcer disease (13–15). In contrast to the other animal models, which primarily allow studies concerning colonization, the *H. mustelae* infection of the ferret represents a disease model and seems thus of special interest for the study of pathogenesis and the role of host immunity in gastric *Helicobacter* infections.

H. mustelae flagella are ultrastructurally very similar to those of *H. pylori*; however, they extend from both bacterial poles and from the lateral parts of the bacteria (20, 40). Only one flagellin molecule has been purified to date. The amino-terminal sequence of this protein was strongly homologous with that of the major *H. pylori* flagellin (49). So far, none of the virulence-associated genes of this organism has been cloned. However, genetic studies in this organism are of interest, not only because sequence information will show which domains of a protein are characteristic for the gastric *Helicobacter* species and, thus, could be of importance for pathogenicity, but also because cloned antigens and genetically defined mutants will provide important tools for pathogenesis and vaccination studies in this model.

We report here on the cloning and sequencing of two homologous new flagellin genes of *H. pylori* and *H. mustelae* that code for the minor flagellin proteins and are called *flaB*. *H. pylori* mutants in the *flaA* and *flaB* genes were constructed by means of reverse genetics to test the effect of those mutations on motility.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. The cloning experiments were performed with genomic DNA prepared from *H. pylori* 85P (29) and *H. mustelae* NCTC 12032 (National Collection of Type Cultures, Colindale, London, United Kingdom). *H. pylori* N6 (11) was used as the recipient strain for the electroporation experiments because of its favorable transformability. A urease-negative mutant of *H. pylori* N6 disrupted in the *ureB* gene (11) was used as a control in the motility tests. *Escherichia coli* MC1061 (3) was used as the host for cloning and subcloning experiments. Vectors and recombinant plasmids used in this study are listed in Table 1. *H. pylori* and *H. mustelae* strains were grown on horse blood or sheep blood agar plates, respectively, supplemented with vancomycin (10 mg/liter), polymyxin B (2,500 U/liter), trimethoprim (5 mg/liter), and amphotericin B (4 mg/liter). Plates were incubated at 37°C under microaerobic conditions. *E. coli* strains were grown in L broth without glucose (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter; pH 7.0) or on L agar plates (1.5% agar) at 37°C. Antibiotic concentrations for the selection of recombinant clones were as follows (in milligrams per liter): kanamycin, 20; spectinomycin, 100; carbenicillin, 100.

Preparation of DNA. Genomic DNA from *H. mustelae* was prepared by the method of Majewski and Goodwin (35). Cosmid and plasmid DNAs were prepared by an alkaline lysis procedure and then purified in cesium chloride-ethidium bromide gradients as previously described (11).

DNA analysis and cloning methodology. Restriction endonucleases, T4 DNA ligase, and DNA polymerase I large (Klenow) fragment were purchased from Amersham or Boehringer Mannheim, T4 DNA polymerase was from Biolabs, and calf intestinal phosphatase was from Pharmacia. All enzymes were used as described in the instructions of the manufacturers. DNA fragments were separated on agarose gels run in Tris-acetate buffer. The 1-kb ladder from Bethesda Research Laboratories was used as a fragment size standard. When necessary, DNA fragments were isolated by electroelution from agarose gels as previously described (28) and recovered from the migration buffer by means of an Elutip-d minicolumn (Schleicher & Schuell, Dassel, Germany). Basic DNA manipulations were performed by the protocols described by Sambrook et al. (44).

Hybridization. For Southern blot hybridizations, DNA fragments were transferred from agarose gels to nylon membranes (Hybond N; Amersham Corp.) by capillary blotting (47) and baked for 2 h at 80°C. Southern blot hybridizations were performed under low-stringency conditions (2× SSPE–0.05% sodium dodecyl sulfate [SDS], 45 to 55°C) (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.5]). Colony blots for screening of the *H. pylori* cosmid bank and identification of subclones were prepared on nitrocellulose membranes (Schleicher & Schuell) by the protocol of Sambrook et al. (44). Hybridizations were performed under low-stringency conditions (2× SSC–0.1% SDS–30% formamide, 37°C) (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate [pH 7.0]). Radioactive labelling of purified restriction fragments and polymerase chain reaction (PCR) products was performed by random priming (10). Hybridization was revealed by autoradiography with Amersham Hyperfilm-MP.

Cosmid cloning. The construction of the cosmid gene bank of *H. pylori* 85P in *E. coli* HB101, which was used for the cloning of the *H. pylori flaB* gene, has been described previously (29).

DNA sequencing. Appropriate fragments of plasmid DNA were subcloned into M13mp18/19 vectors (37). Single-stranded DNA was prepared by phage infection of *E. coli*

TABLE 2. Oligonucleotides used for PCR and primer extension experiments

Name	Position[a]	Strand	Length	Sequence (5'–3')	Reference
OLpBR322-1	2,085–2,104	+	20	GTGATGACGGTGAAAACCTC	50
OLpBR322-2	2,834–2,853	–	20	GGTTGGACTCAAGACGATAG	50
OLKm-1	699–724	+	25	CTGCTAAGGTATATAAGCTGGTGGG	53
OLKm-2	1,305–1,329	–	25	CATACTGTTCTTCCCCGATATCCTC	53
OLHMflaB-2	51–75	+	25	AATCAAAGATAAGGAATTAAGCGGC	This study
OLHMflaB-3	—[b]	–	25	CTGAAATGAAAATAACCCCAAAGTC	This study
OLHPflaA-1	225–249	+	25	GGAGAGATTGAGTTCAGGTTTAAGG	32
OLHPflaA-2	1,602–1,626	–	25	ACTCATCGCATAGCTGCCTGATTGC	32
OLHPflaB-2	775–798	–	24	CAGCGCTCGTAGAAATGCGCACGG	This study
OLHPflaB-7	—	+	21	GTTTATACCTATTAATGAATG	This study
OLHPflaAPE-2	169–198	–	30	GAGTTCGGATTGCACATGCGCATTCATCGC	32
OLHPflaBPE-1	184–210	–	27	GGTCTCTGTTGTTTTGAACCCCTACCG	This study

