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The *Helicobacter pylori flaA1* and *wbpB* Genes Control Lipopolysaccharide and Flagellum Synthesis and Function

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***flaA1* and *wbpB* are conserved genes with unknown biological function in *Helicobacter pylori*. Since both genes are predicted to be involved in lipopolysaccharide (LPS) biosynthesis, flagellum assembly, or protein glycosylation, they could play an important role in the pathogenesis of *H. pylori*. To determine their biological role, both genes were disrupted in strain NCTC 11637. Both mutants exhibited altered LPS, with loss of most O-antigen and core modification, and increased sensitivity to sodium dodecyl sulfate compared to wild-type bacteria. These defects could be complemented in a gene-specific manner. Also, *flaA1* could complement these defects in the *wbpB* mutant, suggesting a potential redundancy of the reductase activity encoded by both genes. Both mutants were nonmotile, although the *wbpB* mutant still produced flagella. The defect in the flagellum functionality of this mutant was not due to a defect in flagellin glycosylation since flagellins from wild-type strain NCTC 11637 were shown not to be glycosylated. The *flaA1* mutant produced flagellins but no flagellum. Overall, the similar phenotypes observed for both mutants and the complementation of the *wbpB* mutant by *flaA1* suggest that both genes belong to the same biosynthesis pathway. The data also suggest that *flaA1* and *wbpB* are at the interface between several pathways that govern the expression of different virulence factors. We propose that FlaA1 and WbpB synthesize sugar derivatives dedicated to the glycosylation of proteins which are involved in LPS and flagellum production and that glycosylation regulates the activity of these proteins.**

Helicobacter pylori is a spiral-shaped gram-negative microaerophilic bacterium that was first isolated from the human stomach in 1984 (42). It is estimated that 70% of the worldwide population is infected by this bacterium. Most infections are asymptomatic, but they can also lead to gastric ulcers and cancers (26, 51, 66, 69). The relationship between colonization by *H. pylori* and the onset of the disease is not fully understood. Hence, it is important to identify essential bacterial virulence factors and elucidate their contribution to disease development.

Several virulence factors contribute to the stringent host and tissue specificities exhibited by *H. pylori* (37). Among them, urease helps neutralize the acidic pH surrounding the bacteria and allows their survival in the gastric environment (21, 45). In addition, the spiral shape and unipolar flagella of *H. pylori* confer on the bacterium a corkscrew motion that enhances motility in the viscous gastric mucus (32, 33, 63) and is essential for host colonization (22, 23, 35). Lipopolysaccharide (LPS) is also important for the virulence of *H. pylori* since strains lacking the O antigen are significantly impaired in their capacity to colonize the murine stomach (40). The *H. pylori* O antigen is composed of *N*-acetyl-D-glucosamine, L-fucose, and D-galactose (4, 5, 46), which form structural motifs that are identical to human blood group antigens Lewis X, Y, and b (4, 5, 46–48). This host mimicry might allow the bacteria to evade human immune defenses and to establish long-term host colonization (54, 61).

This study focuses on two genes of unknown biological function in *H. pylori*: *flaA1* (HP0840) and *wbpB* (HP0679). They exhibit significant homologies to LPS (9), capsule (8, 57, 58), and/or flagellar biosynthetic genes found in medically relevant bacteria. For example, FlaA1 shows 52% homology to the C-terminal half of WbpM, which is essential for LPS synthesis in *Pseudomonas aeruginosa* (9). The sequence homologies result in functional equivalence between these proteins, since *flaA1* can support O-antigen biosynthesis in a *wbpM* mutant (15). Additional homologues identified in *Campylobacter jejuni* (PglF and CJ1293) (25, 64) and *Caulobacter crescentus* (FlmA) (38) are involved in protein glycosylation and/or influence flagellum production. In contrast to FlaA1, which seems widely distributed in the bacterial world, WbpB has only two homologues in bacterial genomes. One ($WbpB_{Pa}$, 63% homology) is found in the LPS biosynthetic cluster of *P. aeruginosa* serotype O5 (9), and the other (WlbA, 52% homology, N-terminal half only) is found in the LPS biosynthetic cluster of *Bordetella pertussis* (1). The homologies described above strongly suggest that *flaA1* and *wbpB* might also be involved in LPS and flagellum biosynthesis in *H. pylori*. Interestingly, in bacteria where a homologue for each gene is found, these homologues are found together within a cluster of genes that are dedicated to the same biological function, suggesting a potential functional link for the *H. pylori* genes despite their presence in distinct areas of the chromosome.

Both genes encode sugar-nucleotide-modifying enzymes. FlaA1 is a UDP-GlcNAc C-6 dehydratase/C-4 reductase (15, 16), and WbpB is predicted to be an oxidoreductase. Consis-

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tent with the potential functional link between *flaA1* and *wbpB* mentioned above, WbpB might be involved in the reduction of the UDP-4-keto-6-deoxy-GlcNAc intermediate generated by FlaA1. Interestingly, neither the 4-keto intermediate nor its reduced derivative are present in the LPS of *H. pylori* (4, 5, 46). In addition, all genes involved in the biosynthesis of the precursors necessary for LPS assembly have been identified (30, 36, 44) and are distinct from *flaA1* and *wbpB.* Consequently, although it is expected that *flaA1* and *wbpB* might affect LPS synthesis based on sequence homologies, such an effect is not anticipated to occur directly via production of LPS sugar precursors.

The sequence homologies described above also suggested that *flaA1* and *wbpB* might be involved in protein glycosylation. This is relevant since flagellin glycosylation has been detected in *H. pylori* (34, 60) and it is possible that additional proteins—other than the flagellins—are glycosylated, as is the case in the closely related *C. jejuni* (64, 72).

Using a gene disruption strategy, we investigated the biological function of *flaA1* and *wbpB* in *H. pylori.* In light of their homologies to LPS biosynthetic and flagellum modification genes found in other bacteria, the study was focused on the role of both genes on LPS biosynthesis and outer membrane barrier function, as well as on their role in flagellum synthesis and function.

MATERIALS AND METHODS

Bacterial strains and common procedures. The *H. pylori* strains used in this study were 26695, J99, NCTC 11637, and SS1. Unless stated otherwise, all strains were grown for 36 h in brain heart infusion-yeast extract (0.25%)-agar media (BHI-YE) supplemented with 0.05% sodium pyruvate, 10% fetal calf serum, 5 μg of trimethoprim per ml, 4 μg of amphotericin B per ml, and 10 μg of vancomycin per ml. When necessary, selection with 5 μg of kanamycin per ml or 4 μg of chloramphenicol per ml was applied. Incubations were performed at 37°C under microaerophilic conditions using sealed jars and gas packs (CampyGen; Oxoid).

Cloning experiments were performed with *Escherichia coli* DH5α unless stated otherwise, using 30 μg of kanamycin per ml, 34 μg of chloramphenicol per ml, or 100 μg of ampicillin per ml when necessary. Each construct was checked by restriction analysis and sequencing. All kits were used as specified by their manufacturer. DNA sequencing was performed at the Robarts Institute Sequencing Facility (London, Canada).

Cloning and sequencing of the *flaA1* and *wbpB* genes from strains SS1 and NCTC 11637. *wbpB* and *flaA1* were amplified from chromosomal DNA using *Pwo* (Roche) DNA polymerase and primers HPWB5 (GCTCTCCATGGGTA TGCTTTTTGCGATGATTG) and HPWB3 (AAGCAGGATCCTCAAGCCA ATTTGACAGACG) for *wbpB* and Flatop (ACTGTACATGTCAATGCCAA ATCATCAAAAC) and Flabot (AAGCTGGATCCTCATAATAATTTCAAC AAA) for *flaA1.* Both PCR products were cloned in Topo-PCR2.1 (Invitrogen) using Top10F′ cells. The constructs were sequenced on both strands using M13 forward and reverse primers.

Cloning and sequencing of the flagellin genes, *flaA* and *flaB*, from strain NCTC 11637. *flaA* and *flaB* were amplified from chromosomal DNA using primers based on the sequences of HP0601 and HP0115 from strain 26695. Note that *flaA* (HP0601, encoding the flagellin) is distinct from *flaA1* (HP0840, encoding the enzyme under study). The primers were HPFlaAP3 (GCTCTCCAT GGCTTTTCAGGTCAATAC) and HPFlaAP5 (AAGAAGATCTCCTAAGTT AAAAGCCTTAAG) for *flaA* and HP0115P1 (GCTCTCCATGGGCATGAGT FTTAGGATAAATAC) and HP0115P2 (AAGAGGATCCTTATTGTAAAAG CCTTAAGA) for *flaB.* The PCR was performed using an Expand long-range template (Roche) with annealing at 46°C. Both PCR products were cloned in the pET23a derivative (13, 49) with an N-terminal histidine tag using NcoI and BglII for *flaA* and NcoI and BamHI for *flaB.* The constructs were sequenced using T7 promoter primer and primers HPFlagAP3 (GTGAATGATGTAACTTTA GAG) and HPFlagBP3 (CTTATAATGTCATGGCTACC).

