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Cloning and Characterization of the *Helicobacter pylori flbA* Gene, Which Codes for a Membrane Protein Involved in Coordinated Expression of Flagellar Genes†

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Flagellar motility has been shown to be an essential requirement for the ability of *Helicobacter pylori* to colonize the gastric mucosa. While some flagellar structural components have been studied in molecular detail, nothing was known about factors that play a role in the regulation of flagellar biogenesis. We have cloned and characterized an *H. pylori* homolog (named *flbA*) of the *lcrD/flbF* family of genes. Many proteins encoded by these genes are known to be involved in flagellar biogenesis or secretion of virulence-associated proteins via type III secretion systems. The *H. pylori flbA* gene (2,196 bp) is capable of coding for a predicted 732-amino-acid, 80.9-kDa protein that has marked sequence similarity with other known members of the LcrD/FlbF protein family. An isogenic strain with a mutation in the *flbA* gene was constructed by disruption of the gene with a kanamycin resistance cassette and electroporation-mediated allelic exchange mutagenesis. The mutant strain expressed neither the FlaA nor the FlaB flagellin protein. The expression of the FlgE hook protein was reduced in comparison with the wild-type strain, and the extent of this reduction was growth phase dependent. The *flbA* gene disruption was shown to downregulate the expression of these flagellar genes on the transcriptional level. The *flbA* mutants were aflagellate and completely nonmotile. Occasionally, assembled hook structures could be observed, indicating that export of axial flagellar filament components was still possible in the absence of the *flbA* gene product. The hydrophilic part of the FlbA protein was expressed in *Escherichia coli*, purified, and used to raise a polyclonal rabbit antiserum against the FlbA protein. Western blot experiments with this antiserum indicated that the FlbA protein is predominantly associated with the cytoplasmic membrane in *H. pylori*. The antiserum cross-reacted with two other proteins (97 and 43 kDa) whose expression was not affected by the *flbA* gene disruption and which might represent further *H. pylori* homologs of the LcrD/FlbF protein family.

Helicobacter pylori is now recognized as one of the most prevalent human pathogens. It is the causative agent of chronic type B gastritis, a prerequisite for duodenal ulcers and most gastric ulcers and a major risk factor for the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. Numerous bacterial factors have been suggested to play a role in the pathogenesis of *H. pylori* infection (see references 5 and 52 for reviews), but so far only for few of those (including urease, motility, and the vacuolating cytotoxin) has this been demonstrated by experiments with animal models (6, 8, 53).

The flagella are among the best-studied structural components of *H. pylori* cells, and comparative studies on flagellar genes and proteins have been performed with the closely related animal pathogens *Helicobacter mustelae* and *Helicobacter felis* (48–50). *H. pylori* cells possess a unipolar bundle of sheathed flagella (11, 14). The flagellar filament is a copolymer of two flagellin subunit species, FlaA and FlaB, which under in vitro culture conditions are expressed in very different quantities, FlaA being the major component (24, 28, 50). The flagellar filament is linked to the flagellar basal body by means of the hook, which itself is a polymer of the FlgE hook protein (36). In vitro experiments with isogenic *H. pylori* strains with mutations in the *flaA* and *flaB* flagellin genes have shown that both flagellins are required for full motility (21). Colonization experiments with spontaneous nonmotile mutants as well as isogenic *flaA*, *flaB*, and *flaA flaB* mutants in the gnotobiotic piglet model of *H. pylori* infection have demonstrated that full motility and the presence of both flagellins are required for the establishment of persistent infection (8). Full motility is thus an essential virulence factor of *H. pylori* and a possible target for novel therapeutic substances.

The *Helicobacter* FlaA and FlaB flagellins have only limited amino sequence similarity, which is in sharp contrast to the situation for *Campylobacter coli* and *Campylobacter jejuni*, which express two flagellins that are almost identical (15, 35, 47). The *H. pylori flaA* and *flaB* genes are unlinked on the chromosome and preceded by different promoters (σ^{28} for *flaA* and σ^{54} for *flaB*), and first reporter gene studies have provided evidence that the genes can be regulated differentially by environmental conditions (22). However, expression of the flagellin genes is also strongly dependent on the growth phase, and the overall pattern of growth phase-dependent changes is the same for both flagellin genes. It therefore appears very likely that expression of the flagellar structural components is governed both by master factors that coordinate gene expression in the bacterium and by environmental factors that influence the individual flagellar components differentially. The regulatory mechanisms are not yet known.

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TABLE 1. Vectors and hybrid plasmids used in this study

Plasmid	Vector	Size (kbp)	Antibiotic resistance, comments[a]	Reference
	pILL570	5.3	Mob, Sp^r	26
	pQE30	3.5	Ap^r, QIAexpress expression vector, 6×His tag fusion	Qiagen
	pSUS33	2.7	Ap^r, derivative of pUC19, *Hin*dIII site deleted	This study
pILL600	pBR322	5.7	Ap^r Km^r, source of Km cassette	27
pSUS39	pILL570	9.8	Sp^r, subclone of *flbA* cosmid containing 5′ part of *flbA* gene	This study
pSUS40	pSUS33	4.3	Ap^r, contains PCR-amplified part of *flbA* gene	This study
pSUS42	pSUS33	5.7	Ap^r Km^r, *H. pylori flbA* Ω Km	This study
pSUS207	pILL570	9.0	Sp^r, subclone of *flbA* cosmid containing 3′ part of *flbA* gene	This study
pSUS223	pQE30	5.1	Ap^r, plasmid for expression of hydrophobic FlbA partial protein	This study
pSUS225	pQE30	4.6	Ap^r, plasmid for expression of soluble FlbA partial protein	This study

[a] Ap^r, resistance to ampicillin; Sp^r, resistance to spectinomycin; Km^r, resistance to kanamycin; Mob, mobilizable vector due to the presence of OriT.

In a few other organisms, notably *Escherichia coli*, *Salmonella* spp., and *Caulobacter crescentus*, the regulation of motility, flagellar biogenesis and chemotaxis has been extensively studied (see references 2, 19, 20 and 45 for comprehensive reviews). In these organisms, numerous (at least 40) genes acting on hierarchically arranged levels are involved in motility regulation. There is, however, already significant evidence that the regulation of flagellar gene expression in *H. pylori* differs significantly from the systems characterized so far (see Discussion). Since it appears very likely that the regulation of motility-associated genes has evolved to optimally adapt the flagellar apparatus to different niches that *H. pylori* colonizes during its pathogenic life cycle, a better understanding of this regulatory system might give new insights into the mechanism of adaptation of a pathogen to unusual environments.

The aim of this study was to characterize the *H. pylori* homolog of the *flbF*/*lcrD* family of motility and virulence-associated genes which, by analogy to other organisms, appeared likely to be an important flagellar regulatory gene in *H. pylori*. The *flbF*/*lcrD* homolog was also selected for study because this gene family represents an interesting, albeit so far incompletely understood, contact point between motility and a number of other virulence-associated traits. The *lcrD*/*flbF* gene family comprises two subfamilies. Several proteins encoded by the first subfamily of genes have been shown to be essential for flagellar biogenesis. The best-studied homolog of this type is the *C. crescentus flbF* gene, which acts at the highest level of the hierarchy of flagellar genes in this organism (19, 40). Other homologs that have likewise been shown to be essential for the synthesis, export, and assembly of flagellar components include the *E. coli*, *Salmonella typhimurium*, and *Proteus mirabilis* FlhA proteins and the *C. jejuni* FlbA protein (16, 32, 33).