[a] Numbers refer to nucleotides in the sequences of pBR322 (for OLpBR322-1 and -2), the kanamycin resistance gene (for OLKm-1 and -2), the *H. pylori flaA* gene (for OLHPflaA-1, -2, and OLHPflaAPE-2), the *H. mustelae flaB* gene (for OLHMflaB-2), a sequence close to the rightmost *Bam*HI site in pSUS101 (for OLHMflaB-3), the *H. pylori flaB* gene (for OLHPflaB-2 and OLHPflaBPE-1), and a sequence upstream the reported sequence for *H. pylori flaB* (for OLHPflaB-7), respectively.

[b] —, not published.

JM101 (54). Sequencing was performed by the dideoxynucleotide chain termination method (45) by using the U.S. Biochemicals Sequenase kit. Both the M13 universal primer and additional specific primers were used to sequence both the coding and noncoding DNA strands. Sequencing of double-stranded DNA was performed as described previously (5).

Extraction of RNA and primer extension. Total RNA was extracted from *H. pylori* 85P by the method described by Leying et al. (32), which itself is a modification of a protocol described by Chirgwin et al. (4). For primer extension, oligonucleotides OLHPflaBPE-1 and OLHPflaAPE-2 (Table 2) were end labelled by using the 5'-DNA terminus labelling system of Bethesda Research Laboratories. Twenty micrograms of RNA was precipitated and resuspended in 25 μl of avian myoblastosis virus buffer (1× avian myeloblastosis virus buffer is 50 mM Tris-HCl [pH 8.3], 40 mM KCl, 5 mM $MgCl_2$, and 5 mM dithiothreitol). A 1.5-pmol amount of labelled primer was added, and the mixture was heated to 95°C for 3 min and then allowed to cool to 60°C over a period of 1 h. After the annealing period, deoxynucleoside triphosphates (dNTPs) (final concentration, 0.1 mM of each of the four dNTPs) and 28 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) were added, and the samples were incubated at 42°C for 1 h. To demonstrate that RNA and not DNA was the template for extension, control samples were incubated with RNase (1 mg/ml) for 10 min at 37°C before the reverse transcriptase was added. After extension, samples were extracted once with phenol-chloroform, precipitated, and resuspended in 10 μl of sequencing gel-loading buffer. Sequencing reactions were performed on double-stranded templates by using the same oligonucleotides as those used for the primer extension experiment. Samples were analyzed on a 9% sequencing gel.

Electroporation of *H. pylori*. To construct *H. pylori* mutants, appropriate plasmid constructions carrying the targeted gene disrupted by a cassette containing a *Campylobacter* kanamycin resistance gene were transformed into *H. pylori* N6 by means of electroporation as described previously (11). After electroporation, bacteria were grown on nonselective plates for a period of 48 h to allow for the expression of the antibiotic resistance and then transferred onto kanamycin-containing plates. The selective plates were incubated for up to 6 days. Described mutant strains are descendants of single *H. pylori* colonies.

PCR. PCRs were carried out by using a Perkin-Elmer Cetus thermal cycler with the GeneAmp kit (Perkin-Elmer Cetus). One hundred picomoles of each primer and at least 5 pmol of target DNA were used. The DNA was denatured at 94°C for 1 min, primers were annealed at temperatures between 42 and 55°C (depending on the calculated melting temperatures of the primers) for 2 min, and products were extended at 72°C for 2 min. Twenty-five cycles were performed.

Western blotting (immunoblotting). Flagella were enriched as previously described (49). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Lugtenberg et al. (34). The concentration of acrylamide in the gel was 11%. Sixty micrograms of protein was applied to each slot of the gel. For Western blotting, proteins were transferred to nitrocellulose sheets (Schleicher & Schuell) by the method of Towbin et al. (52). The blots were incubated with rabbit antiserum AK 179 (32) raised against purified flagellar filaments, and bound antibody was visualized by a peroxidase-coupled goat anti-rabbit antibody (Biogenzia, Bochum, Germany).

Motility testing. For motility testing, brain heart infusion agar plates supplemented with 10% fetal calf serum and antibiotics as described above were used. Two-layered plates were poured (the lower layer containing 1.5% agar, the soft overlay containing 0.5% agar). Plates were inoculated by placing small slices of blood agar plates, densely grown with the strain to be tested, on the surface of the motility plate, the grown side of the slice facing the soft layer. Plates were incubated for 3 days under microaerobic conditions.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this article appear in the GenBank data base under the accession numbers L08907 (*H. pylori*) and L08908 (*H. mustelae*).