Production of anti-flagellin A antibody. Flagellin A was overexpressed from the pET23 construct in BL21(DE3)pLys cells with induction by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C. The protein was purified by metal chelation as described previously (13) in the presence of 6 M guanidine-HCl. The protein was dialyzed in 50 mM ammonium bicarbonate in the presence of 1% sodium dodecyl sulfate (SDS), subjected to microelution from SDS-polyacrylamide gel electrophoresis (PAGE) gels (Bio-Rad Electro-Eluter), and redialyzed in the same buffer. The dialyzate was lyophilized, resuspended in 1.7% saline, and used to immunize rabbits after 1:1 (vol/vol) dilution with incomplete Freund's adjuvant. The immunization schedule and procedure for adsorption of the collected serum against an *E. coli* BL21(DE3)pLys extract were as reported previously (14).

To further enhance the specificity of the serum toward flagellins, the serum was passed through a Blue Sepharose 6 Fast Flow column (Pharmacia Biotech) and purified onto a flagellin A affinity column that was made by coupling purified overexpressed flagellin A to CNBr-activated Sepharose (Pharmacia Biotech) in the presence of guanidine-HCl. After being washed with 1 column volume (CV) of 150 mM glycine (pH 8.9)–350 mM NaCl and after acid shock with 1 CV of 100 mM glycine (pH 3.2), flagellin-specific antibodies were eluted with 1 CV of 1 M ammonium hydroxide and immediately neutralized with 0.09 CV of 0.2 M acetic acid. The recovered antibodies were further adsorbed against a lysate from a *H. pylori* FlaA knockout mutant (see below) as described previously (14).

Preparation of the knockout and complementation constructs. (i) *flaA1* knockout construct. *flaA1* (HP0840) and its potential promoter were PCR amplified from genomic DNA using *Taq* DNA polymerase (Invitrogen) and primers FlaTop2 (GCGAGCGCGAATCTTTAT) and FlaA1Top (ATAGAACCGCTC ACGAGC). The PCR product was cloned into Topo-PCR2.1 to generate TopoFla and was subsequently subcloned into the BamHI and ApaI sites of pBluescript II SK. The construct was digested with NcoI and blunted using T4 DNA polymerase. The kanamycin resistance cassette and its promoter were cut out from pHel3 (27) using SmaI and ligated into the blunted *flaA1*-pBluescript fragment to generate the *flaA1* knockout construct.

(ii) *wbpB* knockout construct. *wbpB* (HP0679) and its potential promoter were PCR amplified from genomic DNA using primers WbpBup (AACAGAGCCC ACGAACGA) and WbpBdown (ATCACGCTTGCGATTGGC) and *Taq* DNA polymerase. The PCR product was cloned into Topo-PCR2.1 to generate TopoWbpB and subcloned into the EcoRI site of pBluescript II SK to generate pBSWbpB. The construct was digested with NsiI and blunted using T4 DNA polymerase. The kanamycin resistance cassette was cut from pHel3 (27) using SmaI and ligated into the blunted pBSWbpB fragment to generate the *wbpB* knockout construct.

(iii) *flaA* knockout construct. Inverse PCR amplification of the *flaA*/pET23 construct was performed with primers HPFlagAP3 (GTGAATGATGTAACTT TAGAG) and HPFlagAP4 (GAACGATGTCAGATTGAATC) and Expand long-range template polymerase (Roche). The PCR product was blunted and dephosphorylated before being ligated to the SmaI-extracted kanamycin cassette as described above.

(iv) Complementation constructs. The EcoRI fragment containing either gene with its promoter was cut out from TopoFla or TopoWbpB, blunted with T4 DNA polymerase, and cloned into the EcoRV-cut pHel2 shuttle vector (27) to generate the complementation constructs pHel2-FlaA1 and pHel2-WbpB.

Southern blotting. Southern blotting was performed using the digoxigenin-labeling method with detection with anti-digoxigenin–alkaline phosphatase–CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate) substrate as specified by the manufacturer (Roche). Chromosomal DNA was extracted from *H. pylori* using DNAzol (Invitrogen) and cut with HaeII/HindIII (*flaA1* mutant) or MaeIII (*wbpB* mutant).

Preparation of knockout mutants and complemented clones. The knockout mutants were generated by electroporation-mediated allelic exchange (63) with modifications described by McGee et al. (43) and selection on kanamycin. Potential transformants were analyzed for gene integration by PCR and Southern blotting. Complementation constructs were introduced into the mutants by electroporation, with selection onto kanamycin and chloramphenicol. The plasmids were extracted from the complemented strains and subjected to restriction analysis to ensure their integrity. Transformants harboring intact plasmids were selected for further studies.

LPS analysis. LPS prepared as described previously (28) was analyzed on SDS-PAGE (15% polyacrylamide) or 10 to 20% Tricine gradient (Novex) gels. Detection was performed by silver staining (24) or Western blotting with anti-Lewis Y (Calbiochem) or anti-lipid A monoclonal antibodies (19).

Preparation of soluble cell extracts. Soluble extracts were obtained by lysing *H. pylori* cells harvested from one BHI-YE plate in 100 μl of breaking buffer (20

mM sodium phosphate [pH 7.5], 1 mM EDTA) with acid-washed glass beads (Sigma G-4649). After being vortexed three times for 30 s each, the cells were pelletted for 10 min at 12,000 × *g* at room temperature and the supernatant was used for SDS-PAGE analysis.

Protein deglycosylation. Enzymatic deglycosylation of flagellum preparations obtained by glycine extraction (50) was performed under denaturing conditions using five different enzymes as provided in the Calbiochem glycoprotein deglycosylation kit (no. 362280).

MS identification of proteins. Protein bands cut out from Coomassie-stained gels were subjected to in-gel trypsinolysis. The peptides were analyzed by liquid chromatography mass spectrometry (MS) (Q-TOF2) and MALDI-MS at the Dr. Don Rix Protein Identification Facility of the University of Western Ontario.

RT-PCR. RNA extracted using the RNeasy kit (Qiagen) was treated with RNase-free DNase I (Invitrogen) and subjected to reverse transcription (RT) using Superscript II RNase H^- reverse transcriptase (Invitrogen) and random hexanucleotide primers (Roche). PCR (15 cycles) was subsequently performed with *Taq* DNA polymerase at an annealing temperature of 52°C and elongation time of 45 s, using gene-specific primers. The primers were Fla363 (GCTATC AGTCAGGTTATCGC) and Fla550 (ACGGCACCACGCTCCCAC) to amplify a 187-bp fragment of *flaA1*, HPWB8 (CAGAACACATGGGAGTAGC) and HPWB7 (GCCGTCCGAGCGCCAATTTGACAGACGC) to amplify a 171-bp fragment of *wbpB*, and HP1045P1 (GTCATTATCTATATGCCCAT) and HP1045P2 (CTGGCTTGAGCATGTAAGG) to amplify a 200-bp fragment from HP1045. The PCR products were analyzed on 2% agarose gels with ethidium bromide staining.

SDS, bile salts, and novobiocin sensitivity assays. Harvested bacteria were diluted to an optical density at 600 nm (OD_{600}) of 1 in BHI-YE. A 30-μl volume of cell suspension was added to 175 μl of BHI-YE supplemented with the appropriate antibiotics and/or detergents, with concentrations up to 15 μg/ml for novobiocin, 0.02% for SDS, and 0.18 mg/ml for bile salts (50% sodium cholate, 50% sodium deoxycholate (Sigma) (29). The assays were performed three times with independent cultures in 96-well plates incubated for 4 to 5 days with agitation at 37°C under microaerophilic conditions. On each plate, each strain was tested in duplicate. A representative example of the SDS-sensitivity experiment is provided in Fig. 6.

Sensitivity of *H. pylori* NCTC 11637 to serum killing. Assays were performed as described by Bacon et al. (6), using 0, 0.1, 0.5, 4, and 10% fresh or heat-inactivated (60 min, 56°C) rabbit serum in saline.