The second subfamily comprises a number of homologous proteins that have been shown to be involved in the secretion of other virulence-associated proteins by the so-called type III secretion pathway (41). Prototypes of those proteins are *Yersinia pestis* LcrD (38, 39), which together with the Ysc group of secretion proteins plays a part in the low-calcium response of *Yersinia* sp. (see reference 46 for an overview), and *S. typhimurium* InvA, recently shown to be part of the secretory machinery required for assembly of the invasome, a transient filamentous structure formed in the process of adhesion to and invasion of epithelial cells (10, 13; see also reference 17 for an overview of the homologies between flagellar proteins and type III secretion pathway proteins). Because of our continued interest in *H. pylori* motility, flagellar gene regulation, and the specific adaptations of the motility apparatus to the unique habitat of *H. pylori*, we have cloned and characterized the *H. pylori flbA* gene and studied the effect of a knockout mutation in the *flbA* gene on the expression of different flagellar components.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Vectors and recombinant plasmids used in this study are listed in Table 1. *H. pylori* N6 (9) was used for the electroporation-mediated mutagenesis. The isogenic *flaA* and *flaB* mutants that were used as controls in motility assays and Western blot experiments have been described previously (21, 50).

H. pylori strains were cultured on blood agar base 2 plates (Oxoid, Wesel, Germany) supplemented with 10% horse blood and the following antibiotics: 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. When necessary for selection of *H. pylori* allelic exchange mutants, kanamycin (20 mg/liter) was used in addition to the other antibiotics.

Recombinant plasmids were transformed and maintained in *E. coli* MC1061 (4) and DH5α (Bethesda Research Laboratories). *E. coli* TG1 (12) was used as the host strain for the expression of parts of the FlbA protein in *E. coli*. M13 DNA for the production of single-stranded sequencing templates was propagated in *E. coli* JM101 (55). *E. coli* strains were grown on standard media, such as Luria-Bertani agar (1.5% agar) or Luria or 2× YC broth, at 37°C (42). For the selection of plasmids in *E. coli*, media were supplemented with spectinomycin (100 mg/liter), ampicillin (100 mg/liter), or kanamycin (20 mg/liter).

DNA techniques. All standard methods of DNA manipulation were performed according to the protocols of Sambrook et al. (42). *H. pylori* genomic DNA was prepared as described by Majewski and Goodwin (30). Large-scale plasmid preparations were purified with the Qiagen Midi column plasmid purification kit (Qiagen Inc.). Single DNA restriction fragments or PCR amplification products for cloning or sequencing purposes were purified from agarose gels with a QiaEX DNA purification kit (Qiagen). DNA restriction and modification enzymes were obtained from BRL Life Technologies or from Boehringer Mannheim Biochemicals and were used according to the directions of the manufacturers. For screening of the cosmid gene bank and plasmid minibank of the cosmid containing the *flbA* gene, a previously described radioactive low-stringency colony blot hybridization protocol was used (51).

DNA sequence determination. The DNA sequences were obtained by the dideoxy-chain termination method (44). Sequencing was performed either by a radioactive protocol using [α-^{35}S]dATP (Amersham) and a Sequenase version 2.0 kit (U.S. Biochemicals, Cleveland, Ohio) or a nonradioactive dye terminator protocol using the Applied Biosystems Prism kit and an automated Applied Biosystems model 373 sequencer. The first sequences were obtained by cloning of suitable restriction fragments of clones pSUS39 and pSUS207 into M13mp18 and M13mp19 vectors (31) and sequencing of single-stranded templates by using both the M13 universal primer and custom-synthesized oligonucleotide primers. Additional sequence information was obtained by sequencing of double-stranded DNA templates by the nonradioactive method. Both strands of the DNA fragment shown in Fig. 1 were completely sequenced independently. Sequence processing and interpretation were done with the help of the GENMON sequence analysis program (Gesellschaft für Biologische Forschung, Braunschweig, Germany) and the HUSAR program package (Publish, ClustalW, Clustree, and Prettybox) of the European Molecular Biology Laboratory, Heidelberg, Germany. Prediction of transmembrane helices is based on the TMpred program (18) as provided by the bioinformatics server of the Swiss Institute for Experimental Cancer Research.

DNA amplification by PCR. PCRs were performed in a Perkin-Elmer Cetus TC1 thermal cycler with a Gene Amp kit (Perkin-Elmer Cetus). In each reaction mixture, at least 5 pmol of target DNA, 100 pmol of each primer (1,000 pmol for degenerate primers), and standard concentrations of deoxynucleotides and $MgCl_2$ were included. As target DNA, whole-cell lysates of *Helicobacter* strains

or purified genomic DNA or plasmid DNA preparations were used. The PCR mixtures were denatured at 94°C for 1 min, annealed at temperatures between 40 and 55°C (depending on the calculated melting temperatures of the primers) for 1 to 2 min, and extended at 72°C for 1 to 3 min. A total of 35 cycles was performed.

RNA methods. RNA was extracted from *H. pylori* N6 and the isogenic *flbA* mutant by the method of Leying et al. (28). Primer extension was performed with the oligonucleotides OLHPFlbA-PE1 (5′-GAA AGT CTT TTT AAA AGC TAA TTT GGA GCG-3′, corresponding to positions 262 to 291 of the sequence in Fig. 1) and OLHPFlbA-PE2 (5′-CGA GAC GGG TTC TTG TGA AGT GCA AGT GGC-3′, positions 26 to 55) exactly as described previously (50). To determine the transcription start site, a sequencing reaction was performed with plasmid pSUS39 as the double-stranded target and the same primers used for the extension reactions. These sequencing reactions were run in parallel with the extension reactions.

For the RNA slot blot hybridizations, slot blotting onto positively charged nylon membranes (Boehringer Mannheim) was performed by using a Bio-Dot SF slot blotting device (Bio-Rad). Samples containing 1 and 5 μg of the RNAs to be tested were precipitated and resuspended in 500 μl of denaturation buffer (10 mM NaOH, 1 mM EDTA). The membrane was prewetted in 6×SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer and assembled in the blotting device, vacuum was applied, and slots were washed twice with 100 μl of denaturation buffer. Subsequently the sample solutions were applied, the slots were washed with 500 μl of denaturation buffer, and the device was disassembled. The membrane was then blotted dry on filter paper and baked for 30 min at 120°C. Afterwards, hybridization was performed by using nonradioactive digoxigenin-DNA chemiluminescence labeling and detection kits (Boehringer Mannheim) and high-stringency hybridization conditions as described previously (21). The DNA probes used for the RNA slot blot hybridizations were generated by PCR using the following primer combinations: *H. pylori flaA* gene probe, primers OLHPFlaA-1 (5′-GGA GAG ATT GAG TTC AGG TTT AAG G-3′) and OLH PFlaA-2 (5′-ACT CAT CGC ATA GCT GCC TGA TTG C-3′, based on sequences from reference 31); *H. pylori flaB* gene probe, primers OLHPFlaB-2 and OLHP FlaB-7 (50); *H. pylori flgE* gene probe, primers OLHPFlgE-1 (5′-CTC AAG TCA AAC TCA TCG-3′) and OLHPFlgE-2 (5′-CTT GCC GTC TAA ATA AGC-3′, based on sequences from reference 36); and *H. pylori* 16S rRNA gene probe, primers OLHP16S-1 (5′-GCT AAG AGA TCA GCC TAT GTC C-3′) and OLHP16S-2 (5′-TGG CAA TCA GCG TCA GGT AAT G-3′, based on sequences from reference 37).

Electroporation of *H. pylori* for the construction of isogenic mutants. Plasmid pSUS42 containing a part of the *flbA* gene disrupted by a kanamycin resistance cassette (*aph3′*-III) was purified from *E. coli* MC1061 and electrotransformed into *H. pylori* N6 as previously described (50). After electroporation, the bacteria were grown on nonselective plates for 48 h and then transferred to plates containing kanamycin. These plates were incubated for 4 to 6 days before single colonies were isolated.

Phenotypic characterization. Motility testing in 0.3% soft agar by stab agar and single-colony motility tests and transmission electron microscopy of negatively stained samples were performed as previously described (21).