RESULTS

Cloning of a flagellin gene from *H. mustelae* NCTC 12032. To clone a flagellin gene from *H. mustelae*, genomic DNA was prepared from *H. mustelae* NCTC 12032 and restricted with various endonucleases. Restriction fragments were hybridized with a 2.1-kb *Bgl*II-*Bam*HI restriction fragment of plasmid pHL319-2-4 (which contains the 5' end of the *H. pylori flaA* gene encoding the *H. pylori* major flagellin) under

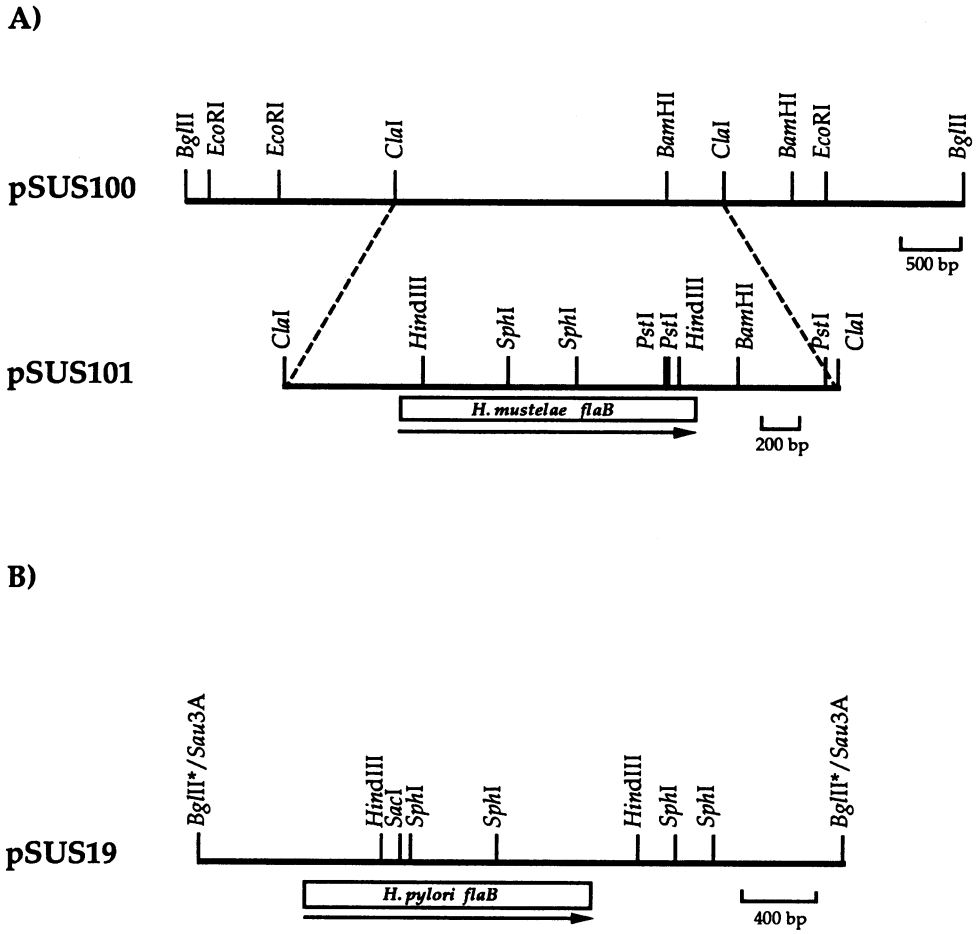

FIG. 1. Linear restriction maps of the recombinant plasmids pSUS100 and pSUS101 (A) and pSUS19 (B), harboring the *H. mustelae* and *H. pylori flaB* genes, respectively. Only the inserts are depicted. Plasmid pSUS101 is a subclone of the 3.0-kb *Cla*I restriction fragment of pSUS100. The location of the ORFs is indicated by the open boxes, and the sense of transcription is indicated by arrows below the boxes. Asterisks mark restriction sites of the vector no longer present in the recombinant plasmids.

low-stringency conditions. Hybridizations of this probe with a complete *Bgl*II digest of genomic *H. mustelae* DNA revealed two hybridizing bands of 7 and 7.5 kb. Hybridizations with probes corresponding to other fragments of the *H. pylori flaA* gene suggested that the homologous gene was not restricted by *Bgl*II but that each of the two hybridizing fragments contained one complete copy of a homologous gene. Chromosomal DNA fragments of 6.5 to 8 kb were electroeluted from a preparative agarose gel, ligated into the *Bgl*II-restricted and dephosphorylated pILL570 plasmid vector, and transformed into *E. coli* MC1061 cells. Plasmid DNA was prepared from 50 recombinant clones, cut with *Bgl*II, and subjected to Southern blot hybridization with the 2.1-kb *H. pylori flaA* probe. Three of the 50 clones contained identical inserts that hybridized with the probe; one of them (pSUS100) was selected for further analysis. Figure 1A shows the restriction map of pSUS100. Further Southern blot analysis indicated that the hybridizing DNA segment was internal to a 3.0-kb *Cla*I restriction fragment that was subcloned into the plasmid vector pILL570 and mapped in more detail. The physical map of the resulting plasmid pSUS101, which contained the presumed flagellin gene, is also shown in Fig. 1A.

