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Comparative Ultrastructural and Functional Studies of *Helicobacter pylori* and *Helicobacter mustelae* Flagellin Mutants: Both Flagellin Subunits, FlaA and FlaB, Are Necessary for Full Motility in *Helicobacter* Species

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Helicobacter mustelae **causes chronic gastritis and ulcer disease in ferrets. It is therefore considered an important animal model of human *Helicobacter pylori* infection. High motility even in a viscous environment is one of the common virulence determinants of *Helicobacter* species. Their sheathed flagella contain a complex filament that is composed of two distinctly different flagellin subunits, FlaA and FlaB, that are coexpressed in different amounts. Here, we report the cloning and sequence determination of the *flaA* gene of *H. mustelae* NCTC12032 from a PCR amplification product. The FlaA protein has a calculated molecular mass of 53 kDa and is 73% homologous to the *H. pylori* FlaA subunit. Isogenic *flaA* and *flaB* mutants of *H. mustelae* F1 were constructed by means of reverse genetics. A method was established to generate double mutants (*flaA flaB*) of *H. mustelae* F1 as well as *H. pylori* N6. Genotypes, motility properties, and morphologies of the *H. mustelae* flagellin mutants were determined and compared with those of the *H. pylori flaA* and *flaB* mutants described previously. The flagellar organizations of the two *Helicobacter* species proved to be highly similar. When the *flaB* genes were disrupted, motility decreased by 30 to 40%. *flaA* mutants retained weak motility by comparison with strains that were devoid of both flagellin subunits. Weakly positive motility tests of the *flaA* mutants correlated with the existence of short truncated flagella. In *H. mustelae*, lateral as well as polar flagella were present in the truncated form. *flaA flaB* double mutants were completely nonmotile and lacked any form of flagella. These results show that the presence of both flagellin subunits is necessary for complete motility of *Helicobacter* species. The importance of this flagellar organization for the ability of the bacteria to colonize the gastric mucosa and to persist in the gastric mucus remains to be proven.**

Helicobacter mustelae is a close relative of the human pathogen *Helicobacter pylori* and naturally colonizes the stomachs of ferrets (17, 39). It has raised particular interest as an animal model for human *H. pylori* infection because its colonization patterns and histology and the resulting chronic lifelong disease bear a close resemblance to the human type B gastritis caused by *H. pylori* (16, 18). Older animals have been observed to develop gastric ulcers, probably because of colonization with *H. mustelae* (19). Infection of the human stomach with *H. pylori* has been shown to be a major risk factor contributing to the development of peptic ulcers and gastric carcinoma and a prerequisite for duodenal ulcers (7, 40). Since the basis of the ferret animal model is an infection that occurs naturally at an early age in an animal with a relatively long life span, this model permits observations of *Helicobacter* colonization over a long period of time, which is a significant advantage of the ferret model over the two other most widely used animal models of *Helicobacter* infection, the *Helicobacter felis* mouse model (32) and the gnotobiotic piglet model of experimental colonization with *H. pylori* (29). Since the discovery of *H. mustelae*, some of the most important virulence factors of *H. pylori* (e.g., urease and motility) have been shown to exist in the ferret organism as well (9, 10, 48, 53).

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All gastric *Helicobacter* species are highly motile organisms, a property that has been shown to be of major importance for the colonizing ability of *H. pylori* in the piglet model (11, 12). Similar to what was described for *Campylobacter jejuni* (15), *Helicobacter* species exhibit much higher motility in viscous media than other enteric bacteria, such as salmonellae or *Escherichia coli* (27). Motility is supposed to be necessary for both colonization and persistence of the bacteria in the gastric mucus (4). *H. pylori* possesses a bundle of unipolar flagella, while *H. mustelae* exhibits polar or subterminal flagella and, in addition, a small number (two to four) of lateral flagella (17, 39, personal observations). In contrast to those of most other bacteria, each flagellum of *Helicobacter* species is enveloped by a sheath that is thought to serve as a protective shield against gastric acidity for the acid-labile flagellar filament (21).

The flagellar filaments of *H. pylori* and *H. mustelae* have been characterized in several recent publications. Geis et al. (20) first published a report on the purification of the major component of the *H. pylori* flagellar filament, the FlaA flagellin. Kostrzynska et al. (28) demonstrated that the filament also contains a small amount of a second flagellin subunit (FlaB) that seems to be located mainly at the proximal part of the filament.

We began to investigate the flagellar organization of *H. mustelae* in order to study the similarities in the flagellar organizations of both gastric *Helicobacter* species. We attempted to clone *H. mustelae* flagellin genes, using the *H. pylori flaA* sequence (33) as a probe, and first identified the gene encoding

TABLE 1. Vectors and plasmids used in this study

Plasmid	Vector	Size (kb)	Characteristics[a]	Reference or source
	pILL570	5.3	Rep_{Ec}, Mob, Sp^r	30
	pILL575	10	Rep_{Ec}, Rep_{Cj}, Mob, Km^r, cos	30
pILL600	pBR322	5.7	Ap^r, Km^r, source of kanamycin cassette	31
pCM4	pBR322	4.1	Ap^r, source of CAT cartridge	Pharmacia Biotech Inc.
pSUS101	pILL570	8.3	Sp^r, *H. mustelae flaB*	49
pSUS110	pILL570	6.7	Sp^r, *H. mustelae flaA*	This study
pSUS111	pILL570	6.7	Sp^r, *H. mustelae flaA*	This study
pSUS25	pILL570	9.4	Sp^r, Km^r, *H. mustelae flaB* Ω Km	This study
pSUS112	pILL570	8.1	Sp^r, Km^r, *H. mustelae flaA* Ω Km	This study
pSUS114	pILL570	7.5	Sp^r, Cm^r, *H. mustelae flaA* Ω CAT	This study
pHL319-2-4	pIC20R2	6.3	Ap^r, *H. pylori flaA*	33
pSUS24	pIC20R2	7.1	Ap^r, Cm^r, *H. pylori flaA* Ω CAT	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, Sp^r, Km^r, and Cm^r, resistance to ampicillin, spectinomycin, kanamycin, and chloramphenicol, respectively; cos, presence of lambda cos site.

the second flagellin subunit, FlaB. Subsequently, we also cloned the homologous *H. pylori flaB* gene and constructed isogenic *H. pylori flaA* and *flaB* mutants (49). Both flagellins in *H. mustelae* as well as in *H. pylori* have similar molecular masses of about 53 (FlaA) and 54 (FlaB) kDa. FlaB in both species is produced in much smaller amounts in vitro than FlaA. The two different flagellin proteins of *H. pylori* are only 56% homologous. The existence of two flagellins that are coexpressed in one filament but whose expression is probably regulated independently is unusual and raises questions concerning the function of this flagellar organization. In our first studies of isogenic *H. pylori flaA* and *flaB* mutants, *flaB* mutants had flagella that could not be differentiated from wild-type flagella, and the motility of the mutants also seemed to be essentially unimpaired (49). *flaA* mutants, however, seemed to be completely nonmotile under the motility testing conditions used, and no flagella could be detected. Thus, a defined function of the FlaB protein was not obvious. We decided to continue our work with *H. mustelae* because this organism grows considerably faster than *H. pylori* and exhibits higher motility, so the motility phenotype can be assessed more easily.

The major objective of our investigation was to identify the function of the FlaB subunit in *Helicobacter* flagella. Therefore, we constructed the first isogenic *H. mustelae flaA* and *flaB* motility mutants and *flaA flaB* double mutants. One prerequisite for this project was the cloning of the *H. mustelae flaA* gene.

A secondary aim was to establish an electroporation technique to construct isogenic *H. mustelae* mutants in order to provide a tool for the investigation of motility, other *Helicobacter*-specific virulence mechanisms, and possible vaccination targets in the ferret animal model. Characterization and motility testing of the constructed isogenic *H. mustelae* motility mutants yielded results that diverged from those of our previous characterization of *H. pylori* mutants and led us to reexamine these mutants in comparative tests. We found that not only FlaA but both flagellin subunits of the two gastric *Helicobacter* species are necessary for expression of fully functional flagella in vitro.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Vectors and recombinant plasmids used in this study are listed in Table 1. *H. mustelae* NCTC12032 (National Collection of Type Cultures, London, United Kingdom) and *H. pylori* 85P (30) were used for the preparation of DNA and for the cloning experiments. For the electroporation-mediated mutagenesis, *H. pylori* N6 (14) and *H. mustelae* F1, a ferret isolate provided by D. S. Tompkins, Leeds, United Kingdom, were used because of the high electroporation efficiency of these strains. The *H. pylori* N6 mutants disrupted in the *flaA* and *flaB* genes, respectively (49), and an isogenic urease-negative mutant of *H. pylori* N6 disrupted in the *ureB* gene (14) have been described previously. *H. mustelae* and *H. pylori* strains were cultured on blood agar-base 2 plates (Oxoid, Wesel, Germany) supplemented with 10% sheep or 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.

Recombinant plasmids were transformed and maintained in *E. coli* MC1061 (6) and HB101 (5). 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 in Luria or 2× YT broth at 37°C. When necessary for the selection of allelic replacement mutants, the following additional antibiotics were included in the media: chloramphenicol (25 mg/liter) and kanamycin (20 mg/liter). 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, such as plasmid isolation by alkaline lysis, restriction endonuclease digestion, filling of 3′-recessed termini for blunt-ended cloning, and ligation, were performed according to the protocols of Sambrook et al. (44). *H. mustelae* and *H. pylori* genomic DNAs were prepared as described by Majewski and Goodwin (35). Briefly, cells were harvested from four to eight densely grown plates and washed subsequently in 0.9% NaCl and 0.1 M Tris–0.1 M EDTA, pH 8.5. The cells were lysed in Tris-EDTA containing 30 mg of lysozyme per ml and 2% sodium dodecyl sulfate (SDS). After subjection to proteinase K and $NaClO_4$ treatment, DNA was extracted twice with phenol-chloroform and once with chloroform, precipitated with ethanol, and finally suspended in Tris-EDTA buffer at a concentration of 100 ng/μl. Large-scale plasmid preparations were purified by CsCl-ethidium bromide density gradient centrifugation (14) or 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 the QiaEX glassmilk DNA purification kit (Qiagen Inc.). DNA restriction and modification enzymes were obtained from BRL Life Technologies or Boehringer Mannheim Biochemicals and were used according to the directions of the manufacturers.