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Lugtenberg et al. (29). The acrylamide concentration in the gel was 11%. Samples corresponding to 80 μg of protein were applied to the gels. For Western blot experiments, the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by the method of Towbin et al. (54). The blots were incubated with rabbit antiserum AK179 (28) raised against purified *H. pylori* flagellar filaments or with the anti-FlbA antiserum. Both antisera were used in a dilution of 1:2,000. Bound antibodies were visualized with a peroxidase-coupled goat anti-rabbit antibody (dilution of 1:2,000; Biogenzia, Bochum, Germany).

Isolation of membranes by differential solubilization with sarcosyl. Differential solubilization of cytoplasmic and outer membranes by using the detergent *N*-lauryl sarcosinate (sarcosyl) was performed by a modification of the method described by Achtman et al. (1). Bacteria from five densely grown blood agar plates were harvested and resuspended in 25 ml of 0.9% NaCl. The bacteria were pelleted by centrifugation (4,000 rpm, 4°C), resuspended in 6 ml of 0.9% NaCl, and subsequently lysed by sonication. After removal of unlysed bacteria by low-speed centrifugation (3,000 rpm, 4°C), the membranes were pelleted by ultracentrifugation (48,000 × *g*, 60 min, 10°C). The pellet containing the membrane vesicles was resuspended in 1.0 ml of 12.5 mM Tris-HCl (pH 7.6). The cytoplasmic membranes were solubilized by addition of 8.0 ml of a 1.88% solution of sarcosyl in 12.5 mM Tris-HCl buffer (pH 7.6). The mixture was shaken for 60 min at room temperature, and outer and cytoplasmic membranes were subsequently separated by ultracentrifugation (48,000 × *g*, 90 min, 20°C). The pellet was resuspended in water prior to further analysis; the supernatant containing the cytoplasmic membranes was concentrated and dialyzed by using Microsep filtration devices (exclusion size, 30 kDa; Filtron Technology Corp., Northborough, Mass.).

Expression of recombinant FlbA protein. Plasmid constructs pSUS223 and pSUS225 containing the DNA fragments to be expressed, cloned into the expression vector pQE30, were transformed into *E. coli* TG1. For purification of the expression product, 50 ml of Luria broth containing ampicillin (100 mg/liter) was inoculated with 6 ml of an overnight culture of the freshly transformed expression strain and incubated at 37°C with vigorous shaking. When the culture reached an optical density at 600 nm of 0.7, the induction period was started by addition of isopropylthiogalactopyranoside (IPTG; 0.5 mM), and the culture was incubated for further 3 h. The bacteria were then harvested and resuspended in the appropriate buffers for sonication (see below).

Purification of recombinant proteins. Expression proteins that carried a C-terminal six-histidine (6×His) tag were purified as follows. The hydrophilic partial FlbA protein from *E. coli* TG1 containing the plasmid pSUS225 was purified under denaturing conditions, using a modification of the protocol suggested by Qiagen. After harvesting, the bacteria were resuspended in sonication buffer (50 mM NaH_2PO_4, 300 mM NaCl [pH 7.8]) containing protease inhibitors (aprotinin, 0.015 μM; leupeptin, 1 μM; Pefabloc, 50 μM; and pepstatin, 50 μM). The whole cell lysate was centrifuged (10,000 × *g*, 15 min, 4°C), and the pellet was resuspended in 2 ml of Ni^{2+}-nitrilotriacetic acid agarose, which had been equilibrated with buffer A (6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl [pH 8.0]). The mixture was gently stirred for 1 h at room temperature to allow binding of the recombinant protein to the resin. Subsequently, the mixture was transferred to a column and allowed to sediment for 30 min. Three washing steps with 2 ml of buffer A, 2 ml of buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl [pH 8.0]), and 3 ml of buffer C (like buffer B but pH 6.3) were performed, and the protein then eluted with 3 ml of buffers D (like buffer B but pH 5.9) and E (like buffer B but pH 4.5). The protein content of the elution fractions was analyzed by SDS-PAGE. The recombinant protein was found in both the buffer D and buffer E elution fractions. For concentration of the samples, 3.5 ml of the protein-containing fractions was applied to a Microsep microconcentrator (exclusion size, 30 kDa) and centrifuged overnight at 2,500 × *g* (4°C). After centrifugation, the sample reservoir of the Microsep contained a volume of approximately 200 μl. To this, a 500-μl volume of 50 mM ammonium hydrogen carbonate buffer (pH 7.9) was added, and the sample was removed from the Microsep device. The Microsep membrane was washed with a further 500 μl of ammonium hydrogen carbonate buffer. Both fractions were combined, another 1.3 ml of the same buffer was added, and the remaining urea in the sample was then removed with a Pharmacia PD-10 column.

For purification of the hydrophobic FlbA partial protein, the following protocol was used. After harvesting, bacteria were resuspended in 4 ml of lysis buffer (50 mM Tris, 5% glycerol, 5 mM $MgCl_2$, 150 mM NaCl [pH 8.0], protease inhibitors as in the sonication buffer). Bacteria were then lysed by sonication. The whole-cell lysate was centrifuged, and the sediment was resuspended in 200 μl of guanidine hydrochloride buffer (6 M guanidine hydrochloride, 50 mM Tris-HCl, 5% glycerol, 5 mM $MgCl_2$, 150 mM NaCl [pH 8.3]). The solution was homogenized by means of repeated aspiration into a syringe with a fine needle. The protein was renatured by slow dropwise addition of 10 ml of lysis buffer while the solution was continuously stirred on ice. Subsequently, the solution was applied to a Ni^{2+}-nitrilotriacetic acid-agarose column (500 μl of resin). The protein was allowed to bind to the column for 15 min (4°C). Subsequently, two washing steps with 3 ml of lysis buffer and 3 ml of washing buffer (50 mM Tris, 5% glycerol, 5 mM $MgCl_2$, 300 mM NaCl [pH 6.5]) were performed, and the protein was then eluted with 600-μl volumes of elution buffer containing increasing concentrations of imidazole (0.09 to 0.5 M in washing buffer). The highest concentration of the recombinant 60-kDa protein was present in the fractions containing 0.16 and 0.23 M imidazole.

Nucleotide sequence accession number. The sequence reported in Fig. 1 has been assigned accession number L38925 in the Genbank/EMBL databases.

RESULTS

Cloning and nucleotide sequence determination of the *H. pylori flbA* gene. Based on two short amino acid sequences (MPGKQM, amino acids 151 to 156 of *Y. pestis* LcrD, and MDGAMKF, amino acids 189 to 195 of LcrD) that are well conserved between *Y. pestis* LcrD, *C. crescentus* FlbF, and *S. typhimurium* InvA, two degenerate oligonucleotide primers, OLFlbA-1D (5′-ATG CCTCGA GGTCGA AAAG CAAG ATG-3′; underlined nucleotides indicate wobble positions) and OLFlbA-2D (5′-GAAA TCTT CAT ACTGGC AGCTCC AGTC-3′), were designed. These primers permitted us to amplify a 130-bp fragment of the *H. pylori flbA* gene, using purified genomic DNA of strain *H. pylori* N6 as a template. Direct nucleotide sequence determination of the fragment confirmed that it was part of a novel *flbF*/*lcrD*-homologous gene. The 130-bp fragment was used as a probe to screen a cosmid library of *H. pylori* 85P DNA in the cosmid vector pILL575, which was constructed and previously described by Labigne et al. (26). One of 400 cosmid clones hybridized with the probe under high-stringency conditions. DNA of this cosmid clone was purified and subjected to partial restriction with *Sau*3A. Frag-

ments with sizes of 2.5 to 5 kb were purified and cloned into the *Bgl*II site of the plasmid vector pILL570. One hundred clones of the resulting minibank were screened for hybridization with the 130-bp probe, and nine clones gave a positive hybridization signal. The complete nucleotide sequence of the *H. pylori flbA* gene was obtained by using two plasmid clones from the minibank with overlapping insert fragments, pSUS39 and pSUS207. None of these clones nor of five additional clones identified in a second round of screening contained the complete sequence; therefore, it appears likely that there is strong selection against cloning of the complete *flbA* gene in vectors with high copy numbers.