Sequencing of the *H. mustelae flaB* flagellin gene. Appropriate restriction fragments of pSUS101 were cloned into M13mp18/19 vectors and sequenced by the dideoxynucleotide chain termination method. The nucleotide sequence obtained is shown in Fig. 2A. The sequenced DNA fragment contained one large open reading frame (ORF) of 1,542 nucleotides coding for a protein with a predicted molecular mass of 54.0 kDa. The deduced protein, in its amino-terminal and carboxy-terminal parts, shared marked homologies with several bacterial flagellins. The highest degrees of homology were found with *Campylobacter* flagellins and the *H. pylori* FlaA (Fig. 3). The N-terminal amino acid sequence of the protein deduced from the cloned gene was not identical to the sequence previously determined for the major *H. mustelae* flagellin (49) (Table 3). It shared, however, very strong homology with the N-terminal amino acid sequence of the minor flagellin of *H. pylori* purified by Kostrzynska et al. (26). The gene was named *flaB* so that the name *flaA*, in analogy to the nomenclature in *H. pylori*, can be given to the gene coding for the major flagellin. It is preceded by a ribosome-binding site similar to the consensus sequence for *E. coli* (46). A stem-loop structure (free energy, $\Delta G = -10.8$ kcal [ca. -45.2 kJ]/mol) consistent with a rho-independent transcriptional stop signal (43) is located downstream of the ORF. Upstream of the ORF there is a putative σ^{54}-depen-

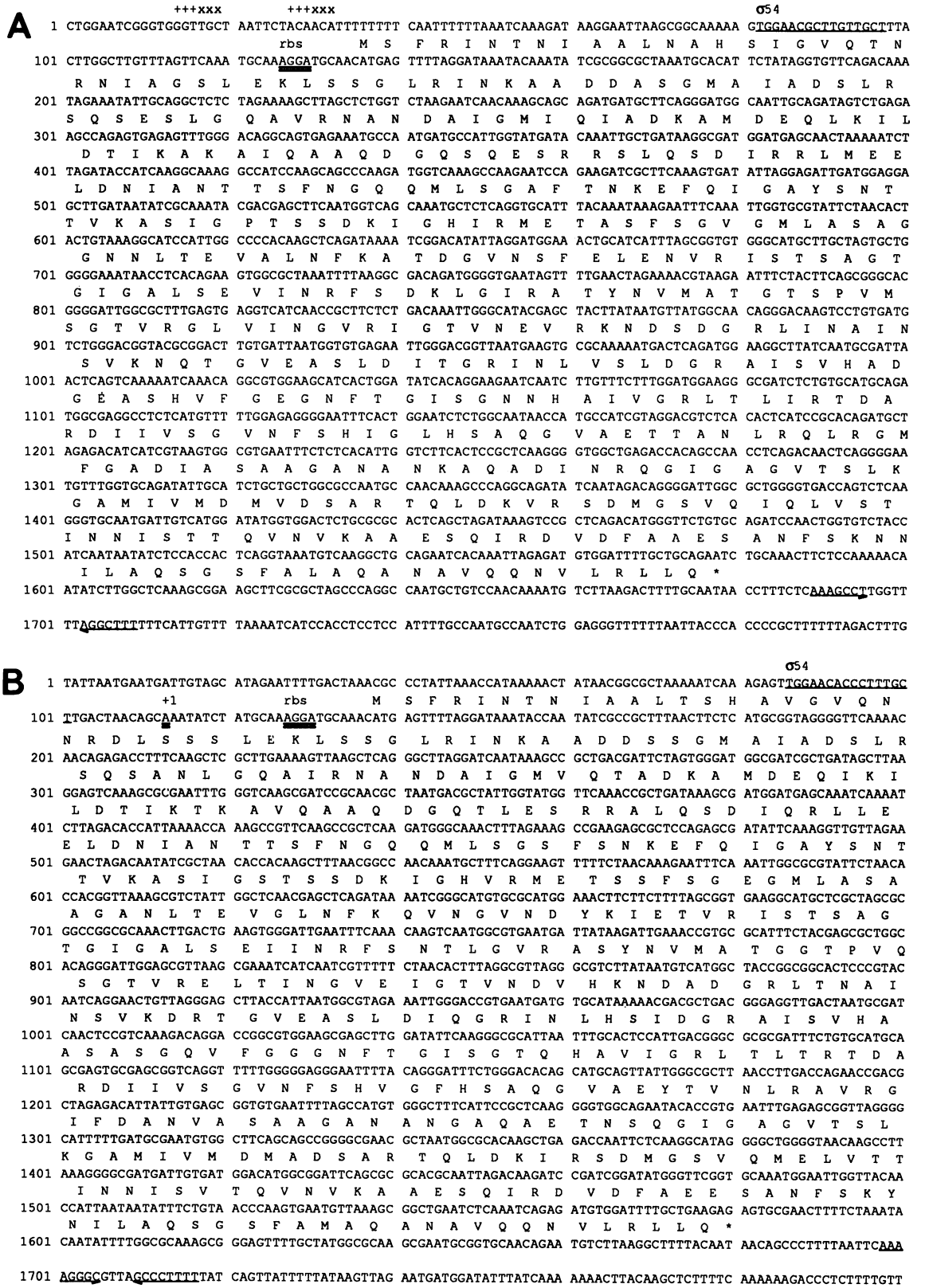

FIG. 2. Nucleotide sequences of the *H. mustelae* (A) and *H. pylori* (B) *flaB* flagellin genes. Numbers on the left represent the nucleotide positions of the first base in each line. The deduced amino acid sequences are given by letters positioned above the first nucleotide of the codons. Asterisks mark the stop codons. Bases marked with an + or an × in panel A belong to two putative upstream enhancing elements located directly adjacent to each other. Sixteen underlined bases marked σ^{54} represent the σ^{54}-dependent promoters. The ribosome-binding sites (rbs) are indicated by double underlining, and putative transcription terminators are indicated by arrows. In panel B, the transcription start site is indicated by double underlining and marked +1.