DNA sequence determination. The DNA sequences of the PCR products, double-stranded DNA, and single-stranded M13 DNA (36) were obtained by the dideoxy chain termination method described by Sanger et al. (45). The reactions used synthetic oligonucleotide primers, α-^{35}S-dATP (Amersham), and the Sequenase version 2.0 kit (U.S. Biochemicals, Cleveland, Ohio). For sequence determination of the *H. mustelae flaA* gene, the complete inserts of the clones pSUS110 and pSUS111 (Table 1) were cut out by restriction with *Bam*HI and *Hin*dIII and were subcloned into M13mp18 and -19 vectors. Overlapping sequence information was generated with the help of custom-synthesized oligonucleotides. The two *H. mustelae flaA* clones pSUS110 and pSUS111 that were originally obtained by cloning of a PCR product were both sequenced completely to exclude sequence errors due to the PCR reaction. Both strands of each clone were sequenced independently. The second strand was sequenced with double-stranded plasmid DNA being used as a template. Direct sequence analyses of PCR products were obtained by including dimethyl sulfoxide (1% final concentration) in the reaction mix. 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 (MULTalign and Publish) of the European Molecular Biology Laboratory, Heidelberg, Germany.

Southern blotting and hybridization. Southern blotting (46) was performed in order to characterize the isogenic mutants of *H. mustelae*. Genomic DNA of the *H. mustelae* strains was digested with restriction enzymes for at least 24 h. After 18 h, fresh enzyme was added to ensure complete digestion. The DNA was

TABLE 2. Oligonucleotides used for PCR amplification

Oligonucleotide	Type and source[a]	Strand	Sequence (5′ to 3′)[b]	Reference
OLHMDFlaA1	D, derived from amino acids 5–11, positions 51–70 of *H. pylori* FlaA	+	CA(AG)GT(TCGA)AA(TC)AC(TCGA)AA(TC)AT(TCA)AA(TC)GC	This study
OLHFFlaA3	ND, *H. felis flaA*	−	GGGTATTCTGACCATCCTGAGCTGC	47
OLHMFlaA3	ND, positions 187–208 of *H. mustelae flaA*	+	GCTGATAAGGCGATGGATGAGC	This study
OLHMDFlaA4	D, derived from amino acids 496–503 of *H. pylori* FlaA	−	TT(TC)TG(TC)TG(AGCT)AC(AGCT)GT(AG)TT(AGCT)GC(TC)TG	This study
OLHMFlaA7S	ND, positions 1425–1444 of *H. mustelae flaA*	−	atcgcggatccTGTCCAAGGATGTTATACTTG	This study
OLHMFlaA8S	ND, positions 41–59 of *H. mustelae flaA*	+	aaggaagatctCTTGAAAAGTTGAGCTCAGG	This study
OLKm1	ND, positions 699–724 of the Km^r gene	+	CTGCTAAGGTATATAAGCTGGTGGG	49, 52
OLKm2	ND, positions 1305–1329 of the Km^r gene	−	CATACTGTTCTTCCCCGATATCCTC	49, 52
OLHMFlaB2	ND, positions 51–75 of *H. mustelae flaB*	+	AATCAAAGATAAGGAATTAAGCGGC	49
OLHMFlaB3[c]	ND	−	CTGAAATGAAAATAACCCCAAAGTC	49

[a] Positions refer to nucleotides in the respective sequences. ND, nondegenerate; D, degenerate; Km^r, kanamycin resistance.
[b] Lowercase letters indicate sequences not homologous to the *H. mustelae* sequence. Underlined nucleotides represent the *Bam*HI (OLHMFlaA7S) and *Bgl*II (OLHMFlaA8S) restriction sites used to clone the PCR product. Nucleotides in parentheses represent wobble nucleotides.
[c] The source sequence of this oligonucleotide is close to the rightmost *Bam*HI site in pSUS101.

separated on a 0.8% agarose gel. The gel was vacuum-blotted onto a positively charged nylon membrane (Boehringer Mannheim Biochemicals), and the DNA was fixed by baking for 15 min at 120°C. Hybridization was performed nonradioactively with the DIG-DNA labelling and detection kit (Boehringer Mannheim Biochemicals) under high-stringency conditions. Hybridizations were carried out with a hybridization solution containing 50% formamide, the hybridization step was performed at 42°C, and consecutive washing steps were carried out twice for 5 min each time at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and twice for 45 min each time at 68°C in 0.1× SSC–0.1% SDS. Blots were stripped for reprobing by subsequent washing in 2× SSC for 5 min at room temperature and two washes in 0.2 M NaOH–0.1% SDS for 15 min each time at 37°C.

DNA amplification by PCR. PCR reactions were performed in a Perkin Elmer-Cetus TC480 thermal cycler with the GeneAmp kit (Perkin Elmer-Cetus). In each reaction, at least 5 pmol of target DNA, 100 pmol of each primer, and standard concentrations of deoxynucleoside triphosphates and $MgCl_2$ were included. Degenerate primers were used at final concentrations of 1 μM. As the target DNA, whole-cell lysates of *Helicobacter* strains or purified genomic DNA or plasmid DNA preparations were used. The PCR reactions 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 exact annealing temperatures, see Results) for 2 min, and extended at 72°C for 2 min. A total of 25 to 35 cycles were performed.

Electroporation of *H. mustelae* and *H. pylori* strains for the construction of isogenic mutants. Plasmids containing the targeted gene that had been disrupted by a DNA fragment conferring antibiotic resistance (kanamycin or chloramphenicol) were purified from *E. coli* by large-scale preparation. These plasmids were electrotransformed into *H. mustelae* and *H. pylori* strains basically as described elsewhere (14). In our experience, electroporation efficiencies in *H. mustelae* were considerably increased when the concentration of bacteria in the electroporation slurry was not too high (approximately 10^9 cells per ml, compared with an optimum of 10^{11} cells per ml for *H. pylori*). One electroporation reaction was carried out in 80 μl of bacterial slurry to which 300 ng of freshly dialyzed plasmid DNA in 5 μl of H_2O had been added. After electroporation, the bacteria were grown on nonselective plates for a period of 48 h and then transferred to plates containing the selective antibiotic(s). The selective plates were incubated about 6 days for the growth of single-gene mutants and up to 9 days to obtain colonies of double mutants. In different *H. mustelae* host strains, considerable variation in electroporation efficiencies was observed. We chose *H. mustelae* F1 because of its good transformability. Efficiencies also varied according to the plasmid construct used. We obtained colony counts ranging from 1 single colony (*H. pylori flaA flaB* double mutants) and 9 transformant colonies (*H. mustelae flaA flaB* double mutants) to about 30 and 50 colonies for *H. mustelae flaB* and *flaA* mutants, respectively.

Motility testing of *Helicobacter* strains. To serve as a medium to evaluate motility, brucella broth (Difco Inc., Detroit, Mich.) was supplemented with 10% fetal calf serum, the antibiotics described above, and 0.3 or 0.4% Bacto agar. We used two different methods for the testing. One method, a stab agar test, is commonly used as a standard motility test to examine the general motility of a certain bacterial strain within the agar, and the second method evaluates the single-colony motility of the bacteria. For the stab agar test, the motility medium was poured into a petri dish in a very thin layer. Bacterial strains were stab-inoculated into the agar with a pipette tip. To examine single-colony motility, about 10^8 bacterial cells were harvested in 1 ml of 0.9% NaCl and diluted $1:10^3$ in 0.9% NaCl. The motility agar was cooled to a temperature of about 40°C. Bacteria were diluted to a final dilution of $1:10^6$ in the lukewarm motility agar and poured into the support in a thin layer. The plates were predried for 15 min under a hood and incubated up to 9 days at 37°C in a microaerobic atmosphere. Single-colony morphology was examined with a phase-contrast microscope (magnification, 10- to 40-fold). As a positive control for kanamycin resistance and full motility, the *ureB*-negative mutant of *H. pylori* N6 was included in the tests.

Western blotting (immunoblotting). Whole-cell sonicates of the *H. mustelae* and *H. pylori* double mutants were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the protocol of Lugtenberg et al. (34). The acrylamide concentration in the gel was 11%. Samples (80 μg each) were applied to the gel. For immunodetection, the gels were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) by the method of Towbin et al. (50). The blots were incubated with rabbit antiserum AK179 (33) raised against purified native flagellar filaments of *H. pylori* (dilution, 1:2,000). Bound antibodies were visualized with a peroxidase-coupled goat anti-rabbit antibody (dilution, 1:2,000; Biogenzia, Bochum, Germany).

Electron microscopy. Bacteria were harvested from blood agar plates and gently resuspended in 0.9% NaCl. Samples were applied to Formvar-carbon-coated copper grids (300 mesh; Plano, Marburg, Germany), immediately fixed for 1 min in 1% glutaraldehyde (pH 7.0), and negatively stained for 5 to 10 sec with 1% phosphotungstate titrated to pH 7.0 with potassium hydroxide. For very gentle preparation, the grids were directly placed upside down on blood plates on which bacteria had been grown (3) and negatively stained immediately. The grids were examined with a Zeiss EM 10 B or Zeiss EM 900 or a Philips 320 EM electron microscope.