A total of 2,600 nucleotides were sequenced. The nucleotide sequence is shown in Fig. 1. One large open reading frame (ORF) of 2,196 nucleotides was identified. The predicted translation product of this ORF was a protein with a molecular mass of 80.9 kDa and a theoretical pI of 6.48. The N-terminal part of the protein is highly hydrophobic and contains seven predicted transmembrane domains (the locations of the transmembrane helices as predicted by the TMpred program are marked in Fig. 1), while the C-terminal part is much more hydrophilic. The predicted protein exhibited a high degree of similarity with other members of the FlbF/LcrD protein family. Figure 2 shows a multiple alignment of the *H. pylori* FlbA sequence with selected sequences of other known members of the FlbF/LcrD protein family.

The most closely related homologs of *H. pylori* FlbA are *C. jejuni* FlbA (51.7% amino acid identity) and *C. crescentus* FlbF (40.4% identity). The *H. pylori* gene was named *flbA* (*flbA* for flagellar biogenesis gene A). The overall sequence similarity with those members of the protein family that are not involved in the regulation of motility is lower (e.g., 32.8% identity with LcrD and 29.3% with InvA).

The most conserved part of the protein is the hydrophobic N-terminal part. The region that contains the transmembrane helices (amino acids 1 to 332) shares 61.4% identical amino acids with *C. jejuni* FlbA and 38.8% with *Yersinia enterocolitica* LcrD. The C-terminal part (amino acids 321 to 732) is significantly less conserved (43.4% identity with *C. jejuni* FlbA and 23.1% with LcrD).

In the nucleotide sequences upstream and downstream of the *flbA* ORF, no conspicuous promoter consensus sequences or transcription terminators were identified. Likewise, no ORFs corresponding to known flagellar genes were found in the immediate vicinity of the *flbA* gene. Specifically, no sequences with homology to the *flhB* family of genes, which in enteric bacteria are organized in an operon with the *flhA* genes, were found when 1-kb DNA sequences upstream and downstream of *flbA* were sequenced.

Determination of the transcription start point of the *H. pylori flbA* gene. Since no consensus promoter sequences could be identified in the upstream region of the *flbA* gene, a primer extension experiment with two different primers was performed. RNA was isolated from *H. pylori* N6. Several primer extension experiments performed with the primer HPFlbAPE-1 consistently showed two extension products corresponding to two transcription start points, 64 and 66 nucleotides upstream of the start codon (nucleotides 183 and 185 of the sequence in Fig. 1) (Fig. 3). No extension product was seen in a control reaction where RNase had been added prior to the addition of reverse transcriptase. The experiment with primer HPFlbAPE-2 that binds 240 bp upstream of primer HPFlbAPE-1 gave no visible extension product.

Construction of an isogenic *flbA* mutant by allelic exchange mutagenesis. For the construction of isogenic *flbA* mutants, a fragment of the *flbA* gene was amplified by PCR using primers OLHPFlbA-7 (corresponding to positions 679 to 698 of the sequence in Fig. 1) and OLHPFlbA-8 (positions 2256 to 2275 of the sequence in Fig. 1), which at their 5′ ends contained restriction sites for *Bam*HI. The positions of the primers were chosen such that the amplification product contained only a single *Hin*dIII restriction site. The resulting 1,600-bp PCR fragment was cut with *Bam*HI and ligated to the plasmid vector pSUS33 that had been linearized with *Bam*HI (pSUS33 is a derivative of pUC19 in which the *Hin*dIII restriction site has been removed). The resulting plasmid, pSUS40, was linearized with *Hin*dIII, the ends were blunted by treatment with Klenow enzyme, and the fragment was then ligated with a *Campylobacter* kanamycin resistance cassette that had been cut out of the plasmid pILL600 with *Sma*I. For the electroporation, we chose a plasmid clone (pSUS42) in which the orientation of transcription of the kanamycin resistance cassette was the same as that of the *flbA* gene. This cassette and orientation were chosen to minimize the risk of polar effects, because shuttle vectors to perform complementation experiments with *H. pylori* are not yet available. Since this cassette does not contain a transcription terminator, it permits the expression of genes situated downstream of the disrupted gene and thus avoids or greatly reduces potential polar effects. Plasmid pSUS42 was then used to electroporate *H. pylori* N6 as previously described (50). Out of several hundred single kanamycin-resistant colonies obtained, four mutant clones were isolated, passaged, and further characterized.

Characterization of a *H. pylori flbA* mutant. The genotypes of the mutants were characterized by PCR using different combinations of primers binding to sequences flanking the insertion site of the cassette and to sequences in the cassette (primers OLKm-1 and OLKm-2 [50]). The sizes of the PCR products obtained in these experiments confirmed that a double crossover had occurred, leading to the replacement of the intact allele by the allele disrupted with the kanamycin resistance cassette, and that vector sequences had been completely eliminated (data not shown).

Phenotypic characterization of the *H. pylori flbA* mutant. All mutant strains grew well and were not significantly affected in viability or growth characteristics. Semiquantitative assessment of urease activity in cytoplasmic fraction, membranes, and water extract did not show a change of urease activity, secretion, or membrane association when the *flbA* mutant was compared with a *flaA* mutant containing the same kanamycin resistance cassette as the *flbA* mutant (both *flbA* and *flaA* mutants had slightly higher urease activities than the wild-type strain, maybe due to the metabolic energy saved when the abundant FlaA flagellin is no longer synthesized).

Motility tests. The motility of the *H. pylori flbA* mutant was assayed by using the previously described methods of motility testing in 0.3% motility agar (single-colony motility and stab agar tests [21]). In the single-colony motility test, the *flbA* mutant, in contrast to the wild-type strain N6, which forms colonies with a large diffuse spreading halo, produced small, sharply delineated colonies that were indistinguishable in the colony morphology from nonmotile *flaA flaB* double mutants (data not shown; see reference 21 for a detailed description of the respective colony morphologies). Likewise, in the stab agar test, no swarming halo was visible. The *H. pylori* N6 *flbA* mutant was thus found to be completely nonmotile in both assays.

Electron microscopy. The N6 *flbA* mutant (Fig. 4B and C) was analyzed by transmission electron microscopy using negative staining and compared with the wild-type strain (Fig. 4A). No flagella could be detected in the mutant. Rarely, short structures about 30 to 100 nm in length that probably repre-