FIG. 3. Comparison of the deduced amino acid sequence of the *H. pylori flaB* gene with those deduced from the *H. mustelae flaB*, *H. pylori flaA*, and *C. coli flaA* genes. The amino-terminal methionine residues have been omitted in correspondence with the situation in the mature proteins. The letters on the left represent the sources of the sequence (HmB, *H. mustelae flaB*; HpB, *H. pylori flaB*; HpA, *H. pylori flaA*; CcA, *C. coli flaA*); the numbers on the right represent the positions of the last amino acid in each line. Asterisks mark amino acids identical with those in the *H. pylori flaB* sequence. Underlining indicates residues conserved in all four compared sequences.

dent promoter sequence (14 of 16 nucleotides identical with the *E. coli* consensus sequence) (51). About 100 nucleotides upstream of the ribosome-binding site, two potential upstream enhancing sequences (GGT-N10-ACA and TGC-N10-ACA) were found.

Cloning and sequencing of the *flaB* flagellin gene from *H. pylori* 85P. To clone the gene coding for the minor *H. pylori* flagellin, we used the cloned *H. mustelae flaB* gene as a probe to screen the previously described cosmid gene bank of *H. pylori* 85P (29). To obtain a probe free of vector contamination, two oligonucleotides (OLHMflaB-2 and -3) that permitted the amplification of the entire gene from the genomic DNA of *H. mustelae* by PCR were synthesized. The purified PCR product was used to screen 400 cosmid clones by low-stringency colony blot hybridization. Seven cosmids were found to hybridize, and one of them (pILL683) was used to subclone the *flaB*-related gene. Fragments with sizes of 3 to 4 kb were generated by partial restriction of the cosmid DNA with endonuclease *Sau*3A, purified, and ligated into the *Bgl*II site of plasmid vector pILL570. One hundred clones were screened with the *H. mustelae flaB* probe, and 1 of the 12 hybridizing clones (pSUS19) was mapped in detail (Fig. 1) and sequenced. The nucleotide sequence is shown in Fig. 2B. The clone contained one large ORF of 1,542 nucleotides coding for a protein of 53.9 kDa. The N-terminal amino acid sequence of the deduced protein was completely

TABLE 3. Alignment of the N-terminal amino acid sequence deduced from the cloned *H. mustelae* flagellin gene with available sequences obtained by protein sequencing

Source	N-terminal amino acid sequence[a]
H. mustelae FlaB	M S F R I N T N I A A L N A H S I G V
H. pylori FlaB[b]	S F R I N T N I A A L T S H A V G V
H. mustelae FlaA[c]	A F Q V N T N I N A L T T X A
H. pylori FlaA[d]	A F Q V N T N I N A M N A H V Q S A

[a] Asterisks indicate residues identical with those of the cloned *H. mustelae* flagellin gene.
[b] From Kostrzynska et al. (26).
[c] From Suerbaum et al. (49).
[d] From Leying et al. (32). Residues 1 to 13 were obtained by protein sequencing, and residues 14 to 18 were deduced from the nucleotide sequence.

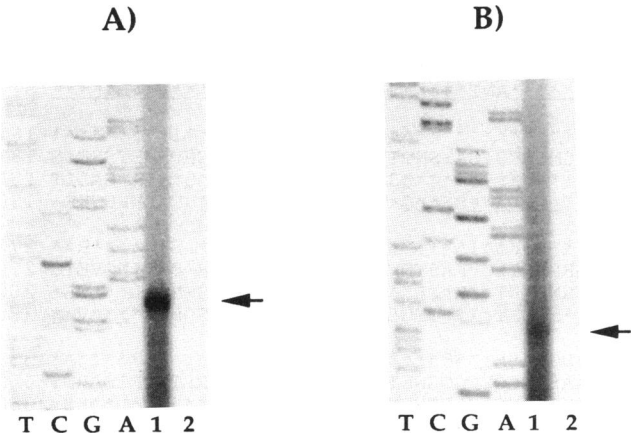

FIG. 4. Mapping of the transcription start point of the *H. pylori flaA* (A) and *flaB* (B) genes by primer extension. Lanes: T, C, G, and A, DNA sequencing reactions performed with the same primer as that used for primer extension; 1, primer extension reactions; 2, control reactions with addition of RNase prior to extension. Arrows mark the major primer extension products.

identical to the N-terminal sequence of the purified minor *H. pylori* flagellin as reported by Kostrzynska et al. (26). The gene was named *flaB*. Upstream of the ORF, a Shine-Dalgarno site and a σ^{54} promoter-like sequence (14 of 16 nucleotides matching the *E. coli* consensus) were found. Downstream, a putative transcription terminator (free energy, $\Delta G = -15.8$ kcal [ca. -66.1 kJ]/mol) was identified.

Mapping of the transcription start point of the *H. pylori flaB* gene. Since the functional activity of σ^{54}-like promoters had not yet been demonstrated in *Helicobacter* species, a primer extension experiment was performed to determine the transcription start point for the *H. pylori flaB* gene. Total RNA was extracted from *H. pylori* 85P, and the mRNA start point of the *flaB* gene was determined by extension of oligonucleotide OLHPflaBPE-1 (Fig. 4B). The transcription of the *flaB* gene starts at the adenine residue (nucleotide 113 of the sequence, as shown in Fig. 2B) 25 nucleotides upstream of the ATG start codon. This residue is positioned 12 nucleotides downstream of the end of the proposed promoter. This result therefore confirms the functional activity of the proposed promoter. The amount of extended product was very low when compared with the amount of product obtained in a parallel experiment using the *flaA*-specific primer OLHPflaAPE-2 (Fig. 4A). No extension product was obtained in the control reactions in which RNase was added prior to extension.