Nucleotide sequence accession number. The sequence of the *H. mustelae flaA* gene has been assigned GenBank accession number L38478.

RESULTS

Cloning of the *H. mustelae flaA* gene. The *H. mustelae flaB* gene had previously been cloned from a partial plasmid bank of genomic *H. mustelae* DNA (49). However, several attempts to clone the *H. mustelae flaA* gene from genomic DNA by a similar approach and by cosmid cloning had been unsuccessful because the recombinant plasmids and cosmids obtained were highly unstable.

Thus, a PCR approach to clone the *H. mustelae flaA* gene was devised. Degenerate oligonucleotide primers were designed according to amino acid sequences near the amino and carboxy termini of the highly conserved *H. pylori* and *H. felis* FlaA proteins. With two primer pairs (Table 2), we were able to amplify fragments of the gene with sizes of about 300 bp (N terminus) and 1.25 kb (C terminus) from genomic DNA of *H. mustelae* NCTC12032 under low-stringency annealing conditions (40°C). For the amplification of the N-terminal 300-bp fragment, the degenerate forward primer OLHMDFlaA1, corresponding to amino acids 5 to 11 of FlaA of *H. pylori* and *H. felis*, and the reverse primer OLHFFlaA3, a nondegenerate primer derived from *H. felis* FlaA (47), were used. This 300-bp fragment was partially sequenced first and was clearly identi-

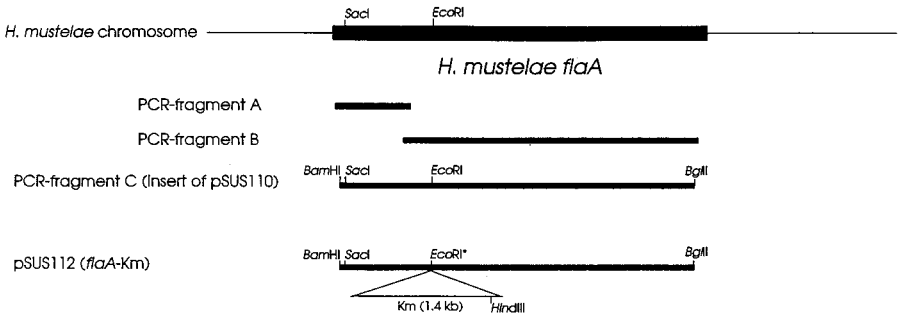

FIG. 1. Strategy for the cloning and gene disruption of the *H. mustelae flaA* gene. PCR fragment A was obtained with primers OLHMDFlaA1 and OLHFFlaA3, and the overlapping PCR fragment B was obtained with primers OLHMFlaA3 and OLHMDFlaA4 (see the text and Table 2 for the origin of the primer sequences). On the basis of the sequences of these fragments, primers OLHMFlaA7S and OLHMFlaA8S were designed, which permitted the amplification and cloning of PCR fragment C (*flaA* gene). For the construction of *H. mustelae flaA* mutants, the *flaA* gene was disrupted by introduction of a kanamycin resistance cassette (Km) into the unique *Eco*RI restriction site of plasmid pSUS110. The *Bam*HI and *Bgl*II sites present in plasmids pSUS110 and pSUS112 were introduced by PCR and are not present in the *H. mustelae* chromosome. The asterisk denotes a restriction site deleted in the cloning process.

fied as part of a novel *flaA* gene. Subsequently, C-terminal sequence information could be obtained from an overlapping 1.25-kb fragment amplified with the nondegenerate forward primer OLHMFlaA3 and situated at the end of these first 300 bp of *H. mustelae flaA* and with the degenerate reverse primer OLHMDFlaA4 corresponding to amino acids near the C terminus of *H. pylori* FlaA (for the location of the primers and PCR fragments, see Fig. 1). On the basis of these two specific *H. mustelae flaA* sequence fragments, we designed specific nondegenerate primers, OLHMFlaA7S and OLHMFlaA8S (Table 2), for the amplification of the *H. mustelae flaA* gene under high-stringency annealing conditions (55°C). These primers included *Bgl*II (N terminus) and *Bam*HI (C terminus) restriction sites in order to clone the amplified fragment into the polylinker of plasmid vector pILL570. The resulting 1.4-kb PCR product containing nearly the complete *H. mustelae flaA* gene was cut with these enzymes, purified, and ligated into the *Bgl*II-digested, dephosphorylated pILL570 plasmid. Several identical clones were identified. Two of them, pSUS110 and pSUS111, were purified for further restriction and sequence analyses.

DNA sequence analysis of the *H. mustelae flaA* gene. The nucleotide sequence of *H. mustelae flaA* was determined with the *H. mustelae flaA* plasmids pSUS110 and pSUS111. The whole PCR-cloned fragment was excised from the plasmids with enzymes *Bam*HI and *Hin*dIII and subcloned into M13mp18 and -19. Overlapping sequence information of both strands was generated with a set of custom-designed oligonucleotide primers. The cloned 1.4-kb fragment of *H. mustelae flaA*, amplified by PCR, contains nearly the complete *flaA* gene. At the 5′ end of the gene, 27 codons were missing. Codons 15 to 27 were obtained from the separately amplified, small 300-bp fragment at the N terminus. Thus, only 14 N-terminal codons have not been sequenced. However, the first 15 N-terminal amino acids had previously been determined by N-terminal amino acid sequencing of the FlaA protein (48), and this amino acid sequence overlaps with that derived from the obtained DNA sequence. At the 3′ terminus, about 22 codons are missing, because we had not been able to amplify *H. mustelae* DNA at the extreme 3′ end of the *flaA* gene with degenerate primers. Overall, we sequenced 1,446 nucleotides of the gene, corresponding to 482 amino acids (Fig. 2). As the protein comigrates at 53 kDa with *H. pylori* FlaA in SDS-PAGE (48), both proteins should have about the same size (510 amino acids). The peptide sequence is 72% identical to that of *H. pylori* FlaA (Fig. 3). The *H. mustelae* FlaA protein has 56% and 53% amino acid identities with the FlaB proteins of *H. mustelae* and *H. pylori*, respectively. The N- and C-terminal portions of the protein are well conserved between the species, whereas a large stretch of the sequence in the middle of the protein is less conserved when compared with that of *H. pylori* FlaA (N-terminal amino acids 1 to 182, 83% identity; middle domain, amino acids 183 to 398, 51% identity; C-terminal amino acids 399 to 497, 81% identity). This is in concordance with the common "three-domain" model of bac-

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      10                  30                  50 SacI             70                  90
ACTTCAGCGGGTGCAACTCAATTGGGTTTAAAAAACTCTCTTGAAAAGTTGAGCTCAGGTTTGAGAATTAACAAGGCAGCAGATGATGCTTCTGGCATGA
T S A G A T Q L G L K N S L E K L S S G L R I N K A A D D A S G M T

     110                 130                 150                 170                 190
CCATTTCAGATAGCTTGCGTTCACAAGCAAGTGCTTTGGGACAAGCAATTAGTAATGCTAATGATGGTATTGGAATCATACAAGTTGCTGATAAGGCGAT
  I S D S L R S Q A S A L G Q A I S N A N D G I G I I Q V A D K A M

     210                 230                 250                 270                 290
GGATGAGCAGCTCAAGATTCTTGACACTATTAAAGTGAAGGCTACACAGGCAGCACAAGATGGACAGTCTCTAGAATCTAGAAAGGCGATCCAGTCTGAT
  D E Q L K I L D T I K V K A T Q A A Q D G Q S L E S R K A I Q S D

     310                 330                 350                 370                 390 EcoRI
ATTATCCGACTGATTCAAGGGCTTGATAACATAGGGAATACGACTTCTTACAATGGTCAGTCATTGCTTTCTGGTCAGTGGACAAATAAGAATTCCAAA
I I R L I Q G L D N I G N T T S Y N G Q S L L S G Q W T N K E F Q I

     410                 430                 450                 470                 490
TCGGTACGTATTCTAATCAGAGCATTAAGGTTTCTGTTGGCTCTACAACTTCTGATAAAATTGGACAGGTGAGAATCAATACAGGTGCGATGATCACAGC
  G T Y S N Q S I K V S V G S T T S D K I G Q V R I N T G A M I T A

     510                 530                 550                 570                 590
GGCTTCAGAGGCCACTTTGACATTCAAGCAAATCAATGGTGGAGGAACATCACCTCTTGAGGGTGTAAAAATCTCTCACTCTGTAGGCACAGGTTTGGGA
  A S E A T L T F K Q I N G G G T S P L E G V K I S H S V G T G L G

     610                 630                 650                 670                 690
GTTTTGGCCGAAGTCATCAACAAAAACAGCGATAAAACTGGCATCAGAGCAAAGGCGAGTGTCGAAACCACATCAGATAAAGAGATTATGTCAGGAAATT
V L A E V I N K N S D K T G I R A K A S V E T T S D K E I M S G N L

     710                 730                 750                 770                 790
TGAAAAACTTGACAATCAATGATGTAAATATTGGGAATATTGTTGACATTAAAAAAGGAGATGCTGATGGTCGTTTGGTTCAGGCAATCAATGCTCTAAC
  K N L T I N D V N I G N I V D I K K G D A D G R L V Q A I N A L T

     810                 830                 850                 870                 890
TTCAAGTACGGGAGTTGAGGCTTCCACAGATTCTAAGGGTCGCTTGAATTTGAGAAGTGTGGATGGACGTGGTATTGTGTTAAAAGCAGATGCTTCAGAG
  S S T G V E A S T D S K G R L N L R S V D G R G I V L K A D A S E