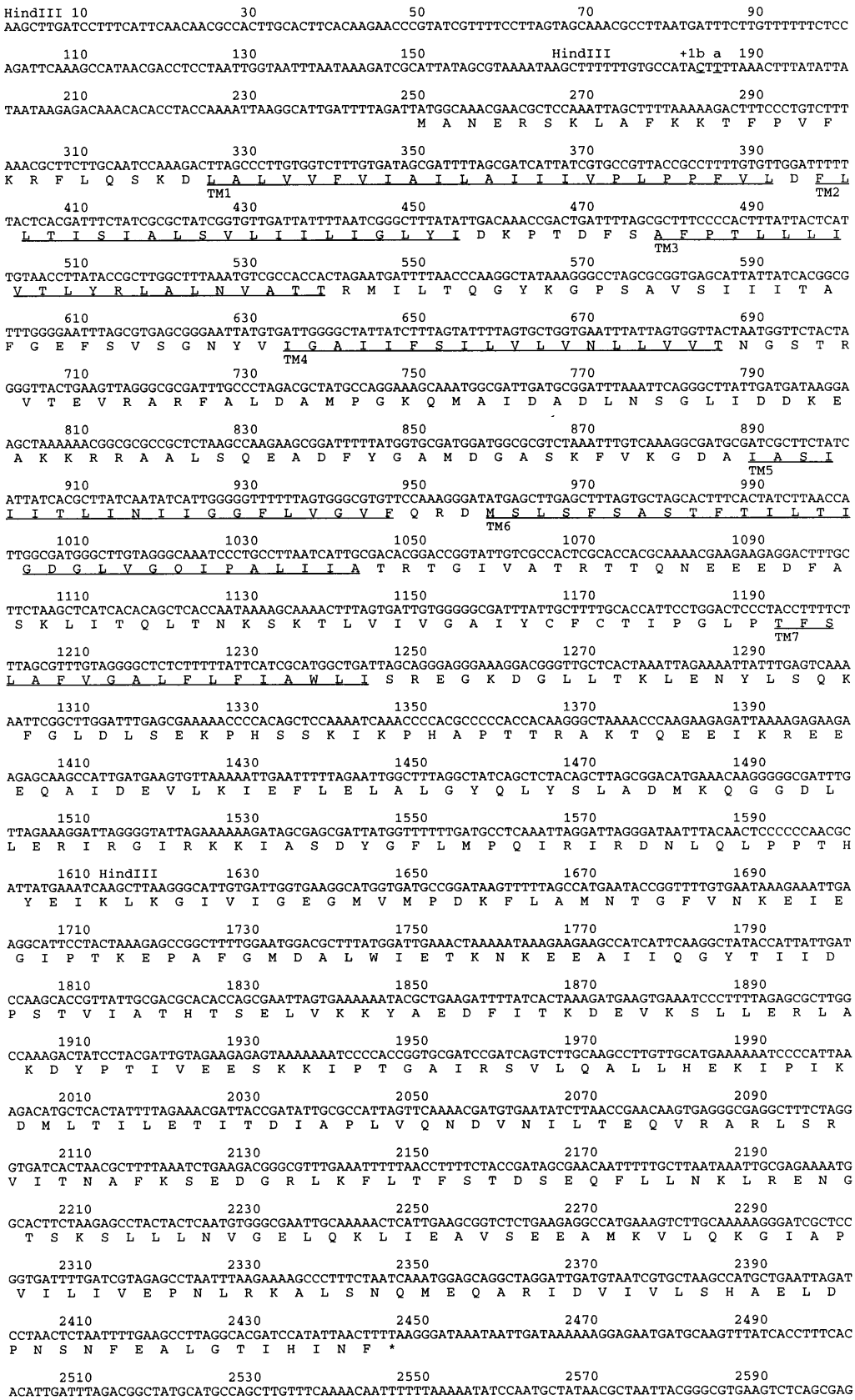

FIG. 1. Nucleotide and derived protein sequences of the *H. pylori flbA* gene. Letters below the sequence indicate the predicted amino acids in the single-letter code. The stop codon is marked by an asterisk. The seven underlined stretches of amino acids marked TM1 through TM7 are potential transmembrane helices as predicted by the TMpred program. The two transcription start points determined by primer extension experiments are underlined and marked +1a and +1b. The positions of *Hin*dIII restriction sites are also indicated above the nucleotide sequence. The *Hin*dIII restriction site at nucleotides 1612 to 1617 is the position where the kanamycin resistance cassette was inserted for construction of an isogenic *H. pylori flbA* mutant strain.

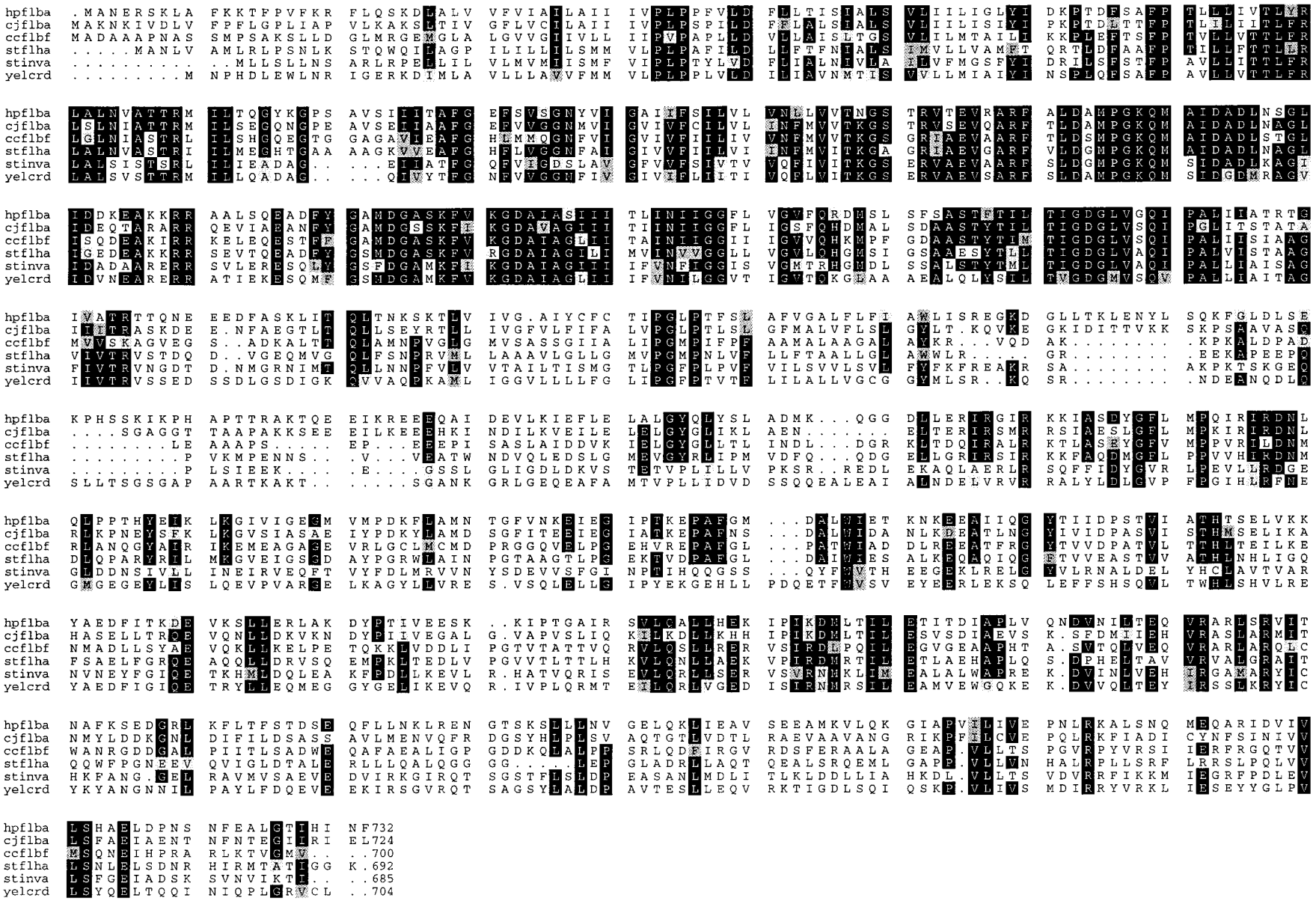

FIG. 2. Amino acid sequence alignment of the predicted *H. pylori* FlbA protein with selected homologs of the LcrD/FlbF protein family, from both the flagellar regulator and the type III secretion system subfamilies. Letters on the left indicate sequence names; numbers on the right indicate amino acid positions. hpflbA, *H. pylori* FlbA; cjflbA, *C. jejuni* FlbA (accession number A49217); ccflbF, *C. crescentus* FlbF (Q03845); stflhA, *S. typhimurium* FlhA (P40729); stinva, *S. typhimurium* InvA (A42888); yelcrd, *Y. enterocolitica* LcrD (P21210).