Motility assays. Bacteria were harvested from a 2-day-old plate into 400 μl of BHI-YE or from a 1-ml overnight liquid culture, diluted 1:10 in BHI-YE saturated with 10% CO_2–85% N_2–5% O_2, and allowed to grow for 7.5 h. The cultures were then diluted to an OD_{600} of 0.3 or 0.9 and were used to inoculate motility plates (0.4% agar in BHI-YE) by stabbing. Each dilution was spotted in triplicate, and dilutions were made in triplicate for each strain. The growth of the swimming halo was monitored after 4 days of incubation at 37°C. The remaining diluted culture was used to inoculate regular BHI-YE plates to estimate bacterial viability by colony counting and microscopic observation.

Suspension-clearing assays. Liquid cultures (1 ml) in BHI-YE saturated with 10% CO_2–85% N_2–5% O_2 were prepared by inoculation from 2-day-old plates. After overnight growth, the cultures were diluted 1:10 in 2 ml of BHI and grown again for 18 to 24 h. The cultures were then diluted to an OD_{600} of 1 (total volume, 2 ml) in BHI and left to sit at room temperature. The rate of suspension clearance was measured over 8 h by carefully removing 70 μl from the top of the suspension and measuring the OD_{600} using a microcell (path length, 1 cm). Two independent experiments using two dilutions of each strain were performed.

Microscopic observations. Cells harvested from plates or liquid cultures were examined as wet mounts under a phase-contrast microscope (Zeiss; oil immersion, ×400 magnification). For electron microscopy, cells were harvested from 1- to 2-day-old plates and resuspended in 1% glutaraldehyde in 20 mM HEPES buffer (pH 7). They were analyzed in the negative staining mode using 1% uranyl acetate.

Urease activity assay. A 10-μl volume of a bacterial suspension at an OD_{600} of 0.4 in saline was mixed with 100 μl of 0.33 M urea–0.001% phenol red in 0.005 M sodium phosphate–0.15 M NaCl (pH 6.7) (11). Urease activity was assayed by monitoring the OD_{565} over time in a 96-well plate. All experiments were done in triplicate. The data were normalized to the total amount of proteins present in the sample (Bio-Rad assay).

Growth curves. Bacterial suspensions (1 ml at an OD_{600} of 0.055) in BHI-YE were grown in 24-well plates with agitation under microaerophilic conditions, and the OD_{600} was monitored over 70 h. Data were recorded from four different plates with two repeats per strain on each plate. The data presented (see Fig. 2A) are the average of all the readings.

TABLE 1. Conservation of the FlaA1 and WbpB proteins among several strains of *H. pylori*

H. pylori strain	FlaA1 protein		WbpB protein	
	Size (aa)[a]	Identity (%)[b]	Size (aa)	Identity (%)
26695	333	100	289	100
J99	333	98	315	96
NCTC 11637	333	98	315	95
SS1	333	99	315	97

[a] aa, amino acids.
[b] Identity with respect to 26695 (set at 100%).

Acid resistance assays. For acid shock resistance assays, 10 μl of bacterial suspension at an OD_{600} of 9 in BHI-YE was diluted in 90 μl of PBS buffer adjusted to different pHs and containing 0 to 20 mM of urea in a 96-well plate. After 1 h of incubation at 37°C under microaerophilic conditions, 15 μl of the bacterial suspension was transferred to 195 μl of BHI-YE broth and the bacteria were incubated for 3 to 5 days before the OD_{600} was recorded. The data presented (see Fig. 3, left panels) are the average of two experiments, with duplicates for each condition tested in each experiment.

For long-term acid exposure, 75 μl of bacterial suspension at an OD_{600} of 1 in BHI-YE was added to 1 ml of BHI-YE adjusted to pH 5, 6, or 7 with 0 or 10 mM urea in 24-well plates. Bacterial growth was measured at 20 and 39 h by monitoring the OD_{600}. The assay was performed twice with independent cultures, with two plates per experiment and duplicates for each condition tested on each plate. The data presented (see Fig. 3, right panels) are the average of the results obtained at 39 h.

Nucleotide sequence accession numbers. The GenBank accession numbers for the genes in this study are as follows: *flaA1* (NCTC 11637), AY319294; *flaA1* (SS1), AY319295; *wbpB* (NCTC 11637), AY319297; *wbpB* (SS), AY319296; *flaA* (NCTC 11637), AY319298; and *flaB* (NCTC 11637), AY319299.

RESULTS

The *flaA1* and *wbpB* genes are highly conserved and expressed in several strains of *H. pylori*. Southern blotting experiments performed using the *flaA1* and *wbpB* genes from strain 26695 as probes indicated that strains SS1 and NCTC 11637 contained a single homologue of each gene (data not shown). After PCR amplification and DNA sequencing, the derived protein sequences were compared with those of strains 26695 and J99 (2, 65) (Table 1). Overall, the four strains harbored nearly identical FlaA1 and WbpB proteins. The only noticeable difference between strains was that WbpB from strain 26695 was slightly shorter than all its homologues.

RT-PCR indicated that the *flaA1* and *wbpB* genes were expressed in laboratory strains SS1, 26695, and NCTC 11637 (Fig. 1), since RT-PCR products of the expected size were obtained only in the presence of reverse transcriptase. Preliminary data obtained using formalin-fixed and paraffin-embedded human gastric biopsy specimens indicated that both genes were also expressed in the context of infection in the natural host (data not shown).

General characteristics of *flaA1* and *wbpB* knockout mutants. On plates, the *flaA1* mutant grew less rapidly than the *wbpB* mutant, which, in turn, grew slightly less rapidly than the wild-type strain. This was not dependent on the presence of kanamycin in the growth media. In broth, all strains exhibited similar growth patterns, and the same maximal culture densities could be obtained after 40 h of growth (Fig. 2A). Interestingly, a significant decrease in OD_{600} was observed for the *flaA1* mutant after 40 to 50 h of growth, suggesting that, in

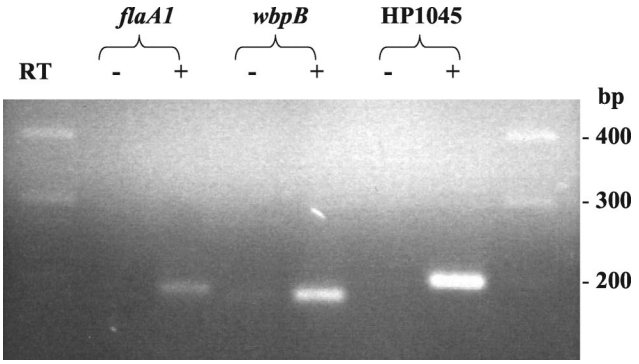

FIG. 1. RT-PCR analysis of expression of *flaA1* and *wbpB* in strain NCTC 11637. HP1045, encoding acetyl coenzyme A synthetase, was used as a positive control for constitutive expression. RT, reverse transcriptase. Signals obtained only in the samples where reverse transcriptase was added indicate expression of the genes of interest. Similar results were obtained with strains 26695 and SS1 (data not shown).

contrast to the wild type, this mutant did not survive in the stationary phase. This could explain the lower growth observed on plates for this mutant. Both mutants still produced urease as judged by the urea-phenol red assay (11). However, the levels of urease produced by the *flaA1* mutant were much lower than those produced by the *wbpB* mutant or by wild-type bacteria (Fig. 2B).

Like the wild type, both mutants were able to resist acid shock in the absence of urea at pH $\geq$ 3 (Fig. 3A to C, bars a). The addition of urea allowed resistance to acid shock of all strains at pH 2 (bars b to e). This indicated that the limited urease activity of the *flaA1* mutant was sufficient to support viability at pH 2. At pH $\geq$ 7, addition of urea became deleterious. In the *flaA1* mutant, this toxic effect inherent to the chaotropic nature of urea appeared at lower urea concentrations (15 mM) (Fig. 3B, bars d and e) than for the wild type and for all pHs of >2. This was consistent with the lower levels of urease activity shown by this mutant (Fig. 2B).

Like wild-type bacteria, both mutants resisted long-term acid exposure in the absence of urea (Fig. 3D to F, bars a). The slight decrease in viability observed for all strains at pH 5 could be eliminated by the addition of 10 mM urea (bars c), indicating again that urease activity supports acid resistance under these conditions too.

The *flaA1* and *wbpB* knockout mutants exhibit altered LPS profiles. SDS-PAGE analysis of wild-type and mutant LPS revealed that the mutants had lost most of their O antigen (Fig. 4A). The *wbpB* mutant produced slightly more Lewis Y-containing O antigen than did the *flaA1* mutant. Also, the O chains present in the *wbpB* mutant appeared slightly shifted compared with the wild-type bands (Fig. 4B), suggesting that the core to which the O antigen was attached might have a slightly different structure. When the core LPS was analyzed on higher-resolution 10 to 20% gradient Tricine gels, the wild-type strain exhibited two well resolved bands, bands 1 and 2 (Fig. 4C). In contrast, in both mutants, the fast-migrating band (band 1) was missing and the slower-migrating band (band 2) was not affected. In addition, a third band (band 3) migrating slightly slower than band 2 appeared in the *wbpB* mutant only. The presence of band 3 was not dependent on the amount of sample loaded on the gel.