     910                 930                 950                 970                 990
GATAATGGTGATGGTAAATCTGCTCCTATGGCAATTGATGCAGTCAATGGCGGTCAAAGCATTACTGATGGTGAGGGCGCTGCTAACTATGGTAGATTAT
D N G D G K S A P M A I D A V N G G Q S I T D G E G A A N Y G R L S

    1010                1030                1050                1070                1090
CTCTTGTAAGACTTGATGCCAGAGACATTGTATTGACTTCAAGCGATAAGCCTGATGAAAATAAATTTAGTGCTATTGGTTTTGGTGATAATAATGTCGC
  L V R L D A R D I V L T S S D K P D E N K F S A I G F G D N N V A

    1110                1130                1150                1170                1190
TATGGCTACAGTGAATTTGCGTGATGTGTTGGGTAAATTCGATGCTTCTGTGAAGTCTGCATCAGGGGCGAATTATAACGCAGTTATTGCCAGTGGTAAT
  M A T V N L R D V L G K F D A S V K S A S G A N Y N A V I A S G N

    1210                1230                1250                1270                1290
TCAAATCTTGGTGCTGGTGTTACTACTTTGGGTGGGTGCGATGCTTGTTATGGATATTGCAGATTCTGCGCGAAAAACACTGGATAAAATCCGCTCCGATC
S N L G A G V T T L V G A M L V M D I A D S A R K T L D K I R S D L

    1310                1330                1350                1370                1390
TTGGTTCTGTTCAAGGTCAGATGGTAAGCACAGTAAATAATATTTCTGTAACACAGGTGAATGTCAAAGCAGCTGAGAGCCGCATGAGAGAAGTTGACTT
  G S V Q G Q M V S T V N N I S V T Q V N V K A A E S R M R E V D F