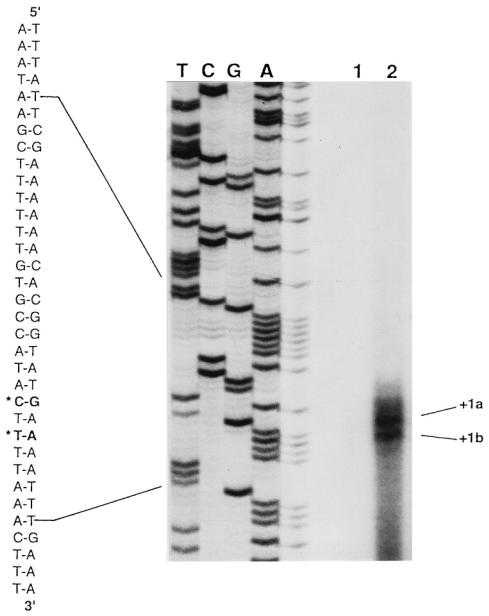

FIG. 3. Mapping of the transcriptional start site of the *H. pylori flbA* gene by primer extension. Lanes T, C, G, and A, DNA sequencing reactions performed with the same primer that was used for primer extension (OLHPFlbAPE-1); lane 1, RNase control; lane 2, primer extension reaction. The nucleotide sequence of the DNA region upstream of the *flbA* gene is given on the left. The identified transcription start sites (+1a and +1b) are indicated by arrows on the right and by asterisks in the sequence.

sented flagellar hooks were seen (Fig. 4C). No further ultrastructural abnormalities of the mutants were observed.

Western blot analysis. The *H. pylori* N6 *flbA* mutant as well as the N6 wild-type strain and isogenic *flaA* and *flaB* mutants were grown on plates for 4 days and harvested, and surface appendages were purified as previously described (50). The resulting material was analyzed by Western blotting with antiserum AK179 (28) raised against native flagellar filaments of *H. pylori* (Fig. 5). In the wild-type strain, the two flagellin bands corresponding to molecular masses of 53 kDa (FlaA) and 54 kDa (FlaB) as well as a 78-kDa band corresponding to the FlgE hook protein (36) were very clearly detected. In contrast, in the *flbA* mutant, neither the flagellin proteins nor the hook protein was detectable. The same result was obtained when whole-cell lysates of the bacteria were used, indicating that expression of flagellar proteins as well as export and assembly were switched off in the mutant (data not shown).

RNA slot blot analysis. To determine whether the absence of flagellin and hook expression in the *flbA* mutant was due to transcriptional regulation, RNA slot blot experiments were performed (Fig. 6). Total RNA was extracted from *H. pylori* N6 and the isogenic *flbA* mutant grown on plates for 2 days. Amounts of 1 and 5 μg of RNA were applied to nylon membranes by using a slot blotting device and hybridized to different DNA probes that had been prepared by PCR. The probes used were designed to detect 16S rRNA and *flaA*-, *flaB*-, and *flgE*-specific mRNAs. Hybridization with the 16S rRNA-specific probe confirmed that equivalent amounts of RNA were present in the different RNA preparations. The *flaA*- and *flgE*-specific probes gave strong signals when hybridized to the RNA isolated from the wild-type strain. The *flaB*-specific probe gave a weaker signal than the *flaA*-specific probe, which was expected because the *flaB* gene is known to be expressed at much lower levels that the *flaA* gene. In the *flbA* isogenic mutant strains, no flagellin-specific mRNAs could be detected. Hybridization with the *flgE*-specific probe gave a signal that was strongly reduced in comparison with the wild-type strain.

The effect of a *flbA* mutation on expression of the FlgE hook protein is growth phase dependent. Since RNA slot blot hybridization with a *flgE*-specific DNA probe (see above) showed the presence of *flgE*-specific mRNA in the mutant strain, wild-type and mutant bacteria were harvested after three different incubation times (Fig. 7). Partially purified flagella were used for a Western blot analysis with antiserum AK179. None of the samples prepared from the mutant contained either FlaA or FlaB flagellin. However, the 78-kDa hook band was clearly present in the earliest sample (1.5 days) and very faintly visible after 3 days of growth. After 6 days, no hook protein could be detected. In the wild-type strain, no decrease of hook protein expression was observed.

Partial overexpression of the FlbA protein in *E. coli*. Two overlapping fragments of the *flbA* gene were amplified by PCR using two different pairs of primers which contained *Bam*HI or *Kpn*I restriction sites. The 1,670-bp fragment obtained by amplification with primers OLHPFlbA-11 (positions 253 to 274 of the sequence in Fig. 1) and OLHPFlbA-13 (positions 1900 to 1921) was able to code for amino acids 2 to 558 of the FlbA protein, which comprised the hydrophobic N-terminal portion of the protein. When expression of this fragment gave only very low protein yields, a second, 1,150-bp fragment was amplified by using primers OLHPFlbA-21 (positions 1300 to 1319 of the sequence in Fig. 1) and OLHPFlbA-22 (positions 2429 to 2449), which coded only for the hydrophilic C-terminal part of FlbA (amino acids 351 to 732). The fragments were cloned into the expression vector pQE30 (Qiagen). In-frame cloning into this vector results in the N-terminal addition of six consecutive histidine residues to the expressed protein, which permits purification of the product by virtue of the high affinity of the 6×His tag to Ni^{2+} ions. The identity of the expression products was confirmed by a Western blot with a polyclonal antiserum raised against the 6×His tag (data not shown). The 60-kDa expression product that contained the N-terminal portion of FlbA was expressed only in very low amounts (the approximate yield of the purification was 0.1 μg/ml of culture). In contrast, the hydrophilic part of the protein could be expressed at very high levels and purified in large amounts (yield, 10 μg/ml of culture [data not shown]). This 46.5-kDa polypeptide was used to raise a polyclonal antiserum against the FlbA protein.

Detection of the FlbA protein in *H. pylori*. The antiserum raised against the hydrophilic part of the FlbA protein was used in Western blot analysis to detect the FlbA protein in *H. pylori* and the isogenic *flbA* mutant. Both the soluble fraction and membrane pellets of whole-cell lysates were analyzed (Fig. 8). Three major bands were recognized by the antiserum: a faint ca. 97-kDa protein that was mainly visible in the soluble fraction and was present in the wild-type strain and in the mutant, a 66-kDa band that was strongly enriched in the membrane pellet and present in the wild-type strain but not in the *flbA* mutant, and a strongly expressed protein of about 43 kDa that was detected in both membrane and soluble fractions and present in the wild-type as well as the mutant strain. No band corresponding to the molecular mass of the expected full-length protein product of the *flbA* gene (80.9 kDa) was visible. The Western blot experiments indicate that, consistent with the predictions made from the sequence, the FlbA protein is mainly associated with membranes. The difference between calculated and observed apparent molecular masses suggests the possibility of a processing event; it may, however also be artifactual and due to the high hydrophobicity of the FlbA protein.