Quantitative analysis performed by anti-Lewis Y and anti-lipid A Western immunoblots confirmed that the *flaA1* mutant produced very little, if any, Lewis Y O antigen and that the *wbpB* mutant produced more Lewis Y O antigen (hatched bar) than the *flaA1* mutant but less than the wild type (Fig. 5A and C). It also revealed that the *wbpB* mutant produced high-molecular-weight O antigen that reacted with anti-lipid A antibody (Fig. 5B and C, white bars) but not with anti-Lewis Y antibody (hatched bars). This material could correspond to other Lewis antigens, which differ from Lewis Y by the number and position of fucose residues (40, 68).

Taken together, these data showed that the inactivation of either *flaA1* or *wbpB* affected LPS synthesis in a similar, although not exactly identical, fashion. It did not affect the syn-

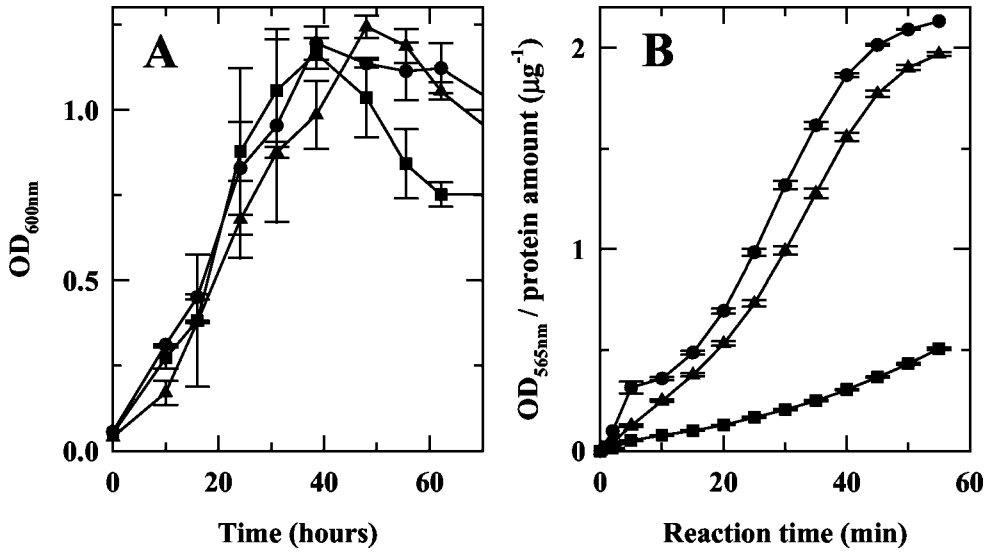

FIG. 2. Effect of *flaA1* or *wbpB* disruption on growth rate (A) and urease activity (B). Circles, wild type; squares, *flaA1*::*kan*; triangles, *wbpB*::*kan*.

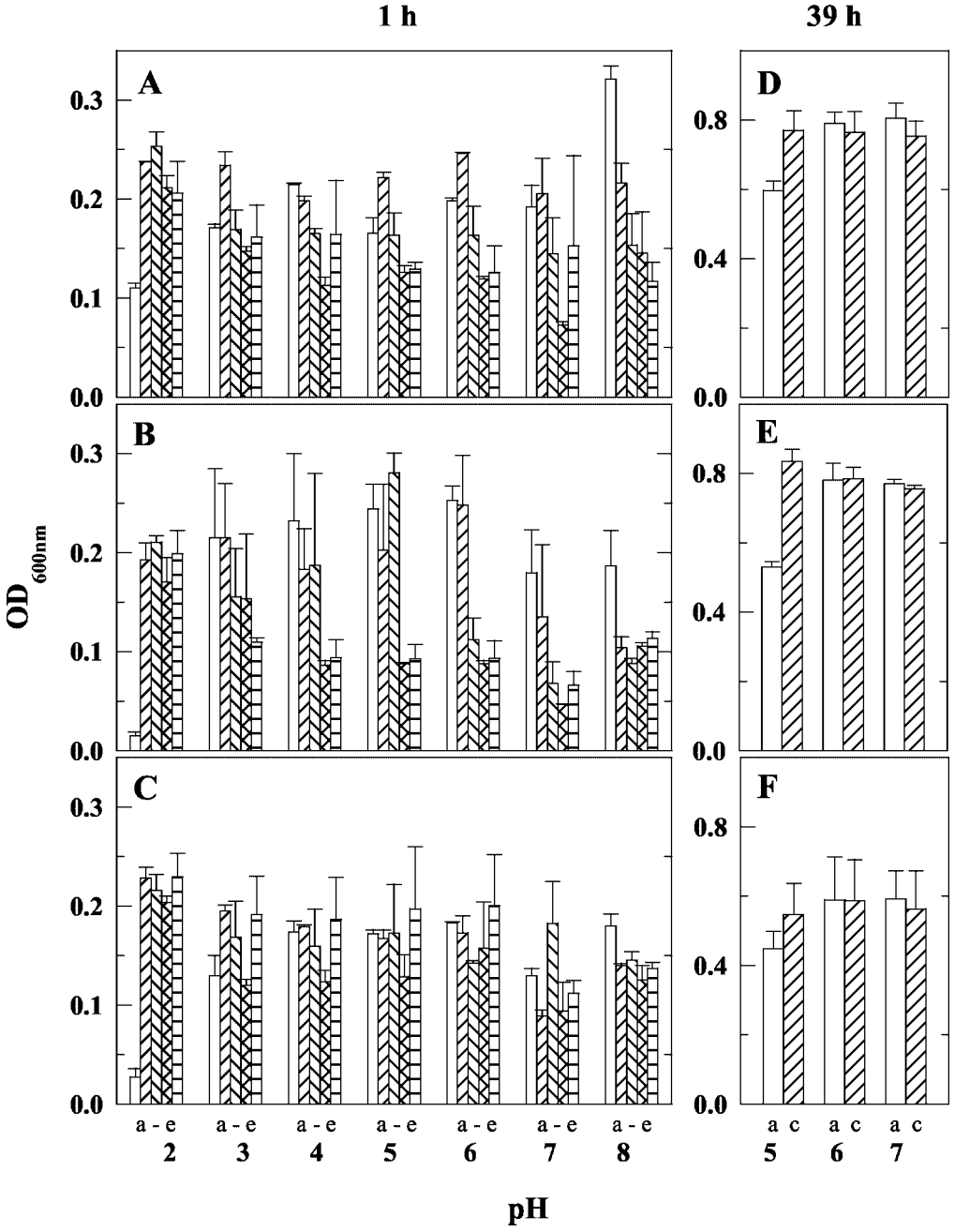

FIG. 3. Effect of gene disruption on the ability of *H. pylori* to resist acid shock (A to C) and long-term acid exposure (D to F) in the presence of variable amounts of urea (a, b, c, d, and e correspond to 0, 5, 10, 15, and 20 mM urea, respectively). (A and D) wild type; (B and E) *flaA1*::*kan*; (C and F) *wbpB*::*kan.*

thesis of LPS precursors per se since low levels of O antigen could still be detected in each mutant. However, it might have affected the transfer of the O units to the lipid A acceptor, as suggested by analysis of the core LPS, and, to a lesser extent, the activity of the transferases responsible for the synthesis of the O units, as shown by the presence of non-Lewis Y O antigens in the *wbpB* mutant.