    1410                1430
TGCTGCTGAATCTGCTGAATTTAACAAGTATAACATCCTTGCACAG
A A E S A E F N K Y N I L A Q
```

FIG. 2. Nucleotide and derived protein sequence of the *H. mustelae flaA* gene. The sequence starts at position 43 of the *flaA* gene. The first 14 codons have not been sequenced. Every twentieth base is numbered. Derived amino acids, starting at position 15 of the *H. mustelae* FlaA protein, are given in single-letter code. This nucleotide sequence is accessible in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under accession number L38478.

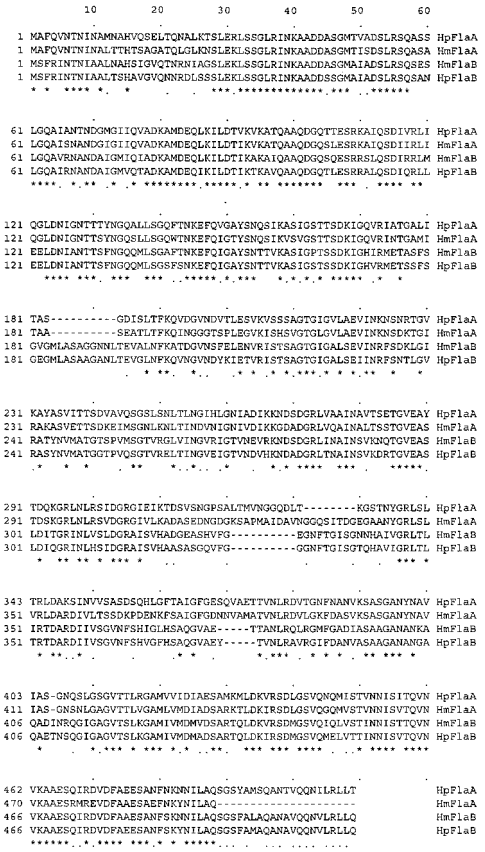

FIG. 3. Sequence alignment of the amino acid sequences of the *H. pylori* and *H. mustelae* FlaA and FlaB protein subunits. The N-terminal methionines have been omitted in correspondence with the situation in the mature proteins. HpFlaA, *H. pylori* FlaA; HmFlaA, *H. mustelae* FlaA; HmFlaB, *H. mustelae* FlaB; HpFlaB, *H. pylori* FlaB. The numbers at the start of each line designate the positions of the first amino acid in each line. Hyphens represent gaps introduced to facilitate alignment. In the line below the sequences, positions marked with asterisks denote totally conserved positions and dots indicate conservative replacements of amino acids.

terial flagellins (54). The alignment of all four *Helicobacter* flagellins shows a stretch of 10 amino acids (185 to 194) that is well conserved (90% identity) in the FlaB proteins and not present in the FlaA proteins. This motif might represent a FlaB-specific flagellar epitope.

Construction of *H. mustelae flaA* and *flaB* mutants by reverse genetics. We constructed *H. mustelae flaA*- and *flaB*-negative mutants by electroporation-mediated allelic exchange. The *flaA* gene of *H. mustelae* in plasmid clone pSUS110 contains a unique *Eco*RI site (nucleotide 391). This plasmid was cut with *Eco*RI, the 3′-recessed ends were filled, and the resulting blunt ends were ligated with the 1.4-kb *Campylobacter* kanamycin resistance cassette (*aph3′*-III) (31) that had been cut out from pILL600 with restriction enzyme *Sma*I. For the mutagenesis of *H. mustelae flaB*, the *flaB* plasmid clone pSUS101 was cut with *Eco*RV to remove a small fragment of 318 bp between nucleotides 1041 and 1359 of the *flaB* gene (49). This fragment was then replaced with the kanamycin resistance cassette (cut out with *Sma*I) by blunt-ended ligation. From the resulting plasmid clones, plasmids pSUS112 (*flaA* Ω Km) and pSUS25 (*flaB* Ω Km) were selected, with both carrying the kanamycin cassette in the same transcriptional orientation as the respective flagellin genes. The plasmids were electroporated into *H. mustelae* F1. The recombinant strains that had acquired kanamycin resistance were characterized to define their respective genotypes and phenotypes.

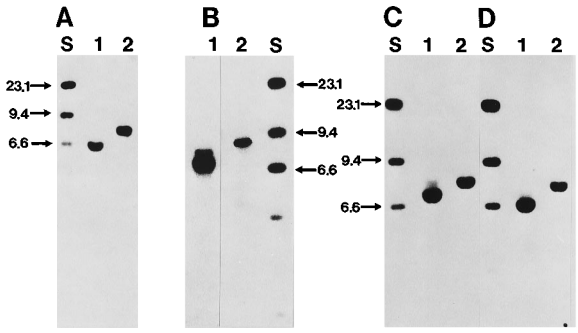

FIG. 4. Southern blots of genomic DNA of the *H. mustelae* F1 wild-type strain compared with those of the *flaA* (B) and *flaB* (A) mutants and *flaA flaB* double mutants (C and D) of the same strain. The DNA was digested with the restriction enzyme *Bgl*II and separated by electrophoresis in 0.8% agarose. Lanes S show DNA size markers (DIG-labelled molecular weight standard II; Boehringer Mannheim Biochemicals). Lanes 1 contain genomic DNA of wild-type *H. mustelae* F1. Lanes 2 contain DNA of the respective flagellin mutants. The blots were hybridized with PCR-generated *flaA* (B and C) and *flaB* (A and D) probes. Hybridizations with probes recognizing the respective resistance genes (kanamycin and chloramphenicol) recognized the same bands as the respective flagellin probes in the mutants and did not hybridize with the wild-type DNA (data not shown). Note the similar sizes of the wild-type *H. mustelae* F1 DNA *Bgl*II fragments that contain the *flaA* (7.5 kb) and *flaB* (6.8 kb) genes. The numbers represent kilobases.

Construction of *H. mustelae* and *H. pylori flaA flaB* double mutants by introduction of two selective markers conferring antibiotic resistance. As a general approach to the construction of double mutants, we used a promoterless chloramphenicol acetyltransferase (CAT) cartridge as a second selective marker for gene disruption of single mutants that were already kanamycin resistant. The natural level of *flaA* expression under the control of the *flaA* σ^{28} promoter in *Helicobacter* species (demonstrated for *H. pylori* and *H. felis flaA* [47, 49] but also *H. mustelae flaA*) was thought to be high enough to ensure sufficient expression of chloramphenicol resistance.

The *H. mustelae flaA flaB* double mutants were generated from the kanamycin-resistant *H. mustelae flaB* mutants as follows. Plasmid pSUS110 with the *H. mustelae flaA* gene was restricted with *Eco*RI. The promoterless CAT cartridge was excised from plasmid pCM4 with *Bam*HI. The ends of both fragments were blunt ended and ligated. The constructed plasmid pSUS115 contained the *cat* gene in the same transcriptional orientation as that of *flaA*. It was electroporated into the *H. mustelae flaB* mutants, and double mutants were selected on plates containing only chloramphenicol in order to facilitate the growth of recombinant cells. Nine independent transformants could be isolated after 9 days of incubation on the selective medium. Afterwards, the colonies were subcultured on plates containing kanamycin and chloramphenicol.

H. pylori flaA flaB double mutants were generated by a similar approach. The CAT cartridge was introduced into the unique *Bam*HI restriction site located in the middle of the *H. pylori flaA* gene on plasmid pHL319-2-4 (33). This plasmid construction (pSUS24) was transferred into the kanamycin-resistant *H. pylori flaB* mutants (49) by electroporation. One single recombinant resistant to kanamycin and chloramphenicol was selected and characterized further.

Characterization of *H. mustelae flaA* and *flaB* and of the *H. mustelae* and *H. pylori flaA flaB* double mutants. The genotype of the constructed mutants was verified by Southern blotting and hybridization (Fig. 4). Genomic DNA of all mutants was

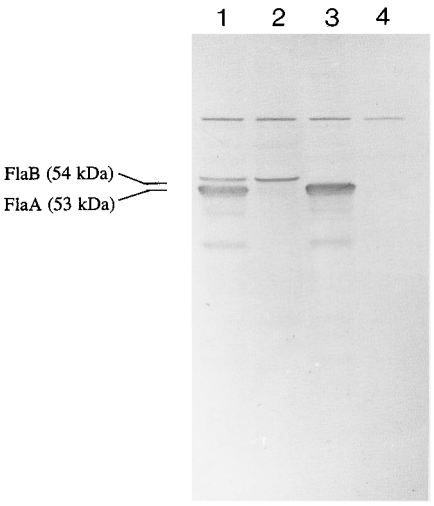

FIG. 5. Western blot of whole-cell lysates of wild-type *H. mustelae* F1 (lane 1), *flaA* (lane 2) and *flaB* (lane 3) mutants, and *flaA flaB* double mutants (lane 4). The blot was developed with a 1:2,000 dilution of antiserum AK179 raised against purified native filaments of *H. pylori.*

prepared and restricted with *Bgl*II. The DNA was analyzed by Southern blotting and hybridization with four different probes: (i) a probe generated by PCR (primers OLKm-1 and OLKm-2) to detect the kanamycin resistance cassette; (ii) plasmid pILL570 to check for the presence of vector sequences; (iii) a probe generated from the *H. mustelae flaA* gene amplified by PCR (primers OLHMFlaA7S and OLHMFlaA8S) to detect *flaA*-containing fragments; and (iv) a probe generated from the PCR-amplified fragment of *H. mustelae flaB* (primers OLHM FlaB2 and OLHMFlaB3) to detect fragments containing the *flaB* gene (49). All hybridization reactions were carried out under high-stringency conditions.

We did not detect any vector sequences introduced into the genomic *Helicobacter* DNA (data not shown). All flagellin mutants had replaced their intact alleles with the disrupted genes on the plasmids by double crossover. The hybridization results with the respective mutant strains confirmed that the kanamycin and chloramphenicol resistance cassettes had been integrated into the genome at the predetermined sites. The respective restriction fragments hybridizing in the wild-type strains were larger in the mutant strains by the sizes of the inserted cassettes (1.4 kb for the kanamycin cassette and about 800 bp for the CAT fragment) (Fig. 4).

To check for flagellin expression, we performed Western blot analyses of whole-cell lysates of the mutants and compared the results with those for the wild-type strains. The blots were developed with antiserum AK179 (33) raised against native purified *H. pylori* filaments because AK179 cross-reacts sufficiently with both *H. mustelae* flagellin subunits. The *H. mustelae flaA* mutants did not express FlaA in detectable amounts, and the *H. mustelae flaB* mutants did not produce FlaB. These findings were consistent with the results for *H. pylori* published earlier (49). The amount of expression from the respective undisrupted flagellin genes seemed to be unchanged in comparison with that for the wild-type strain (Fig. 5). In the *flaA flaB* double mutants of *H. pylori* and *H. mustelae*, expression of both flagellin subunits was completely abolished. All Western blot findings could be reproduced with five separate mutant colonies of every transformation experiment (data not shown).