Construction of isogenic *flaA* and *flaB* mutants of *H. pylori* N6. Isogenic mutants of *H. pylori* N6 deficient in expression of major or minor flagellin were constructed by the following approach: for inactivation of *flaA*, a 1.4-kb *Bam*HI restriction fragment of plasmid pILL600 containing a gene encoding resistance to kanamycin (*aph*3′-III) was cloned into the unique *Bam*HI site of plasmid pHL319-2-4, which is situated in the middle of the *H. pylori flaA* gene, giving rise to plasmid pSUS10. For inactivation of *flaB*, the same kanamycin cassette (cut out with *Sma*I) was inserted into the unique *Sac*I site of pSUS19, situated in the *flaB* gene, after treatment of the linearized pSUS19 with T4 polymerase. The resulting plasmid was called pSUS22. *H. pylori* N6 was transformed with either pSUS10 or pSUS22 by electroporation. More than 50 independent transformants were obtained in transformation experiments with both plasmids. Several mutant strains (defined as descendants of a single colony of kanamycin-resistant *H. pylori*) were analyzed by a PCR approach (Fig. 5). The strains were tested (i) for the absence of vector sequences by using oligonucleotides OLpBR322-1 and -2 derived from the nucleotide sequence of pBR322 shared by pILL570 (not shown), (ii) for the presence of the kanamycin cassette by using primers OLKm-1 and -2 derived from the nucleotide sequence of the kanamycin resistance gene (Fig. 5A), and (iii) for integration of the cassette into the targeted gene by using primers specific for sequences of the targeted gene upstream and downstream of the site of disruption (primers OLHPflaA-1 and -2 for the detection of *flaA* [Fig. 5B]) and OLHPflaB-2 and -7 for amplification of a part of *flaB* [Fig. 5C]). In the mutant strains, under the experimental conditions used, disruption of the targeted gene led to a negative amplification reaction for the particular gene because of the augmentation in size of

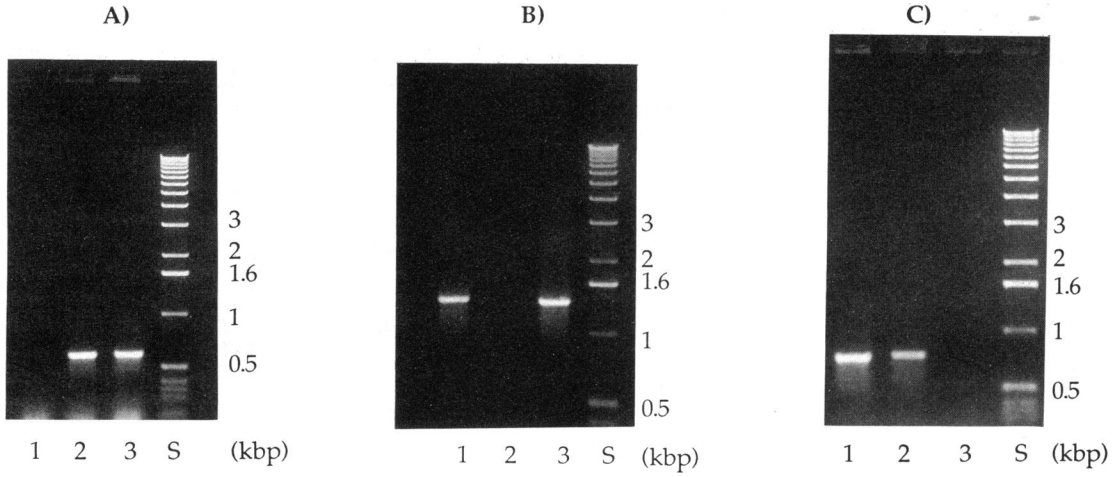

FIG. 5. Genotypic characterization of *H. pylori* wild-type strain N6 and corresponding isogenic *flaA* and *flaB* mutants by PCR. (A) Detection of the kanamycin resistance gene (predicted PCR product, 630 bp); (B) detection of the *flaA* gene (predicted PCR product, 1,400 bp); (C) detection of the *flaB* gene (predicted PCR product, 750 bp). Lanes: 1, wild-type strain *H. pylori* N6; 2, N6 *flaA* mutant; 3, N6 *flaB* mutant; S, DNA size markers.

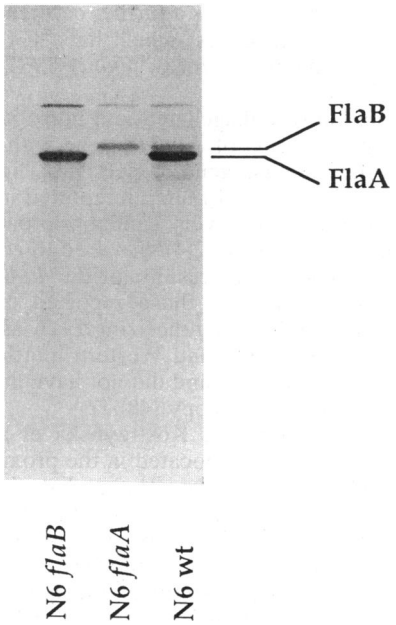

FIG. 6. Western blot of flagella partially purified from *H. pylori* N6 and the isogenic *flaA* and *flaB* mutants developed with an antiserum raised against *H. pylori* flagellar filaments.