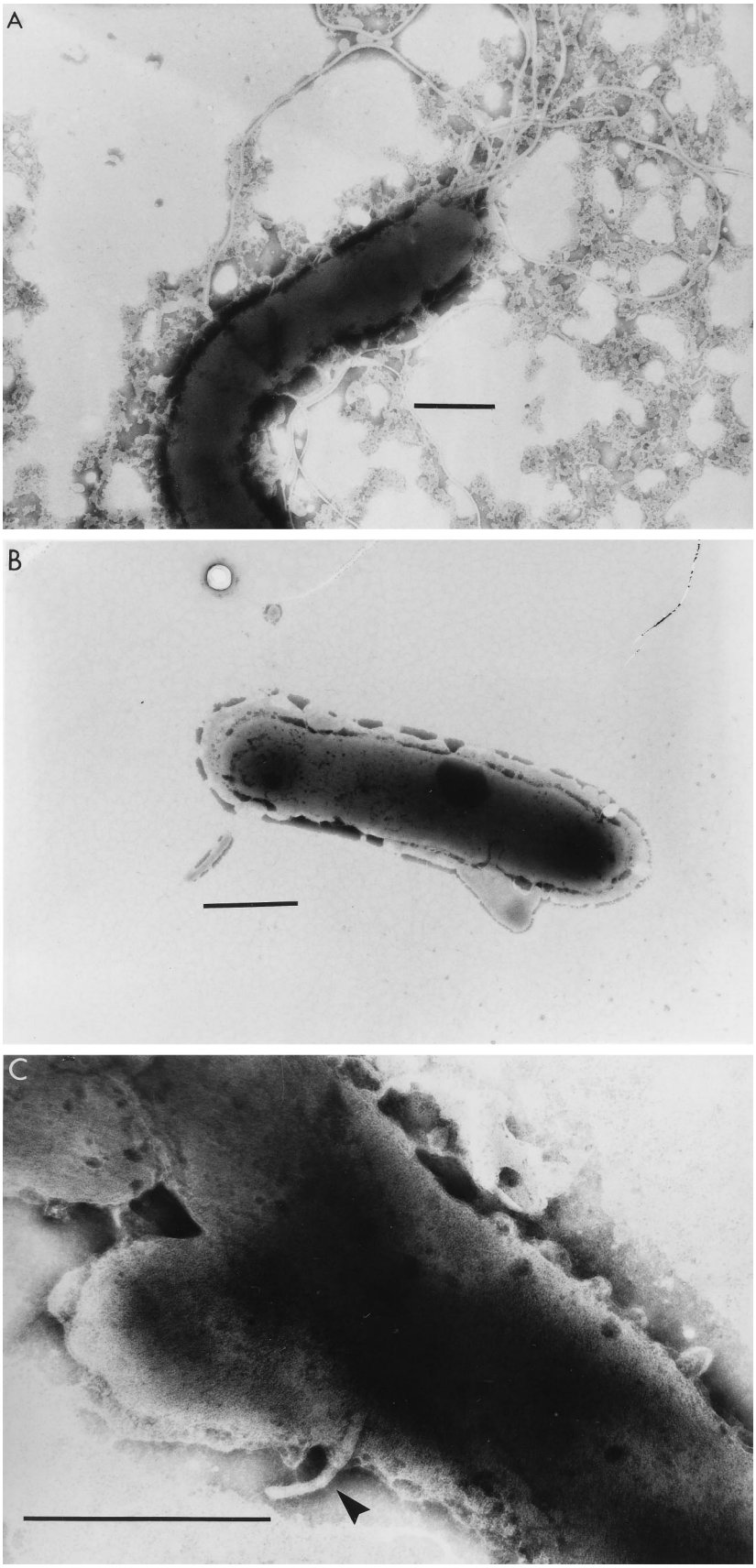

FIG. 4. Electron micrographs of *H. pylori* wild-type strain N6 (A) and the N6 *flbA* mutant (B and C), negatively stained with 1% phosphotungstate (pH 7.0). The specimens were prepared from bacteria grown on agar plates for 3 days. Note the characteristic unipolar sheathed flagella in the wild-type strain. No flagella were visible in the *flbA* mutant strain. Occasionally, structures resembling flagellar hooks (30 to 100 nm in length) were seen in the *flbA* mutant (indicated by an arrowhead in panel C). Bars, 0.5 μm.

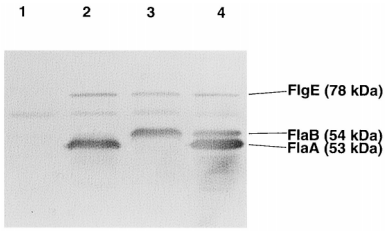

FIG. 5. Western blots of flagella partially purified from the *H. pylori* N6 *flbA* mutant (lane 1), N6 *flaB* mutant (lane 2), N6 *flaA* mutant (lane 3), and wild-type N6 strain (lane 4). The blot was developed with antiserum AK179 raised against purified flagellar filaments of *H. pylori*. The positions of the bands corresponding to the FlaA and FlaB flagellins as well as the hook protein (FlgE) are indicated on the right. Note that the bands corresponding to both flagellin species as well as the hook protein are not present in the *flbA* mutant strain.

To determine the cellular location of the FlbA protein, both sarcosyl-soluble (i.e., cytoplasmic) and sarcosyl-insoluble (i.e., outer) membrane fractions were isolated and analyzed by a Western blot with the FlbA antiserum. The 66-kDa FlbA band was enriched in the sarcosyl-soluble fraction of the membranes, indicating that FlbA is a cytoplasmic membrane protein. The two other bands recognized by the antiserum, whose expression was not affected by disruption of the *flbA* gene, might be homologs of the FlbA protein, a hypothesis supported by the results of low-stringency hybridizations of a *H. pylori* sorted cosmid library with a *flbA* gene probe (see Discussion).

DISCUSSION

Motility has been shown to be a key factor in the ability of *H. pylori* to colonize the gastric mucosa (7, 8). While several structural components of the flagella have been characterized in molecular detail, virtually nothing is known about factors that regulate flagellar biogenesis and motility. In this study, we have cloned and characterized the first *H. pylori* flagellar regulatory gene, the *H. pylori flbA* gene, that codes for a homolog of the FlbF/LcrD family of proteins. Although the results of this study have many parallels with studies performed on other FlbF/LcrD homologs, there are a number of interesting differences between *H. pylori* and other bacteria, which illustrate the diversity of these systems. A similar study of the effect of a *flbA* or *flhA* mutation on the expression of different flagellar genes has not been performed with other bacteria with complex flagella.

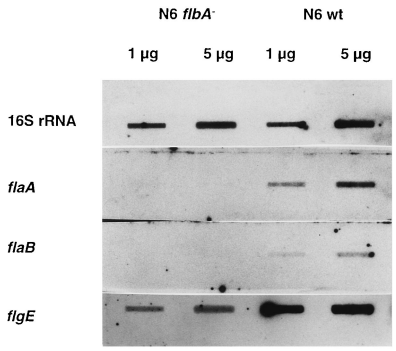

FIG. 6. Slot blot hybridizations of total RNAs isolated from the *H. pylori* N6 wild-type (wt) strain and the N6 *flbA* mutant. One and five micrograms of RNA were blotted onto a nylon membrane and hybridized with PCR-generated DNA probes specific for 16S rRNA (as a control for the RNA concentration in the sample), *flaA*-specific mRNA, *flaB*-specific mRNA, and *flgE*-specific mRNA. There was no detectable transcription product of the *flaA* and *flaB* flagellin genes in the mutant, and the concentration of *flgE*-mRNA was reduced in comparison with the wild-type strain.

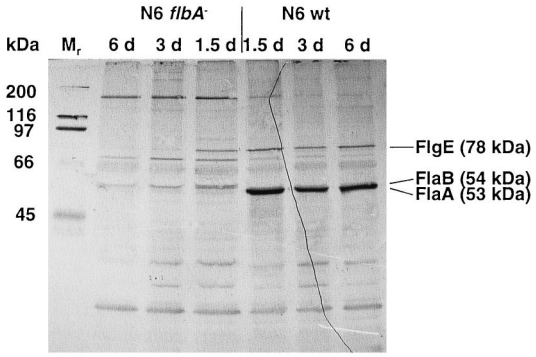

FIG. 7. Growth phase-dependent kinetics of FlgE (hook protein) expression in the *H. pylori* N6 isogenic *flbA* mutant and the corresponding wild-type (wt) strain. Bacteria grown on plates (see Materials and Methods for details) were harvested after 1.5, 3, and 6 days (d) of growth (as indicated above the lanes). At each time point, partially purified flagella were isolated, and component proteins were separated by SDS-PAGE (12% gel) and transferred to nitrocellulose. The Western blot was developed with antiserum AK179 raised against purified flagellar filaments. In wild-type strain N6, all known flagellar proteins were expressed at each time point. In the *flbA* mutant strain, the FlaA and FlaB flagellins were not expressed at any time point. The hook protein FlgE was expressed in small amounts in the early growth phase in the *flbA* mutant. In the late growth phase (day 6), FlgE expression was no longer detectable. Lane M_r, molecular mass standard proteins (molecular masses are indicated on the left). Positions of the bands corresponding to the FlaA and FlaB flagellins as well as the hook protein (FlgE) are indicated on the right.