Electron microscopy of the *H. mustelae* and *H. pylori* mutants. Bacteria of the different wild-type and mutant strains were examined by transmission electron microscopy using negative staining (Fig. 6).

In both *H. pylori* and *H. mustelae*, it was impossible to distinguish the flagella of the isogenic *flaB* mutants from those of the wild-type strains in length and appearance (Fig. 6A and B). Nevertheless, subtle differences in length cannot be ruled out because the flagellar length seems to be subject to considerable variation in the wild-type strains. Moreover, in *H. mustelae*, it is difficult to visualize completely undamaged full-length flagella because of their greater length and ensuing fragility.

The *flaA* mutants of both *Helicobacter* species express only truncated flagella. The *H. mustelae flaA* mutants exhibited short truncated flagella (Fig. 6D) which were about 300 nm to 1.2 μm in length, compared with a length of about 5 to 12 μm for *H. mustelae* wild-type flagella. Interestingly, the *H. mustelae* polar and lateral flagella were equally reduced to a truncated form. The number and localization of flagella in *H. mustelae flaA* mutants did not differ from those of the wild-type strain. When reexamining the *H. pylori flaA* mutants, we also found truncated flagella which were mostly shorter than those of the *H. mustelae flaA* mutants (Fig. 6C). They were usually arranged as a unipolar tuft of three to seven stubby flagellar extensions. The *H. mustelae* and *H. pylori flaA flaB* double mutants did not possess flagella (Fig. 6F).

As with those of the wild-type strains, the stubby flagella of *H. pylori* and *H. mustelae flaA* mutants were enveloped by a sheath (Fig. 6C and D) whose length was equal to the length of the filament. Both on the double-mutated cells as well as on the *flaA* mutants of *H. mustelae*, we could occasionally see structures resembling empty flagellar sheaths extending as membranous tubules (width, about 20 to 40 nm) about 2 to 4 μm from the cell surface (Fig. 6E). Because of our very gentle method of cell preparation, it is unlikely that these structures, which were seen in many independently prepared grids, represent technical artifacts. However, this is difficult to prove without a specific method to visualize flagellar sheath material.

Comparative functional analysis of *H. mustelae* and *H. pylori* flagellin mutants by motility testing. Two different types of motility tests were performed to compare the motility of the different *H. mustelae* and *H. pylori* mutants with that of the wild-type strains. Single-colony motility was assessed with the phase-contrast microscope by comparing the swarming halos surrounding single colonies (Fig. 7A to D). With the microscope, the bacteria could also be examined directly for their motility within the agar. In general, *H. pylori* mutants and wild-type strains were less motile in our testing system than *H. mustelae* strains. In particular, in 0.4% motility agar, the *H. pylori* strains showed nearly no single-colony motility. *H. mustelae* strains, however, remained motile in 0.4% motility agar. All comparative tests were performed in 0.3% agar.

In the standard stab agar test (Fig. 7E), *flaA* mutants and *flaA flaB* double mutants of *H. pylori* and *H. mustelae* both showed no apparent motility. However, in our single-colony testing system, only the double mutants of *H. mustelae* and *H. pylori* were completely nonmotile. This was defined as no apparent motility in motility agar as seen with the phase-contrast microscope and was characterized by colonies with sharp outlines (dense colonies) and without any granularity after 9 days of incubation (Fig. 7D). In contrast, *flaA* single mutants of both *Helicobacter* species possessed some residual motility. They could be observed to move in the motility agar, although they did so much less than did wild-type strains and *flaB* mutants, consistent with the fact that they carry truncated flagella. Their single-colony motility after at least 5 days of incubation in 0.3% motility agar was of a small granular type (Fig. 7C).

Despite possessing flagella of apparently normal length, *H.*

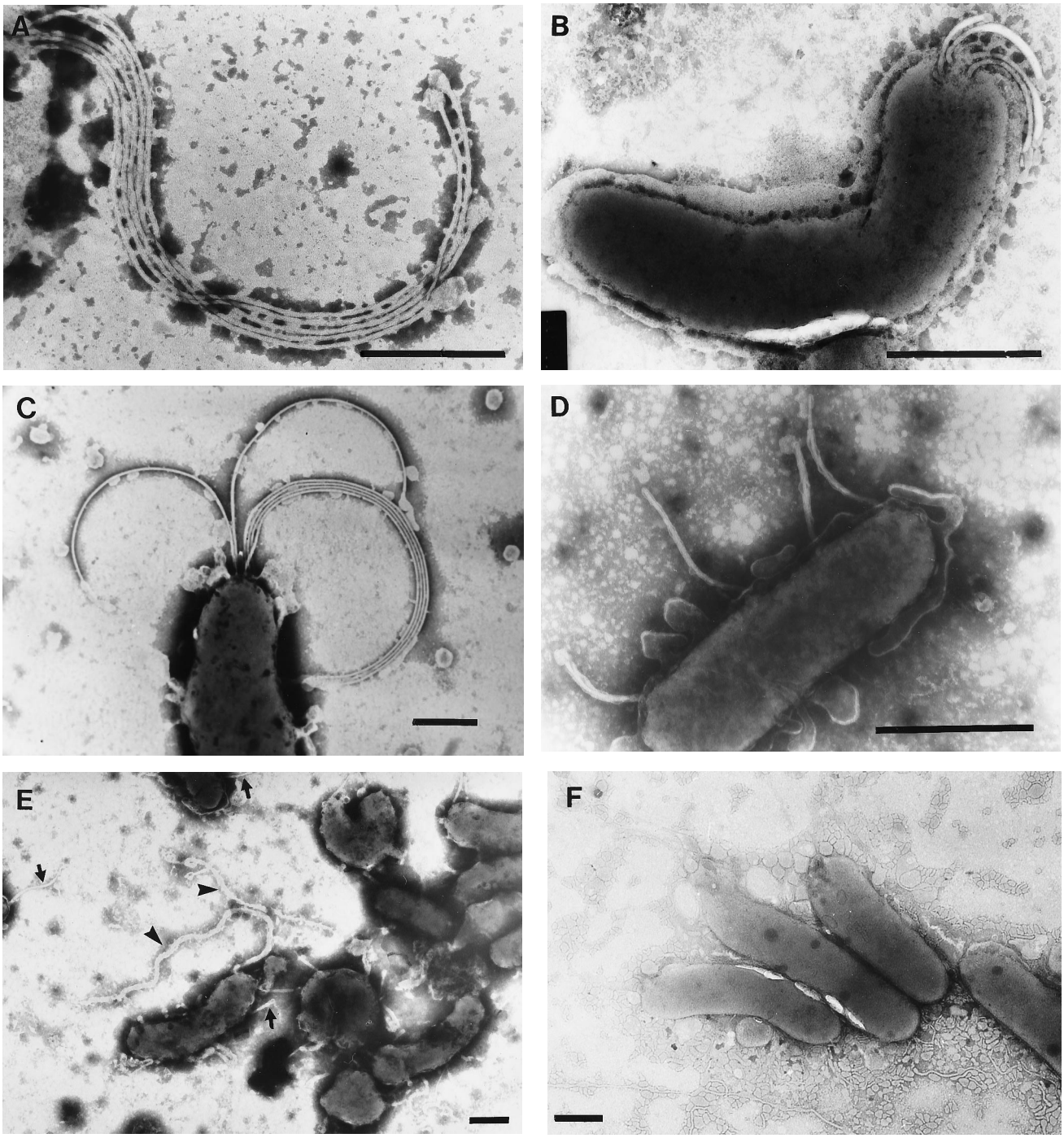

FIG. 6. Electron microscopy of wild-type *H. pylori* and *H. mustelae* and *H. pylori* motility mutants, negatively stained with potassium phosphotungstate (pH 7.0). Bars, 1 μm. The *H. pylori* N6 *flaB* mutants (C) apparently possess full-length sheathed flagella like those of the wild-type N6 strain (A). One *H. pylori* N6 *flaA* mutant cell with a unipolar tuft of truncated sheathed flagella is shown in panel B. The *H. mustelae* F1 *flaA* mutants (D and E) exhibit truncated flagella with sheaths (arrows in panel E) and, occasionally, additional empty flagellar sheaths (arrowheads in panel E). The *H. mustelae* F1 *flaA flaB* double mutants (F) do not possess flagella. All specimens were prepared from plates that had been incubated for 3.5 days. The grids were directly placed upside down on the plates as described in Material and Methods.

mustelae and *H. pylori flaB* mutants were not fully motile. In the stab agar test, *Helicobacter flaB* mutants formed a smaller swarming zone (reduced by about 30 to 40%) (Fig. 7E) in the motility agar. This was equally apparent in the single-colony morphology test, in which some colonies seemed to be fully motile but most were significantly smaller than wild-type colonies and had a granular colony morphology in contrast to the completely diffuse kind of motility seen with the wild-type strains (Fig. 7A and B). Motility was not influenced by the antibiotic resistance (kanamycin) introduced into the mutant strains, since we observed normal motility for a kanamycin-resistant *ureB* mutant of *H. pylori* N6.

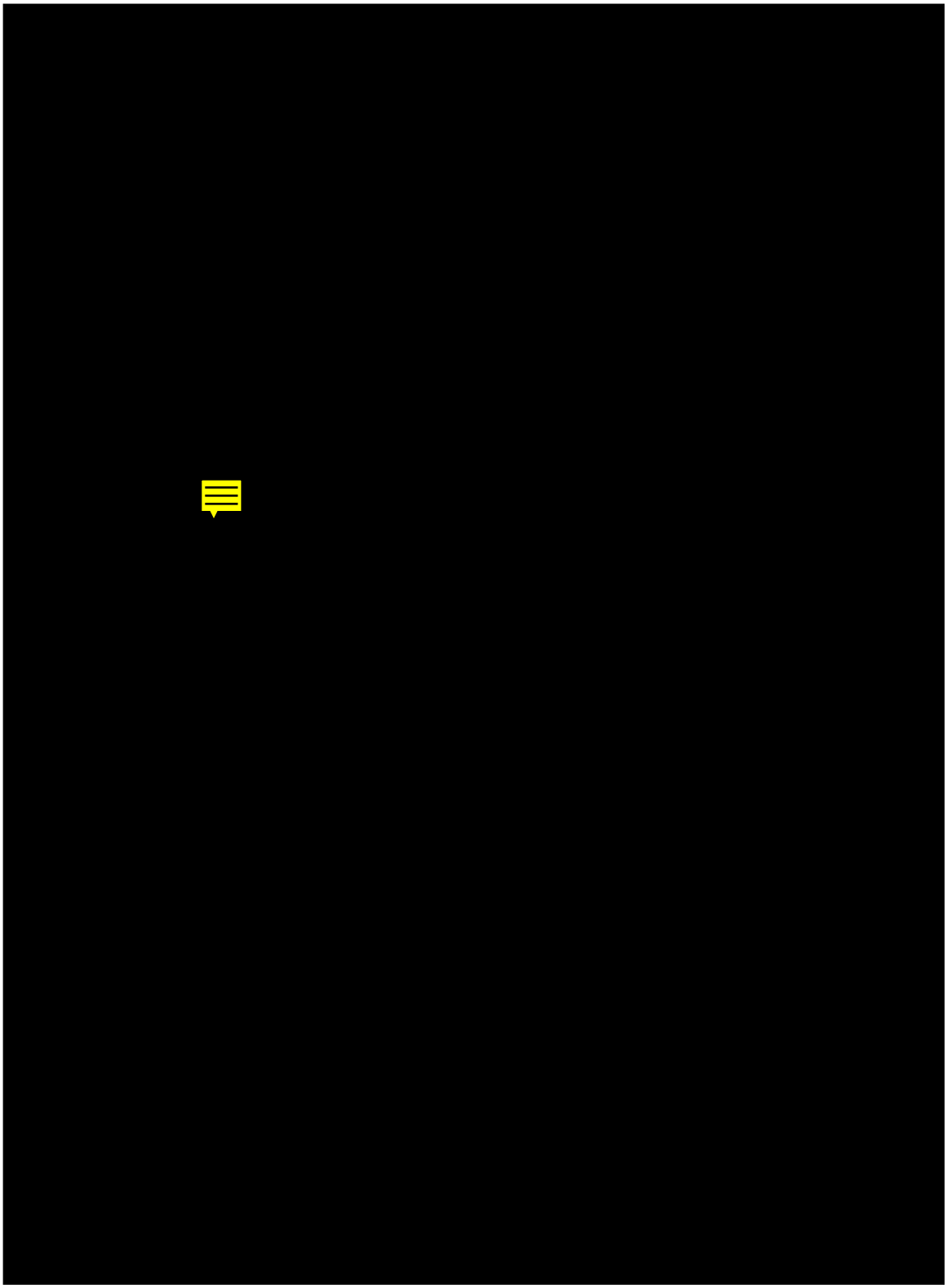

FIG. 7. Motility testing of *H. pylori* flagellin mutants. Panels A to D show the single-colony motility (as described in Materials and Methods) of the three different types of *H. pylori* N6 motility mutants and wild-type N6 strain. The wild-type strain (WT) forms diffuse colonies with large swarming halos (A), the *flaB* mutant (flaB-) has a smaller swarming zone and a slightly granular colony morphology (B), the *flaA* mutant (flaA-) exhibits a small granular colony morphology with only a little bit of residual swarming (C), and the *flaA flaB* double mutants (flaA-B-) do not exhibit motility and form dense colonies with a smooth outline (D). Panel E depicts a stab agar test of wild-type *H. mustelae* F1 (WT) and *flaA* (flaA-) and *flaB* (flaB-) mutants. *flaA flaB* double mutants looked like the *flaA* mutants in this test (data not shown). All shown plates were made of 0.3% agar and incubated for 5 days after inoculation. Stab agar as well as single-colony motility testing gave very similar results with the other *Helicobacter* species, with the exception that *H. mustelae* strains clearly showed higher motility. Bars, 0.5 mm.

DISCUSSION

In experiments with gnotobiotic piglets, it has been demonstrated that *H. pylori* cells strongly rely on their high motility to colonize the gastric epithelium and to persist in the gastric mucus (11). Lately, the molecular basis of motility in *H. pylori* and *H. mustelae* has been studied intensively (28, 33, 49).

The major goal of this study was to perform an in-depth functional and morphological characterization of isogenic *Helicobacter* motility mutants in the *flaA* and *flaB* genes as well as of *flaA flaB* double mutants. Because our first examination of *H. pylori* mutants did not permit us to define a function for the minor flagellin subunit FlaB, we performed parallel studies of *H. pylori* and *H. mustelae*, which grows faster and is more motile in vitro and will also allow for the testing of mutants in the ferret animal model.