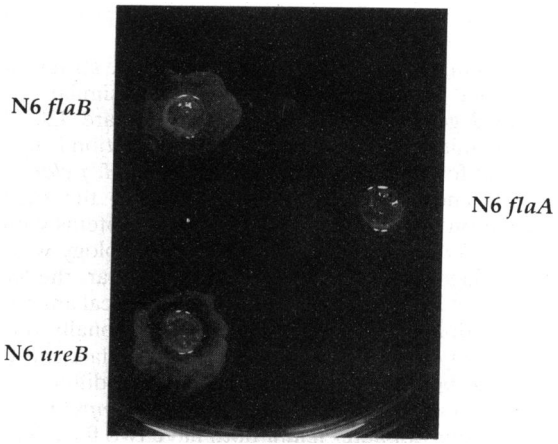

FIG. 7. Motility of isogenic *flaA* and *flaB* mutant strains of *H. pylori* N6 compared with urease-negative *ureB* mutant. The figure shows swarming of the *ureB* and *flaB* strains in a kanamycin-containing soft agar plate. The *flaA* strain exhibits no swarming.

the fragment to be amplified (fragments over 2 kb could not be amplified in our system that uses unpurified bacterial lysates as a source of template DNA). Correct integration was also verified by using combinations of one primer specific for the targeted gene and one specific for the kanamycin cassette (data not shown). For all tested strains, the analysis confirmed that a double crossover event had taken place, leading to the complete elimination of the vector and to rescue of the antibiotic resistance by allelic replacement of the intact copy of the targeted gene by the disrupted copy introduced by electroporation.

Phenotypic characterization of *H. pylori flaA* and *flaB* mutants. The *flaA* and *flaB* mutant strains were characterized by Western blots of partially purified flagella developed with antiserum AK179 (raised against purified flagellar filaments) and by motility testing. The results of the Western blots (Fig. 6) showed disappearance of the 53-kDa FlaA band in the N6 *flaA* mutant and disappearance of a weak band of slightly higher molecular weight in the *flaB* mutant, showing that the introduced mutations selectively abolished the expression of only the targeted flagellin gene, but did not seem to affect the expression of the other gene to a detectable extent.

The motility of the mutant strains was tested by demonstration of swarming on soft agar plates (Fig. 7). Since the plates contained kanamycin to assure the stability of the mutants, a previously described kanamycin-resistant *ureB*-disrupted mutant of *H. pylori* N6 (11) was used as a control. *flaA* mutants were completely nonmotile. *flaB* mutants, however, maintained motility, and the degree of their motility compared with that of the *ureB* mutant appeared unaffected by the mutation.

DISCUSSION

Motility is considered a major virulence factor of *H. pylori* and (together with urease expression) is one of the two bacterial properties for which this hypothesis is supported by experiments in an animal model (7–9).

The *H. pylori* flagellum was therefore among the first structures of this bacterium to be subjected to a detailed molecular analysis. In 1989, Geis et al. (16) reported the purification of *H. pylori* flagellin as a single 51-kDa protein. The N-terminal amino acid sequence of this protein was subsequently determined and proved that the purified molecule was indeed a flagellin (19). Later, Kostrzynska et al. (26) published a more detailed ultrastructural and biochemical study of the *H. pylori* flagellar filament. These investigators found that the filament was composed of two different flagellin species, a predominant one with a molecular mass of 56 kDa and a minor one with a molecular mass of 57 kDa that was expressed in much lower amounts. Comparison of the N-terminal sequences showed that the 51-kDa protein described by Geis et al. and the 56-kDa major flagellin of Kostrzynska et al. were the same proteins. The gene coding for this major flagellin was recently cloned and sequenced by Leying et al. (32) by using an oligonucleotide derived from the N-terminal amino acid sequence of the purified protein as a probe. The predicted molecular mass of the gene product is 53.2 kDa (it is, however, not excluded that the molecular mass of the native protein is higher if it is posttranslationally modified as has been described for *Campylobacter coli* flagellins [33]).

In this study, the cloned *flaA* gene from *H. pylori* was used as a probe to clone a flagellin gene from the closely related animal pathogen *H. mustelae*. The gene that was cloned and sequenced, although clearly a flagellin gene, did not encode the major *H. mustelae* flagellin, which we had purified previously (49). The N-terminal amino acid sequence of the deduced gene product exhibited, however, strong homologies with that of the minor *H. pylori* flagellin as described by Kostrzynska et al. Since the gene encoding the minor *H. pylori* flagellin had not been described to date, we used the cloned *H. mustelae* gene to screen a cosmid gene bank of *H. pylori* 85P and to clone and sequence the homologous *H. pylori flaB* gene. The N-terminal amino acid sequence of the protein deduced from the nucleotide sequence of this gene was exactly identical with that of the purified minor flagellin. Also, its amino acid composition was in good agreement overall with the results of chemical determinations reported

by Kostrzynska et al. (26), except for the proline content, which was significantly lower.