Reintroducing the functional gene in *trans* partly restored the production of the O antigen in each mutant (Fig. 5), indicating that the loss of O-antigen production observed in each mutant was gene specific and not due to polarity effects. The copy number and poor stability of the complementation plasmid in strain NCTC 11637 might explain why complementation was not complete. In the *flaA1*-complemented *flaA1* mutant, the O antigen produced was composed of Lewis Y-containing species, as in the wild type (white and hatched bars of similar size). Interestingly, in the *wbpB*-complemented *wbpB* mutant, the amount of Lewis Y-containing O antigen did not increase (hatched bar) but its proportion of the total O antigen (detected with anti-lipid A [white bar]) did increase, so that all O

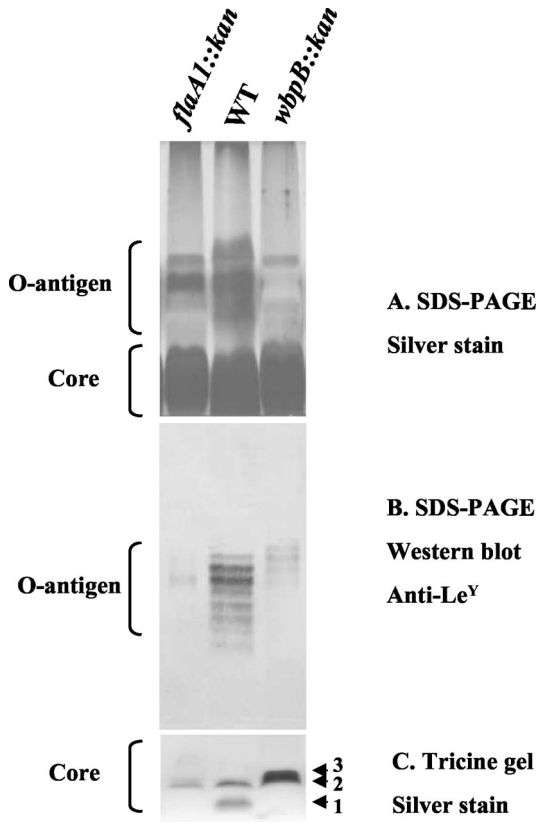

FIG. 4. Gel electrophoresis analysis of the LPS of the *flaA1* and *wbpB* mutants of *H. pylori* strain NCTC 11637. The LPS were analyzed on SDS-PAGE (12% polyacrylamide) gels (A and B) or 10 to 20% Tricine gradient gels (C) and detected by silver staining (A and C) or Western immunoblotting (B) with anti-Lewis Y antibody (Calbiochem). Only 1/10 of the amount of sample used for the SDS-PAGE gels was loaded on the Tricine gel to allow for good resolution of core bands. WT, wild type.

antigen produced was of the Lewis Y type, as in the wild type. Hence, qualitatively, reintroducing the functional *wbpB* gene into the *wbpB* mutant also restored a wild-type like O-antigen production made exclusively of Lewis Y O antigens. The identity of the Lewis Y-reacting band that appears below the O antigen in the *flaA1*::*kan* + *wbpB* and *flaA1*::*kan* lanes (Fig. 5A) is unknown, but it does not appear to be LPS related since it did not react with anti-lipid A antibody.

Disruption of *flaA1* or *wbpB* affects the barrier properties of the outer membrane. Defects in LPS synthesis often correlate with higher sensitivity of bacteria to killing by serum, detergents (SDS or bile salts) (29), or hydrophobic antibiotics (novobiocin) (67) and with decreased virulence properties in animal models (56, 70). Hence, the sensitivity of the *flaA1* and *wbpB* mutants to each of these compounds was investigated.

The extreme sensitivity to serum of the NCTC 11637 strain used for these studies (7) prevented us from detecting any significant differences between the wild type and mutants. However, a significantly higher sensitivity of the mutants than of the wild type to SDS (Fig. 6A) and a slight increase in sensitivity to novobiocin and bile salt were observed (data not shown). These results indicate the higher accessibility of the outer membrane to antibiotic or detergents and its reduced stability, which is consistent with decreased O-antigen production.

Providing the functional gene in *trans* restored wild-type-like SDS sensitivity in each mutant (Fig. 6B and C), demonstrating the gene specificity of the increased SDS sensitivity phenotype.

***flaA1* can cross-complement the *wbpB* inactivation.** We investigated whether the bifunctional dehydratase/reductase FlaA1 could complement the disruption of the putative reductase activity of WbpB by monitoring both the LPS production by and SDS sensitivity of cross-complemented mutants. Providing a functional copy of *flaA1* in *trans* in the *wbpB* mutant did restore wild-type-like SDS sensitivity to the *wbpB* mutant (Fig. 6C). This correlated with an increased production of Lewis Y-containing O antigen in this complemented mutant (Fig. 5, hatched bars). This indicated that FlaA1 and WbpB have a redundant reductase activity and might be involved in the same biochemical pathway. However, providing *wbpB* in the *flaA1* mutant did not restore wild-type levels of SDS sensitivity (Fig. 6B) or O-antigen production (Fig. 5) to the *flaA1* mutant, indicating that the complementation effect observed previously was gene specific. This is consistent with the fact that WbpB is predicted to be monofunctional (reductase only) and cannot complement the lack of dehydratase function of the *flaA1* mutant.

The *flaA1* and *wbpB* disruptions abrogate flagellum-mediated motility. On examination by phase-contrast microscopy, the wild-type bacteria were motile and exhibited corkscrew movements. In contrast, the *flaA1* and *wbpB* mutants only oscillated and showed no corkscrew movement.

Motility assays performed with bacteria in soft agar showed a clear reduction of motility on disruption of the *flaA1* and *wbpB* genes (Table 2). The results were not dependent on the length of incubation or on the density or nature (from broth or plate) of the inoculum.

To further demonstrate the effects of the mutations on bacterial motility, suspension-clearing assays were performed. The wild-type strain exhibited good stability in the suspension, since the OD_{600} only decreased slightly over time (Fig. 7). In contrast, the mutants settled rapidly. These data show that disruption of *flaA1* or *wbpB* resulted in a similar phenotype whereby the ability of the bacteria to maintain themselves in suspension by active movement was impaired. Taken together, the data presented above indicate that both mutants have impaired motility.

Flagellum production is abrogated in the *flaA1* mutant but not in the *wbpB* mutant. The wild-type and mutant strains were examined by electron microscopy to investigate if the gene disruptions had any effect on the production of flagella. The typical unipolar sheathed flagella of the wild-type strain could be readily detected by negative staining (Fig. 8A). In contrast, the *flaA1* mutant was devoid of flagella, indicating that the production and/or assembly of flagellins was impaired in this mutant (Fig. 8B). The *wbpB* mutant still produced flagella (data not shown).

The *flaA1* mutant still produces flagellins. To examine whether the lack of flagellum production observed in the *flaA1* mutant was due to the lack of flagellin production or to a defect in their export and assembly, soluble cell extracts of the *flaA1* mutant were compared with cell extracts from the wild

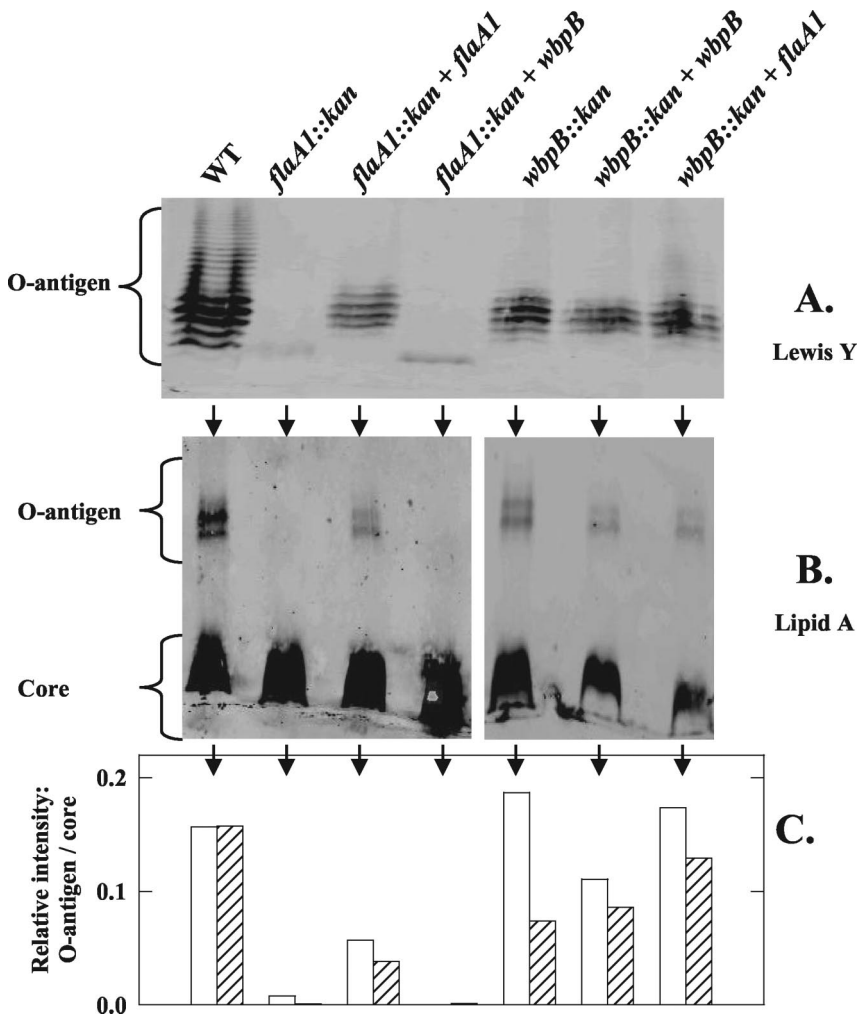

FIG. 5. (A and B) SDS-PAGE analysis of the LPS of knockout mutants and complemented strains detected by Western immunoblotting using anti-Lewis Y (A) or anti-lipid A (B) antibodies. Detection was performed using goat anti-mouse immunoglobulin G conjugated with Alexa Fluor 680 and scanning with a Li-Cor Odyssey infrared imaging system. The anti-Lewis Y antibody detected the O antigen exclusively whereas the anti-lipid A antibody detected the lipid A associated both with the O antigen and with the core LPS. (C) Quantitative data were obtained by normalizing the amount of O antigen detected with either antibody (open bars, anti-Lipid A; hatched bars, anti-Le^Y) by the amount of core LPS detected with the anti-Lipid A antibody. WT, wild type.

type and the *wbpB* mutant in an anti-flagellin Western blotting assay.