Several attempts to clone the homologous *H. mustelae flaA* gene from genomic DNA by different approaches had been unsuccessful, probably because of a gene in the neighborhood that is toxic when expressed in a high-copy-number plasmid in *E. coli* or because of other genetic elements interfering with plasmid stability. In order to construct isogenic *H. mustelae flaA* mutants for the comparative studies, it was sufficient to isolate the *H. mustelae flaA* gene without any regulatory sequences. So we used a PCR strategy to amplify and clone the gene with the help of degenerate primers designed on the basis of the homologous *flaA* genes of *H. pylori* and *H. felis*. With this approach, we obtained a DNA fragment of 1,446 bp, representing almost the complete *H. mustelae flaA* gene. The *H. mustelae* FlaA protein sequence is 72% identical with that of *H. pylori* FlaA, and it shares 56% and 53% amino acid identities with *H. mustelae* and *H. pylori* FlaB, respectively. The interspecies similarity of the *Helicobacter* FlaA and FlaB proteins is very high, whereas the similarity of the FlaA and FlaB subunits within one species does not exceed the homology with other bacterial flagellins (e.g., 55.5% identity of *H. mustelae* FlaB and *Campylobacter coli* FlaB). This situation is different from that of *C. coli* and *C. jejuni*, for which the FlaA and FlaB proteins are more than 90% identical (25, 38), possibly as a result of recombinational activity between the two flagellin genes that are closely linked on the chromosome (2). Alm et al. (2) have shown that the second flagellin gene in *C. coli flaA* mutants can serve as a reserve gene copy to restore high motility by recombination with the disrupted gene. In *Helicobacter* spp., this is unlikely to occur because of the sequence differences between the two flagellin genes; this is confirmed by our observations (data not shown) that all *Helicobacter* flagellin mutants remained stable after several passages on motility agar with and without selective antibiotics. In addition, the *flaA* and *flaB* genes of both *H. pylori* and *H. mustelae* are not genetically linked (49). It seems likely that the separate localizations and sequence divergence of the two *Helicobacter* flagellin genes represent an adaptation of the bacterium to the requirements of its ecological niche.

In other aspects of flagellar organization, such as flagellar promoters and coexpression of two flagellin subunits, the flagellar organization of the *Helicobacter* species is very similar to that of the genus *Campylobacter* (24, 38).

In order to further elucidate the functions of the two different flagellin subunits, we wanted to construct isogenic *flaA* and *flaB* mutants of *H. mustelae* as well as *flaA flaB* double mutants of both *H. mustelae* and *H. pylori*. We therefore adapted the electroporation technique for use with *H. mustelae* and devised a strategy to construct, for the first time, double mutants in both species with a promoterless CAT cartridge being used as second selective marker.

In general, the characterization of wild-type strains and mutants yielded very similar results for both *Helicobacter* species. All *flaB* mutants were less motile than the wild-type strains; they exhibited smaller swarming halos around the colonies and a mostly large granular single-colony morphology in contrast to the large halos and diffuse pattern of motility seen with the wild-type strain. These results parallel descriptions by Eaton et al. (10), who observed a granular pattern of motility in single colonies of spontaneous *H. pylori* mutants with decreased motility. The flagellar morphology and flagellar length of the *flaB* mutants, however, were apparently normal. *flaA* mutants exhibited very weak residual motility (small granular type) that could only be visualized by examination of single colonies in very soft agar. *flaA* mutants of both *Helicobacter* species possess two to seven stubby, truncated flagella with sheaths. In *H. pylori flaA* mutants, the truncated flagella were arranged in unipolar tufts. This is in contrast to earlier descriptions of unflagellated *H. pylori flaA* mutants (26, 49). The reason why these truncated flagella had first escaped attention could be the thick network of fibrils which frequently covers *H. pylori* as well as *H. mustelae* cells (13, 39). A possible influence of growth conditions or growth phase on the expression of truncated flagella cannot, however, be excluded. In *H. mustelae flaA* mutants, polar as well as lateral flagella are truncated. This may indicate that lateral and polar flagella are composed of the same structural elements or are subject to the same regulation mechanisms and do not represent functionally separate motility systems.

Double mutants in *flaA* and *flaB* of both *Helicobacter* species were completely nonmotile and did not exhibit flagella. In both *Helicobacter flaA* mutants and *flaA flaB* double mutants, we occasionally observed empty flagellar sheaths attached to the cells. Thus, the generation of flagellar sheaths appears to be an active process independent of filament production. This is consistent with findings reported by Richardson et al. (43), who similarly observed empty flagellar sheath structures in aflagellate mutants of *Vibrio cholerae*. On the other hand, the FlaA and FlaB polymers both seem to be involved in the determination of the lengths of the filament-containing sheaths because in the *flaA* mutants, the lengths of the sheaths did not exceed the lengths of the truncated filaments.

A remarkable structural feature of *H. pylori* flagella is the terminal bulbs formed by the sheath at the tip of the flagellum (20, 23). Terminal bulbs were seen on the *flaA* and *flaB* mutants of *H. pylori*. In *H. mustelae*, the presence of similar structures has not been unequivocally demonstrated, because undamaged flagella are much more difficult to visualize (see also reference 39). The function of these bulbs remains to be elucidated.

Lately, flagellar filaments consisting of several different flagellin subunits have been found to be present in quite a number of bacteria, such as *Campylobacter* spp. (two flagellins [24, 38]), *Caulobacter crescentus* (three flagellins [8, 37]), *Halobacterium halobium* (five flagellins [22]), and *Rhizobium meliloti* (two flagellins [41, 42]). A physiological function for this complex type of filament organization has not been defined yet. However, Trachtenberg et al. (51) described the complex filament of *R. meliloti* as more brittle and thus supposedly stiffer than plain filaments consisting of one flagellin only. These complex flagella would possibly be able to better withstand the stronger mechanical forces in viscous and semisolid media. Our present study demonstrates that in *H. pylori* and *H. mustelae*, both flagellin subunits are required for the assembly of a fully functional flagellar filament. This conclusion is strengthened by similar findings that have been reported by Guerry et al. about the roles of the *flaA* and *flaB* genes in *C.*

coli (24). Our results indicate that the presence of FlaB is necessary for optimal motility, especially in viscous environments. We hypothesize that in the gastric *Helicobacter* species, the amount of FlaB and the FlaA/FlaB ratio can be regulated in vivo in response to certain environmental conditions (e.g., viscosity) in order to modulate the physicochemical properties of the bacterial filament and thus assure optimal motility in varying environments. This concept is supported by the fact that both in *Helicobacter* spp. and in *Campylobacter* spp., the expression of the two flagellin genes is regulated by different promoters (σ^{28} for the *flaA* genes and σ^{54} for the *flaB* genes [2, 24, 49]). Alm et al. (1) have recently provided evidence for environmental influences on FlaB expression in *C. coli* by means of transcriptional fusions of a CAT cassette with the *flaB* promoter. We are presently studying the transcriptional regulation of both *Helicobacter* flagellar genes, using transcriptional fusions of the promoterless CAT cassette with the *flaA* and *flaB* promoters. Experiments with the *H. mustelae* and *H. pylori* flagellin mutants in the respective animal models of the ferret and gnotobiotic piglet are under way and will help to elucidate the importance of the complex flagellum for colonization and persistence in vivo.

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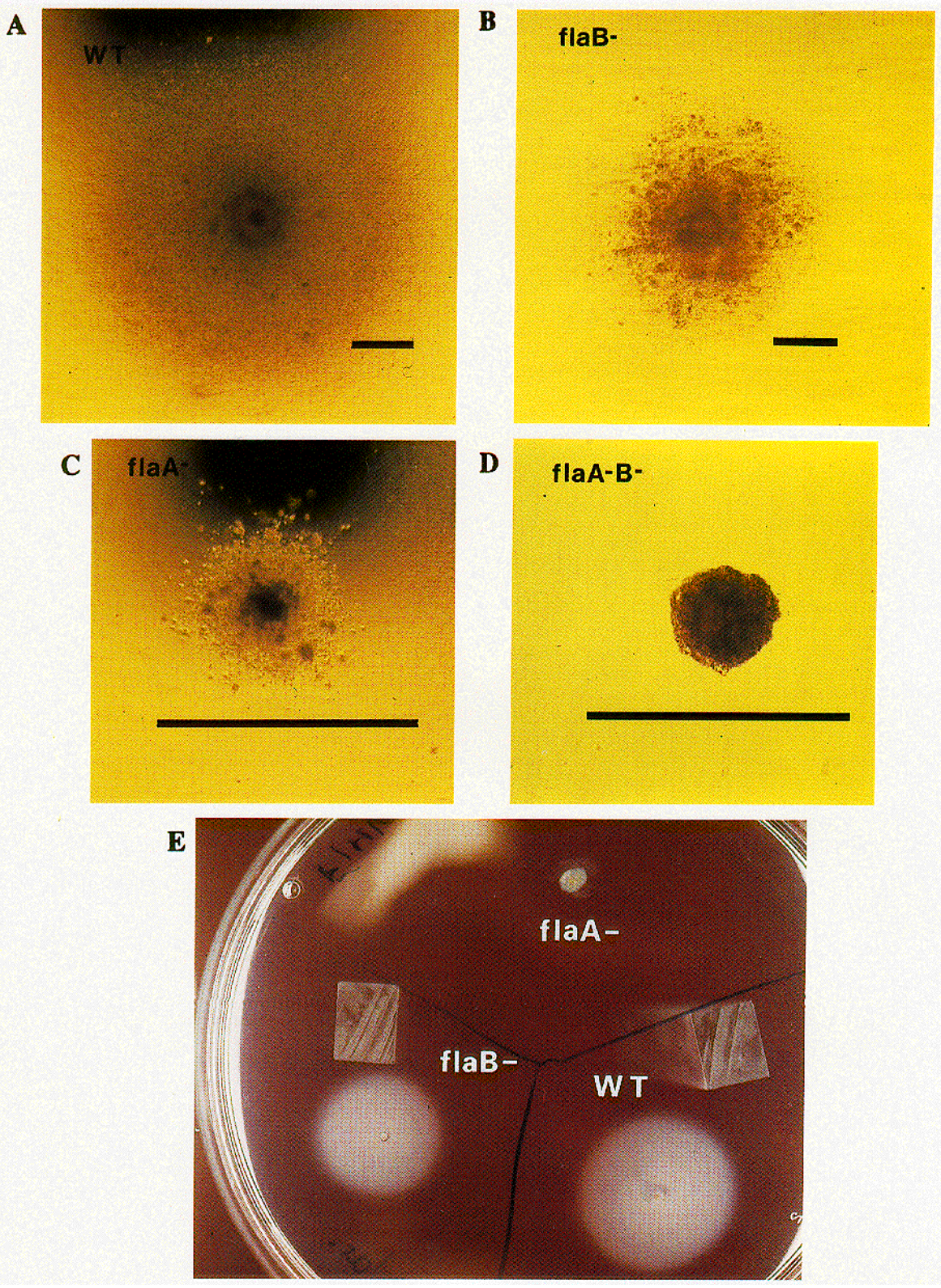
REFERENCES

1. **Alm, R. A., P. Guerry, and T. J. Trust.** 1993. The *Campylobacter* sigma 54 *flaB* flagellin promoter is subject to environmental regulation. J. Bacteriol. **175:**4448–4455.
2. **Alm, R. A., P. Guerry, and T. J. Trust.** 1993. Significance of duplicated flagellin genes in *Campylobacter*. J. Mol. Biol. **230:**359–363.
3. **Beveridge, T. J., T. J. Popkin, and R. M. Cole.** 1994. Electron microscopy, p. 42–71. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