The *H. mustelae flaB* gene is the first entire structural gene to be cloned from this organism. It is very similar to the *H. pylori flaB* gene. The deduced proteins share 420 (81.7%) identical amino acids. This level of conservation is similar to that found for the *ureA* and *ureB* genes of *H. pylori* and *H. felis* (12) and might be representative of the degree of conservation of functionally homologous proteins within the gastric *Helicobacter* sp. The degree of homology with other bacterial flagellins is much lower. In particular, the *flaA* and *flaB* genes of *H. pylori* share only 58% identical amino acids, indicating that both proteins may by functionally different. The relatively low relatedness of *H. pylori* FlaA and FlaB is remarkable because it represents a striking difference from the situation in the closely related genus *Campylobacter*. *C. coli* and *Campylobacter jejuni* both have two flagellin genes. The two genes in these species are, however, almost identical (more than 90% identity on both the amino acid and nucleotide levels) and located almost directly adjacent to each other (22, 39). In *H. pylori*, not only are the genes much less homologous but also there was no evidence for a genetic linkage of the genes, since they were found on completely unrelated cosmids (data not shown). Hybridization studies have not provided evidence for the existence of further flagellin genes homologous with *flaA* or *flaB* (data not shown).

Both genes are preceded by typical AGGA Shine-Dalgarno sequences (46) and begin with an ATG start codon. Upstream of both the *H. mustelae* and *H. pylori flaB* genes, there are sequences which closely (14 of 16 nucleotides) resemble the consensus sequence for the so-called σ^{54}-dependent promoters (5′-TGGYRYR-N4-TTGCA-3′, with Y = T or C and R = G or A [51]). Since the activity of σ^{54}-like promoter sequences had not yet been demonstrated in *H. pylori*, a primer extension experiment that showed that the synthesis of *flaB* mRNA starts 12 nucleotides after the end of the proposed promoter was performed. Since this distance is typical for σ^{54}-dependent promoters (also called −12/−24 promoters), the result confirms that transcription of the *H. pylori flaB* gene is in fact under the control of the proposed promoter. The small amount of extended product in comparison with the amount of product obtained in a parallel experiment using a *flaA*-specific primer (that otherwise confirmed the position of the *flaA* mRNA start point as reported by Leying et al. [32]) suggests that the low expression level of FlaB compared with that of FlaA is due to regulation at the transcriptional level.

σ^{54}-dependent promoters were initially found to regulate the expression of genes involved in the assimilation of nitrogen. More recently, σ^{54}-dependent promoters have also been found associated with genes serving other physiological functions, including pilin genes in *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*, the *H. pylori* urease, and several flagellar genes in different species (5, 29; for reviews, see references 27 and 51).

Although the majority of bacterial flagellin genes have σ^{28}-like promoter elements (including the *H. pylori* and *Campylobacter flaA* genes), σ^{54}-like promoters have been found upstream of several *Caulobacter crescentus* flagellar genes and the *C. coli* and *C. jejuni flaB* genes (25). The precise role of these promoters in the regulation of flagellar assembly has yet to be studied.

Also notable is the relatively high G+C content of both the *H. mustelae* and the *H. pylori flaB* genes (45.3 and 46.8%, respectively) when compared with the reported overall G+C content of the genomes. This is a further difference from the *Campylobacter* flagellin genes, which have G+C contents matching the low (about 35% for *C. jejuni*) G+C contents of these species.

The construction of unflagellated and nonmotile mutants of *H. pylori* by disruption of the *flaA* gene with a chloramphenicol acetyltransferase gene cassette was first reported by Haas et al. (23). The *flaA* mutants reported in this study have been constructed by a very similar approach, and the same flagellin clone (pHL319-2-4) was used to construct the disruption. As expected, the results for the *flaA* mutants of strain N6 were identical to those reported earlier: *flaA* mutants were incapable of synthesizing FlaA as visualized by SDS-PAGE (not shown) and Western blotting (Fig. 6), and mutants were nonmotile and did not have flagella when analyzed by electron microscopy (48).

As for the minor flagellin, Kostrzynska et al. (26) described that it was primarily located in the proximal part of the filament. Our study demonstrates that *flaB* mutants retained motility, and the minor flagellin thus does not seem to be required for motility. Preliminary electron microscopy studies (48) have confirmed that the bacteria are indeed flagellated, but an in-depth study of the morphology and other properties of the *flaB* mutants has yet to be performed and will be published separately. So far, the role of the minor flagellin is unclear and can be only an object of hypotheses. Because of their different promoters, the expression of both flagellins can probably be regulated independently, and it seems therefore conceivable that the relative contribution of both proteins to the filament may vary in vivo and permit an adaptation of the physicochemical properties of the filament to different environmental conditions such as the viscosity of the medium. Antibodies against the major flagellin are produced in most patients with *H. pylori* infection (17), and although it is as yet unknown if those antibodies prevent motility, it seems conceivable that an increase of the relative amount of FlaB in the filament can facilitate bacterial evasion from host immunity. It also remains to be investigated whether one or both *H. pylori* flagellins, by certain domains, interact with the flagellar sheath and contribute to its assembly. Studies are now under way in our laboratories to test these hypotheses and to gain further insight into the relative roles of the two flagellin molecules and the regulation of their expression. Likewise, cloning of the *H. mustelae flaA* gene and of the *H. felis* flagellin genes as well as the construction of mutants in these organisms are under way. Studies of our *H. pylori flaA* and *flaB* mutants in the gnotobiotic piglet model as well as of nonmotile *H. mustelae* and *H. felis* mutants in the respective animal models will permit clarification of the role of flagella and motility in the pathogenesis of gastric *Helicobacter* infections and also evaluation of the usefulness of flagellin mutants for vaccination purposes.

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