First, we demonstrated that strain NCTC 11637 produces two flagellins, FlaA and FlaB, which were >99.5% identical to flagellins found in strain 26695 and 57.2% identical to one another. NCTC 11637 FlaA was 53.3 kDa in size, and FlaB was only slightly larger (54.0 kDa).

Second, we demonstrated that both flagellins could be readily detected by Western immunoblotting using a polyclonal antiserum raised in rabbits against overexpressed and purified NCTC 11637 FlaA flagellin. This was consistent with their high levels of similarity at the protein level. Note that in the gel system used, the two flagellins were not resolved from one another.

Third, the anti-FlaA antibody was used to investigate flagellin production in wild-type and mutant *H. pylori* strains. Figure 9 shows that even after adsorption against an *E. coli* lysate, the anti-FlaA antibody detected several proteins in wild-type *H. pylori* soluble extracts (bands a to d). Band c (53 kDa) had the strongest reactivity with the anti-FlaA antibody and migrated to the same position as pure overexpressed flagellins (data not shown), indicating that it corresponds to the flagellin(s) FlaA and/or FlaB. Band c was not affected by the *flaA1* or *wbpB* disruption, indicating that both mutants still produced flagellin(s), including the aflagelatted *flaA1* mutant. Hence, the data suggest that the export and/or assembly of the flagellins is impaired in the *flaA1* mutant.

Band a (66 kDa) was shown to correspond to the large subunit of urease, UreB, by MS analysis. The cross-reactivity of the anti-FlaA antibody with UreB could be explained by the existence of a 113-amino-acid stretch where the two proteins exhibit 54% homology. Interestingly, a strong reduction in the amount of band a was observed in the *flaA1* mutant only. This was consistent with the reduction in urease activity described above for this mutant (Fig. 2B). MS analysis also indicated that the reduction in UreB production was accompanied by a re-

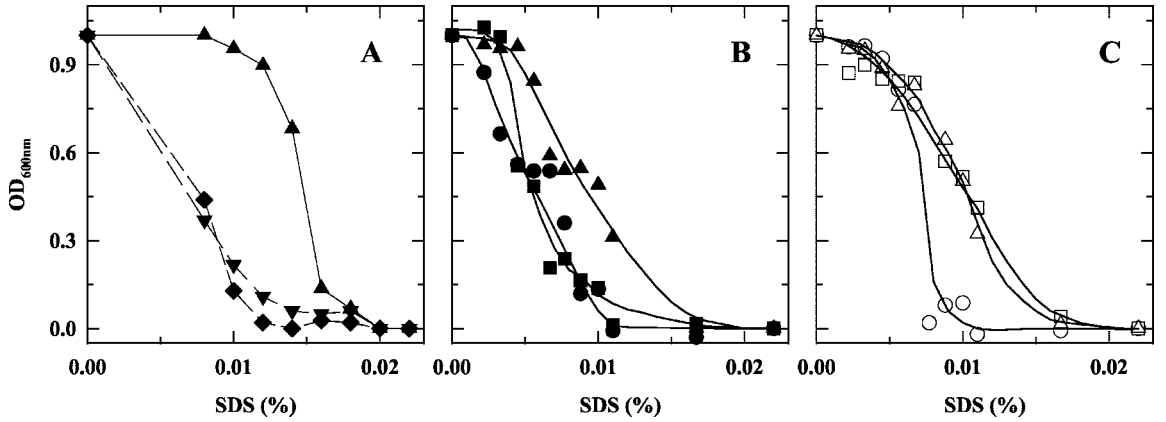

FIG. 6. SDS sensitivity of the wild-type, mutant, and complemented strains. To compare wild-type and mutant strains, the assay was performed in the presence of kanamycin. Resistance to kanamycin was provided by the pHel3 vector in the wild-type strain. Similar results were obtained when the experiment was performed in the absence of kanamycin, using the plasmid-free wild-type strain (data not shown). To compare the mutants and the complemented strains, the assays were performed in the presence of kanamycin and chloramphenicol. In this case, mutants harboring the pHel2 vector were used as references. (A) ▲, wild type plus pHel3; ▼, *flaA1* mutant; ◆, *wbpB* mutant. (B) ●, *flaA1* mutant plus pHel2; ▲, *flaA1* mutant plus pHel2-*flaA1*; ■, *flaA1* mutant plus pHel2-*wbpB*. (C) ○, *wbpB* mutant plus pHel2; □, *wbpB* mutant plus pHel2-*wbpB*; △, *wbpB* mutant plus pHel2-*flaA1*.

duction in the production of the small subunit of urease, UreA, as observed on Coomassie-stained gels (band e, 31 kDa). The production of bands a and e was restored in the complemented mutant, indicating that the effect was gene specific.

MS analysis indicated that band d contained multiple proteins unrelated to flagellins. Since this band was not affected by the *flaA1* or *wbpB* disruptions, its analysis was not pursued further.

Finally, the production of band b (58 kDa) was abrogated in the *flaA1* mutant only, and the effect was gene specific since band b reappeared in the *flaA1*-complemented *flaA1* mutant (Fig. 9). Considering that several strains of *H. pylori* harbour glycosylated flagellins (34, 60), the possibility that band b might correspond to posttranslationally modified flagellins was investigated by MS analysis. However, contamination of band b with catalase (58.6 kDa) that was present both in the wild type and in the *flaA1* mutant (a negative control that does not contain band b) prevented us from demonstrating the presence of flagellins within band b. The experiments below aim at clarifying this question by refining the purification and detection of the flagellins and by investigating the glycosylation status of the flagellins in the band b-producing wild-type and *wbpB* mutant strains.

TABLE 2. Effect of the inactivation of *flaA1* and *wbpB* on the motility of *H. pylori* NCTC 11637 as measured by the soft agar plate motility assay[a]

OD_{600}	Diam[b] (mm) of motility halo of strain:		
	WT	*fla1*::*kan*	*wbpB*::*kan*
0.3	9 ± 1	2 ± 1	3 ± 0
0.9	10 ± 2	3 ± 1	4 ± 1

[a] The motility plates were inoculated with two different dilutions of bacterial culture and were typically read after 4 days of growth at 37°C. Longer incubation times did not result in larger motility halos. All cultures exhibited similar viability when plated on BHI-YE. WT, wild type.

[b] Results are expressed as means ± standard deviations.

The flagellins of wild-type *H. pylori* strain NCTC 11637 are not glycosylated. To examine the glycosylation status of the NCTC 11637 flagellins, flagellins obtained by glycine extraction were analyzed by Western immunoblotting as well as Coomassie and Ponceau staining (Fig. 10). Three major bands (bands A to C) in the size range of interest were detected by Coomassie (Fig. 10C) or Ponceau (Fig. 10A) staining. As described above (Fig. 9), band A is thought to be UreB. A significant reduction in reactivity of the serum with band A was obtained using serum that had been purified by affinity on a flagellin A column and adsorbed against a lysate of a urease-

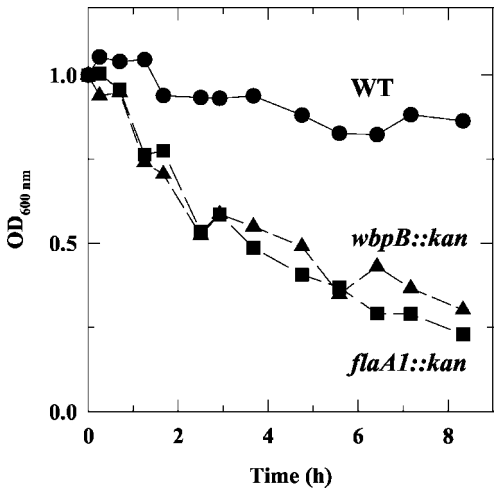

FIG. 7. Suspension-clearing assays comparing the abilities of the wild-type (WT) and mutant strains to maintain themselves dispersed in suspension by active swimming. Aliquots of a bacterial suspension were withdrawn at regular intervals from the top of the suspension, which was maintained without agitation under microaerophilic conditions, and the OD_{600} was recorded as an estimation of the cell density. This is a representative example of three independent experiments performed with different cultures.