4. **Blaser, M. J.** 1994. *Helicobacter pylori*: microbiology of a "slow" bacterial infection. Trends Microbiol. **1:**255–260.
5. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
6. **Casadaban, M., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *E. coli*. J. Mol. Biol. **138:**179–207.
7. **Cover, T. L., and M. J. Blaser.** 1992. *Helicobacter pylori* and gastroduodenal disease. Annu. Rev. Med. **43:**135–145.
8. **Driks, A., R. Bryan, L. Shapiro, and D. J. DeRosier.** 1989. The organization of the *Caulobacter crescentus* flagellar filament. J. Mol. Biol. **206:**627–636.
9. **Dunn, B. E., C.-C. Sung, N. S. Taylor, and J. G. Fox.** 1991. Purification and characterization of *Helicobacter mustelae* urease. Infect. Immun. **59:**3343–3345.
10. **Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **59:**2470–2475.
11. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1989. *Campylobacter pylori* virulence factors in gnotobiotic piglets. Infect. Immun. **57:**1119–1125.
12. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1992. Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. J. Med. Microbiol. **37:**123–127.
13. **Evans, D. G., D. J. J. Evans, J. J. Moulds, and D. Y. Graham.** 1988. *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin of *Campylobacter pylori*: a putative colonization factor antigen. Infect. Immun. **56:**2896–2906.
14. **Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne.** 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. J. Bacteriol. **174:**4212–4217.
15. **Ferrero, R. L., and A. Lee.** 1988. Motility of *Campylobacter jejuni* in a viscous environment: comparison with conventional rod-shaped bacteria. J. Gen. Microbiol. **134:**53–59.
16. **Fox, J. G., E. B. Cabot, N. S. Taylor, and R. Laraway.** 1988. Gastric colonization by *Campylobacter pylori* subsp. *mustelae* in ferrets. Infect. Immun. **56:**2994–2996.
17. **Fox, J. G., T. Chilvers, C. S. Goodwin, N. S. Taylor, P. Edmonds, L. I. Sly, and D. J. Brenner.** 1989. *Campylobacter mustelae*, a new species resulting from the elevation of *Campylobacter pylori* subsp. *mustelae* to species status. Int. J. Syst. Bacteriol. **39:**301–303.
18. **Fox, J. G., P. Correa, N. S. Taylor, A. Lee, G. Otto, J. C. Murphy, and R. Rose.** 1990. *Helicobacter mustelae*-associated gastritis in ferrets. An animal model of *Helicobacter pylori* gastritis in humans. Gastroenterology **99:**352–361.
19. **Fox, J. G., G. Otto, J. C. Murphy, N. S. Taylor, and A. Lee.** 1991. Gastric colonization of the ferret with *Helicobacter* species: natural and experimental infections. Rev. Infect. Dis. **13**(Suppl. 8)**:**S671–S680.
20. **Geis, G., H. Leying, S. Suerbaum, U. Mai, and W. Opferkuch.** 1989. Ultrastructure and chemical analysis of *Campylobacter pylori* flagella. J. Clin. Microbiol. **27:**436–441.
21. **Geis, G., S. Suerbaum, B. Forsthoff, H. Leying, and W. Opferkuch.** 1993. Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*. J. Med. Microbiol. **38:**371–377.
22. **Gerl, L., and M. Sumper.** 1988. Halobacterial flagellins are encoded by a multigene family. Characterization of five flagellin genes. J. Biol. Chem. **263:**13246–13251.
23. **Goodwin, C. S., R. K. McCulloch, J. A. Armstrong, and S. H. Wee.** 1985. Unusual cellular fatty acids and distinctive ultrastructure in a new spiral bacterium (*Campylobacter pyloridis*) from the human gastric mucosa. J. Med. Microbiol. **19:**257–267.
24. **Guerry, P., R. A. Alm, M. E. Power, S. M. Logan, and T. J. Trust.** 1991. Role of two flagellin genes in *Campylobacter* motility. J. Bacteriol. **173:**4757–4764.
25. **Guerry, P., S. M. Logan, S. Thornton, and T. J. Trust.** 1990. Genomic organization and expression of *Campylobacter* flagellin genes. J. Bacteriol. **172:**1853–1860.
26. **Haas, R., T. F. Meyer, and J. P. van Putten.** 1993. Aflagellated mutants of *Helicobacter pylori* generated by genetic transformation of naturally competent strains using transposon shuttle mutagenesis. Mol. Microbiol. **8:**753–760.
27. **Hazell, S. L., A. Lee, L. Brady, and W. Hennessy.** 1986. Campylobacter pyloridis and gastritis: association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J. Infect. Dis. **153:**658–663.
28. **Kostrzynska, M., J. D. Betts, J. W. Austin, and T. J. Trust.** 1991. Identification, characterization, and spatial localization of two flagellin species in *Helicobacter pylori* flagella. J. Bacteriol. **173:**937–946.
29. **Krakowka, S., D. R. Morgan, W. G. Kraft, and R. D. Leunk.** 1987. Establishment of gastric *Campylobacter pylori* infection in the neonatal gnotobiotic piglet. Infect. Immun. **55:**2789–2796.
30. **Labigne, A., V. Cussac, and P. Courcoux.** 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J. Bacteriol. **173:**1920–1931.
31. **Labigne-Roussel, A., P. Courcoux, and L. Tompkins.** 1988. Gene disruption and replacement as a feasible approach for mutagenesis of *Campylobacter jejuni*. J. Bacteriol. **170:**1704–1708.
32. **Lee, A., J. G. Fox, G. Otto, and J. Murphy.** 1990. A small animal model of human *Helicobacter pylori* active chronic gastritis. Gastroenterology **99:**1315–1323.
33. **Leying, H., S. Suerbaum, G. Geis, and R. Haas.** 1992. Cloning and genetic characterization of a *Helicobacter pylori* flagellin gene. Mol. Microbiol. **6:**2863–2874.
34. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K12 into four bands. FEBS Lett. **58:**254–258.
35. **Majewski, S. I., and C. S. Goodwin.** 1988. Restriction endonuclease analysis of the genome of *Campylobacter pylori* with a rapid extraction method: evidence for considerable genomic variation. J. Infect. Dis. **157:**465–471.
36. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene **19:**269–276.
37. **Minnich, S. A., N. Ohta, N. Taylor, and A. Newton.** 1988. Role of the 25-, 27-, and 29-kilodalton flagellins in *Caulobacter crescentus* cell motility: method for construction of deletion and Tn*5* insertion mutants by gene replacement. J. Bacteriol. **170:**3953–3960.
38. **Nuijten, P. J., F. J. van Asten, W. Gaastra, and B. A. van der Zeijst.** 1990. Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. J. Biol. Chem. **265:**17798–17804.
39. **O'Rourke, J., A. Lee, and J. G. Fox.** 1992. An ultrastructural study of *Helicobacter mustelae* and evidence of a specific association with gastric mucosa. J. Med. Microbiol. **36:**420–427.
40. **Parsonnet, J.** 1993. *Helicobacter pylori* and gastric cancer. Gastroenterol. Clin. North Am. **22:**89–104.
41. **Pleier, E., and R. Schmitt.** 1989. Identification and sequence analysis of two related flagellin genes in *Rhizobium meliloti*. J. Bacteriol. **171:**1467–1475.
42. **Pleier, E., and R. Schmitt.** 1991. Expression of two *Rhizobium meliloti* flagellin genes and their contribution to the complex filament structure. J. Bacteriol. **173:**2077–2085.

43. **Richardson, K., L. Nixon, P. Mostow, J. B. Kaper, and J. Michalski.** 1990. Transposon-induced non-motile mutants of *Vibrio cholerae*. J. Gen. Microbiol. **136:**717–725.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
46. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
47. **Suerbaum, S., R. L. Ferrero, and A. Labigne.** Unpublished data.
48. **Suerbaum, S., G. Geis, C. Josenhans, and W. Opferkuch.** 1992. Biochemical studies of *Helicobacter mustelae* fatty acid composition and flagella. Infect. Immun. **60:**1695–1698.
49. **Suerbaum, S., C. Josenhans, and A. Labigne.** 1993. 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. J. Bacteriol. **175:**3278–3288.
50. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
51. **Trachtenberg, S., D. J. DeRosier, and R. M. Macnab.** 1987. Three-dimensional structure of the complex flagellar filament of *Rhizobium lupini* and its relation to the structure of the plain filament. J. Mol. Biol. **195:**603–620.
52. **Trieu-Cuot, P., G. Gerbaud, T. Lambert, and P. Courvalin.** 1985. In vivo transfer of genetic information between gram-positive and gram-negative bacteria. EMBO J. **4:**3583–3587.
53. **Turbett, G. R., P. B. Hoj, R. Horne, and B. J. Mee.** 1992. Purification and characterization of the urease enzymes of *Helicobacter* species from humans and animals. Infect. Immun. **60:**5259–5266.
54. **Wilson, D. R., and T. J. Beveridge.** 1993. Bacterial flagellar filaments and their component flagellins. Can. J. Microbiol. **39:**451–472.
55. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.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