All members of the FlbF/LcrD family have in common a highly hydrophobic N-terminal part with several putative membrane-spanning domains that is believed to reside in the cytoplasmic membrane and a relatively hydrophilic C-terminal part. This basic organization is shared by the *H. pylori* FlbA protein. The hydrophobic N-terminal part of the protein is predicted to be capable of forming six to eight membrane-spanning helices, and differential membrane solubilization experiments support the hypothesis that *H. pylori* FlbA is a cytoplasmic membrane protein. The exact membrane topology of the FlbA protein requires further investigation.

Our finding that the FlbA protein has an apparent molecular mass that is significantly lower than that predicted is in parallel with the findings reported by Plano et al. (38), who reported a molecular mass of 70 kDa for *Y. pestis* LcrD, which is 8 kDa

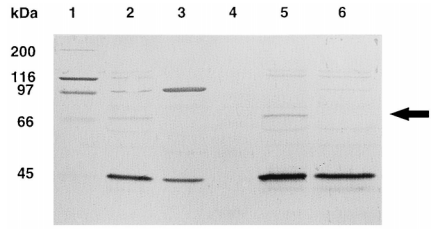

FIG. 8. Detection of the FlbA protein in *H. pylori* by Western blotting with a polyclonal rabbit serum raised against the hydrophilic part of the *H. pylori* FlbA protein. Lane 1, molecular mass standard proteins (molecular masses are given on the left); lane 2, *H. pylori* N6 wild-type strain, whole-cell sonicate; lane 3; *H. pylori* N6 soluble proteins; lane 4, *H. pylori* N6 outer membrane proteins (sarcosyl-insoluble membrane proteins); lane 5, *H. pylori* N6 inner membrane proteins (sarcosyl-soluble membrane proteins); lane 6, *H. pylori* N6 *flbA* mutant inner membrane proteins. Equal amounts of protein (80 µg) were applied to all slots of the gel. The 66-kDa band that is not present in the *flbA* mutant and most likely represents a processed form of the FlbA protein is marked by an arrow.

less than the mass calculated from the amino acid sequence. This finding suggests the possibility of a processing event; however, those differences could also be artifactual and due to the extreme hydrophobicity of those proteins.

The data presented here clearly show that *H. pylori flbA* is involved in the coordinated regulation of flagellar biogenesis. A mutation in *flbA* leads to a complete stop of the expression of the *flaA* and *flaB* genes, which are under the control of σ^{28}- and σ^{54}-type promoters, respectively, and to a growth phase-dependent reduction of expression of *flgE*, which, like *flaB*, has a σ^{54} promoter (36). It appears likely that those effects are due to a block of gene transcription in the *flbA* mutant, but changes of mRNA stability as possible causes of the observed effects cannot be ruled out. Likewise, the possibility that polar effects of the mutation play a role in the observed phenotype of the *flbA* mutant still exists, although the construction of the mutant with a cassette that permits the expression of downstream genes was done in a way to obviate polar effects.

Strains with mutations in genes that belong to the *flbF/lcrD* family have remarkably varied phenotypical characteristics. The proteins that are most highly related to the *H. pylori* FlbA protein are the ones associated with flagellar biogenesis, which form a distinct subfamily within the FlbF/LcrD protein family. The probably best characterized of those homologs is *C. crescentus* FlbF. FlbF is required for transcription of several structural flagellar genes, most of which are controlled by σ^{54} promoters. In *Caulobacer*, FlbF is one of the proteins at the top of the hierarchy of flagellar genes. The *flbF* gene is regulated in a cell cycle-dependent manner, with expression of FlbF occurring earlier in the cell cycle than expression of the structural flagellar genes (40, 43). Preliminary results of experiments with a *H. pylori flbA*-chloramphenicol acetyltransferase reporter gene fusion have provided evidence that the expression of *H. pylori* FlbA is similarly growth phase dependent. The peak expression of the *flbA* gene preceded the peak expression of the flagellin genes. FlbA could thus be responsible for coordinating the growth phase-dependent regulation of flagellin gene expression (23).

Some of the motility-associated *flbF/lcrD* gene homologs (e.g., *C. crescentus flbF* and *P. mirabilis flhA* [16, 43]) have been shown to be controlled by σ^{28} promoters. The transcription start site of the *H. pylori flbA* gene was mapped in this study; however, no conspicuous homologies with known promoter sequences were found upstream of the *flbA* gene, which parallels the situation in *C. jejuni* (32).

The genetic organization of the *H. pylori* flagellar apparatus differs from that of other bacteria in several respects. The first major difference is the presence of two flagellin genes that code for distinctly different flagellin proteins, are preceded by different promoters, and are unlinked on the chromosome (3, 47, 50). Although little is known about the regulation of flagellar gene expression in *H. pylori*, there is evidence that the regulatory network of flagellar biogenesis is significantly different from that in other known systems. The most significant such observation is the finding that strains with mutations in the *H. pylori* hook (*flgE*) gene still express the FlaA and FlaB flagellin proteins, which accumulate intracellularly (36). This is in contrast to the situation in *Salmonella*, where mutations in the hook gene also prevent the expression of the flagellin genes (25), a feedback mechanism that appears to make sense economically. It is presently not known why this feedback mechanism, which prevents flagellar genes of the lower hierarchical levels from being expressed when a fully functional flagellum cannot be assembled, does not exist in *H. pylori*. The observation that the *H. pylori flbA* mutants could still assemble flagellar hooks on the cell surface indicates that export of axial filament components is still possible in those mutants. Taken together with the lack in *H. pylori* of the above-mentioned feedback mechanism, it appears unlikely that the transcriptional effects observed in the *H. pylori flbA* mutants are secondary to a block in the export of axial filament components. It cannot be ruled out, however, that the FlbA protein is involved in secretion of another regulatory component of the system.

The position of the *flbA* gene on the *H. pylori* chromosome has been roughly mapped by hybridization of the ordered cosmid library described by Bukanov and Berg (3) with a *flbA* gene probe. Hybridization revealed that *flbA* is located on cosmid 17, about 100 kbp away from the *flaA* gene. Interestingly, two unrelated cosmids that hybridized to the probe under low-stringency hybridization conditions were identified. These hybridization results are in parallel with the results of the Western blot analyses presented here, which showed the presence in *H. pylori* of at least two proteins strongly cross-reacting with an antiserum raised against the hydrophilic part of FlbA that were not affected by a *flbA* knockout mutation. It seems likely that these cosmids contain other homologs of the FlbF/LcrD family, which might be part of type III secretion systems. Subcloning of the two possible *H. pylori* homologs is under way in our laboratory. Since *H. pylori* is known to secrete several proteins (the most abundant of those being urease (see reference 34 for a review) by yet unknown mechanisms, the elucidation of type III secretion systems in this pathogen and their secreted proteins appears of considerable interest.

It has been shown in experiments with nonmotile mutants in the gnotobiotic piglet model of *H. pylori* infection that even though they can cause only a very transient colonization, these mutants can still elicit a humoral immune response (8). A *flbA* mutant might represent a candidate to test the usefulness of attenuated strains for vaccination against *H. pylori*. This as well as other potential applications of *H. pylori flbA* mutant strains will be further evaluated in the future.

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A. Schmitz and C. Josenhans contributed equally to this study.

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