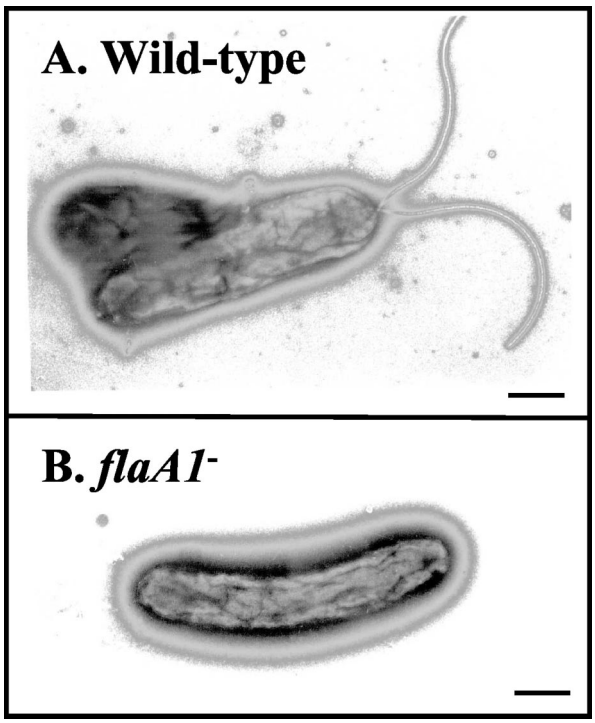

FIG. 8. Electron micrographs of wild-type (A) and *flaA1* mutant (B) strains obtained with 1% uranyl acetate staining. The sheathed unipolar flagella were easily detected in the wild-type strain as well as in the *wbpB* mutant (data not shown), but the *flaA1* mutants were devoid of flagella. Bar, 0.5 μm.

producing *H. pylori* flagellin A mutant (compare the intensity of band B in Fig. 10B and D).

Band C migrated at the expected size for FlaA and FlaB, as seen using purified (nonglycosylated) overexpressed flagellins as controls, and reacted strongly with the anti-FlaA antiserum (Fig. 10B and D), confirming that it corresponds to FlaA and/or FlaB. Consistent with this assignment, band C was very faint in the *flaA* mutant (Fig. 10B), since this mutant produces only the minor flagellin FlaB. Note that although affinity purification and adsorption of the antibody against the *H. pylori flaA* mutant cell extract (Fig. 10B) significantly increased its specificity toward flagellins, it resulted in a significant titer loss as indicated by the lower reactivity of the serum toward purified flagellins (compare Fig. 10D and B) and by the very weak detection of the flagellins in soluble extracts (compare Fig. 10B with Fig. 9B).

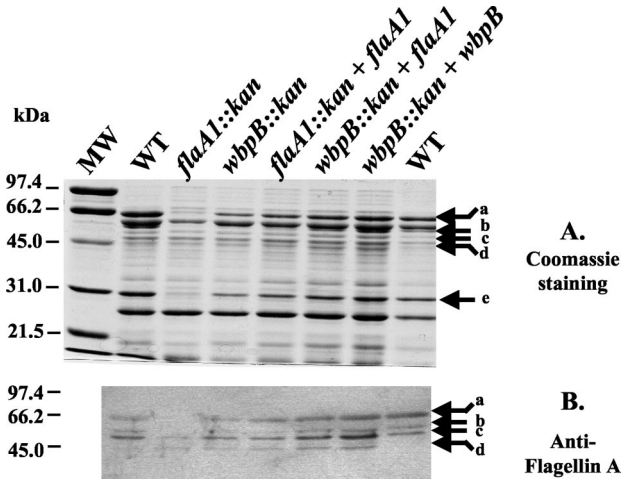

FIG. 9. SDS-PAGE analysis of soluble cell extracts of wild-type (WT), mutant, and complemented strains by Coomassie staining (A) and anti-FlaA Western immunoblotting (B). The gels contained 8% acrylamide. The identities of the bands labeled a to e are described in Results. MW, molecular mass standards.

Enzymatic deglycosylation using a combination of five different enzymes had no effect on the migration or amount of band C, indicating that it corresponds to nonglycosylated flagellins. *C. jejuni* glycosylated flagellines prepared and treated under the same conditions were included as a positive control and showed a significant shift in size after deglycosylation. Band B could be readily deglycosylated enzymatically (Fig. 10C). However, since it did not react with the anti-FlaA antibody and did not comigrate with band C after deglycosylation, it does not correspond to glycosylated flagellins. Note that no equivalent of the anti-FlaA-reacting band b from Fig. 9 was observed in glycine extracts, indicating that the glycine extraction method eliminated cross-reacting bands that were not flagellum related.

Overall, this analysis excludes the existence of any glycosylated flagellins in the wild-type strain and suggests that the *wbpB* mutant produces wild-type-like flagellins. However, these data did not determine whether the lack of motility of the *wbpB* mutant could be due to a lack of production of one of the two flagellins since FlaA and FlaB could not be resolved on these gels, and even after adsorption against a *flaA* knockout mutant, the anti-FlaA antibody still detected both flagellins without discrimination (Fig. 10B). Attempts at refining the purity of the flagellins by ultracentrifugation/acid dissociation and affinity chromatography to answer this question by direct MS analysis were unsuccessful due to the precipitation and therefore the loss of the flagellins.

DISCUSSION

The goal of this study was to determine the biological function of two novel genes encoding sugar-nucleotide-modifying enzymes, *flaA1* and *wbpB*, in *H. pylori.* Whereas *flaA1* exhibits homologues in numerous medically relevant bacteria, *wbpB* is a rather rare gene with only two significant homologues in bacterial genomes. The data provided in this study showed that both genes were conserved and expressed (Fig. 1; Table 1) in all *H. pylori* strains examined, suggesting an important and conserved function. Each gene could be inactivated in strain NCTC 11637 by insertion of a kanamycin resistance cassette, indicating that neither of them was essential in this strain. This is in contrast to a recent report indicating that disruption of HP0840 (FlaA1) might be lethal in other strains (60).

The two genes are distant from one another on the *H. pylori* chromosome (2, 65). The similarity of the phenotypes observed after disruption of either gene suggested that they belonged to the same functional pathway despite the absence of genetic linkage. Indeed, each gene affects LPS synthesis, outer membrane barrier function, and flagellum-mediated motility. However, subtle differences in the phenotypes of each mutant could

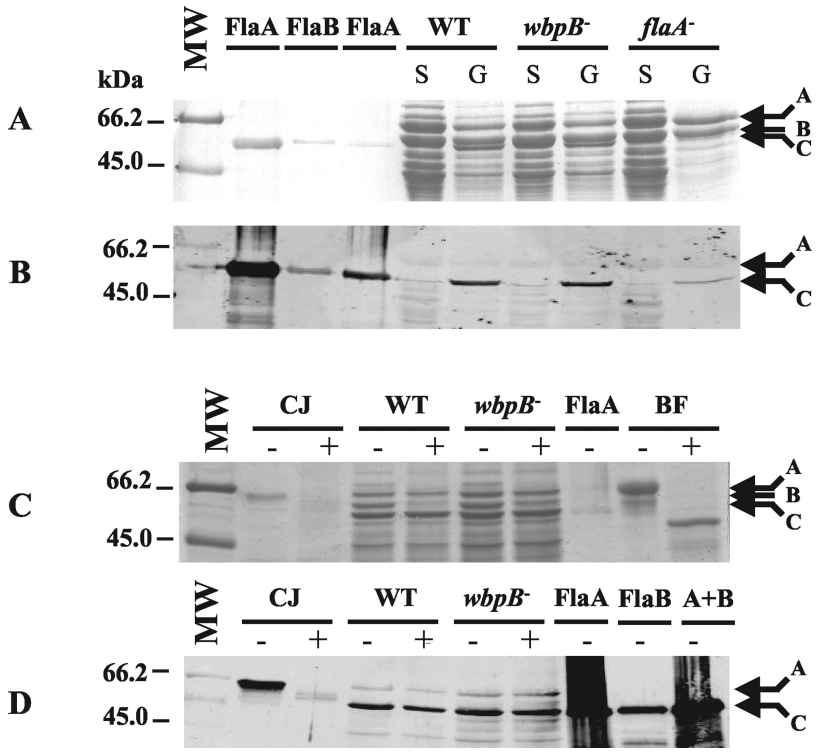

FIG. 10. Analysis of the wild-type (WT) and *wbpB* mutant flagellins by SDS-PAGE and Western immunoblotting. (A and B) Optimization of the sample preparation method showing enrichment of the samples in the flagellin band (band C) in glycine extracts (G) compared to soluble extracts (S); Ponceau red staining (A) and Western immunoblotting with anti-FlaA serum (B) after affinity purification and adsorption against an *H. pylori* flagellin A (*flaA*$^-$) knockout mutant were used. (C and D) Enzymatic deglycosylation of flagellum preparations obtained by glycine extraction. + and − indicate the presence and absence of enzymatic deglycosylation, respectively. Coomassie staining (C) and Western immunoblotting with anti-FlaA antibody adsorbed against an *E. coli* lysate (D) were used. *C. jejuni* (CJ, strain 81–176) flagellum preparations obtained under the same conditions and bovine fetuine (BF) were used as positive controls for deglycosylation. Note that the anti-FlaA antibody readily detects *C. jejuni* glycosylated flagellins. All gels contained 10% acrylamide, and overexpressed and purified flagellins (FlaA and FlaB) were used as positive controls independently or as a mixture (A+B). MW, molecular mass standards.

be observed, and the phenotypes were proven to be gene specific by complementation experiments.

The functional link between the two genes is consistent with the fact that in the few bacteria that harbor a homologue for each gene, both genes were found within a single operon and participated in the same biological function (1, 9). This is also consistent with their biochemical activities, which might be partly complementary. Indeed, the unidirectional complementation of the disruption of *wbpB* by *flaA1* supports the hypothesis that this functional complementation involves the common reductase activity associated with each enzyme. While this awaits further biochemical evidence, the reason for such redundancy of the reductase activity is currently not clear. The chromosomally encoded copy of *flaA1* only partially complemented the *wbpB* mutation (Fig. 5 and 6C), so that the phenotype observed in the *wbpB* mutant was intermediate between those of the wild type and the *flaA1* mutant (with the presence of flagella and higher levels of residual O antigens in the *wbpB* mutant). Full complementation of the *wbpB* mutation was observed only when multiple copies of *flaA1* were provided using a complementation vector. This suggests that the reductase activity of FlaA1 is not very efficient, so that there is no actual functional redundancy in vivo. In addition, 4-keto sugar derivatives can be regarded as "pluripotent," since they can undergo various enzymatic modifications (52, 62, 71). Thus, the 4-keto-6-deoxy-UDP-GlcNAc generated by FlaA1 might be involved in multiple biosynthetic pathways and its reduction by WbpB might serve as a channeling mechanism to prevent its diversion toward other pathways. Because the *wbpB* gene is fairly rare in the bacterial world but is conserved and expressed in all *H. pylori* strains examined, the pathway at stake might be related to specific features of the biology and/or pathogenesis of *H. pylori.*

LPS structure (4, 5, 46) and biochemical data (15, 30, 36, 44) indicated that FlaA1 and WbpB were not LPS biosynthetic enzymes per se in *H. pylori.* Disruption of their genes nevertheless significantly reduced O-antigen production and resulted in the formation of altered core LPS (Fig. 4). In the absence of structural information for these mutants, it is reasonable to assume that band 1 observed on Tricine gels could correspond to the core and that the slower-migrating band 2 could correspond to the "core plus one O-chain unit." Band 3, which migrates only slightly slower than band 2, could represent a "core plus one" structure that carries an additional sugar residue on the terminal galactose of the O-chain unit. This additional sugar residue is likely to be L-fucose since the resulting difucosylated lactosamine motif is commonly found as a terminal structure in the *H. pylori* LPS (40, 46). This incor-

poration of an extra residue would explain the altered reactivity of the O chain produced by the *wbpB* mutant with the anti-Lewis Y antibody (Fig. 4). The activity of the transferase responsible for addition of this terminal sugar residue must be impaired or down-regulated in the *flaA1* mutant so that no band 3 is present in this mutant and all O chains produced are of the Lewis Y type.

Both mutants were nonmotile (Fig. 7; Table 2), and the lack of motility correlated directly with the lack of flagellum production in the *flaA1* mutant whereas the *wbpB* mutant still produced wild-type-like sheathed flagella. Western immunoblotting showed that flagellin(s) was produced by the *flaA1* mutant, suggesting that it was the export or assembly of the flagellins into a flagellum that was impaired in this mutant. Flagellin O glycosylation has been found in other strains of *H. pylori* (34, 60) and has been proposed to be concomitant with its export to the bacterial surface (34). Considering our observation that the O-glycosylation sites described in the flagellin sequence of strain 1061 were conserved in the flagellins of strain NCTC 11637 and considering that glycosylation of surface appendage within one bacterial species is often strain dependent (3, 20, 59), we investigated if flagellins were also glycosylated in strain NCTC 11637. Our results obtained using enzymatic deglycosylation treatment and Western immunoblotting indicated that the flagellins of wild-type strain NCTC 11637 were not glycosylated. Thus, a defect in the posttranslational modification of the flagellins cannot be responsible for the lack of production of flagella in the *flaA1* mutant or for the lack of functionality of the *wbpB* mutant flagella.

It has been reported previously that *H. pylori* mutants lacking the major flagellin, FlaA, do not produce flagella whereas mutants lacking the minor flagellin, FlaB, produce nonfunctional flagella (63). The possibility that FlaA would be missing in the *flaA1* mutant and would prevent flagellum assembly cannot be excluded since the gel system and antibody used for these studies did not allow for discrimination between both flagellins. Similarly, the possibility that FlaB would be missing from the *wbpB* mutant could explain the presence of nonfunctional flagella. Alternatively, the lack of motility of the *wbpB* mutant might be due to a defect within a structural component of the basal body or motor of the flagella that has yet to be identified.

The defects in LPS production, membrane barrier properties, and flagellum-mediated motility associated with inactivation of *flaA1* or *wbpB* suggested that both genes might be important for the virulence of *H. pylori.* In addition, the down-regulation of urease production observed in the *flaA1* mutant (Fig. 2B and 9, bands a and e) might further compromise its survival in vivo, although the residual levels of urease produced were sufficient to sustain acid resistance in vitro (Fig. 3). Hence, FlaA1 and WbpB could represent valuable targets for the development of novel therapeutic agents against *H. pylori.* Since inactivating these enzymes abolishes the production of several virulence factors, it is unlikely that resistance to FlaA1- or WbpB-specific inhibitors could arise easily, in contrast to what is observed with currently available antibiotics (18, 31, 41). Mouse colonization assays are under way to investigate whether inactivation of *flaA1* or *wbpB* results in defects in host colonization or survival in vivo.

The effect of a single gene on the synthesis of several virulence factors has been demonstrated previously (10, 20, 39) and involves direct incorporation of specific sugars within the structure of different virulence factors. In contrast, the sugars produced by FlaA1 (and WbpB) are not integral part of the virulence factors affected by disruption of either gene. We propose that these sugars are instead part of glycosylation motifs that target proteins involved in LPS or flagellum assembly and function and regulate their activity. Such regulation of protein function by their glycosylation has been observed previously in other bacterial species (17, 53, 55).

Several of the *C. jejuni* N-glycosylated proteins carry bacillosamine, a 2,4-diacetamido-2,4,6-trideoxyglucopyranose (72). Based on the biochemical function of FlaA1 (15) and considering the close relationship between *H. pylori* and *C. jejuni*, we propose that bacillosamine-dependent protein glycosylation also occurs in *H. pylori* and that FlaA1 is the first enzyme responsible for bacillosamine biosynthesis. While this work was in progress, it was shown that FlaA1 could complement the function of CJ1293, a homologue found in *C. jejuni*, and that CJ1293 was involved in the production of flagella (25). We also demonstrated that CJ1293 has the same biochemical activity as FlaA1 and performs a UDP-GlcNAc C-6 dehydration consistent with the first step of bacillosamine biosynthesis (12). Hence, these new data corroborate our hypothesis that FlaA1, like CJ1293, is involved in bacillosamine biosynthesis. Potentially glycosylated enzymes involved in LPS and flagellum synthesis are currently being analyzed in the context of the *flaA1* mutation to confirm this hypothesis.

In summary, we demonstrate herein that FlaA1 and WbpB are functionally linked in the dual control of LPS biosynthesis and flagellum production and/or function in *H. pylori* and propose that this dual control involves protein glycosylation. This work is the first report to establish the existence of a regulatory network connecting the LPS and flagellum biosynthesis machineries and suggests that FlaA1- and WbpB-dependent protein glycosylation might be the underlying mechanism.

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