

Functional and Topological Characterization of Novel Components of the *comB* DNA Transformation Competence System in *Helicobacter pylori*

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Helicobacter pylori is one of the most diverse bacterial species known. A rational basis for this genetic variation may be provided by its natural competence for genetic transformation and high-frequency recombination. Many bacterial competence systems have homology with proteins that are involved in the assembly of type IV pili and type II secretion systems. In *H. pylori*, DNA uptake relies on a transport system related to type IV secretion systems (T4SS) designated the *comB* system. The prototype of a T4SS in *Agrobacterium tumefaciens* consists of 11 VirB proteins and VirD4, which form the core unit necessary for the delivery of single proteins or large nucleoprotein complexes into target cells. In the past we identified proteins ComB4 and ComB7 through ComB10 as being involved in the process of DNA uptake in *H. pylori*. In this study we identified and functionally characterized further (T4SS-homologous) components of the *comB* transformation competence system. By combining computer prediction modeling, experimental topology determination, generation of knockout strains, and genetic complementation studies we identified ComB2, ComB3, and ComB6 as essential components of the transformation apparatus, structurally and functionally homologous to VirB2, VirB3, and VirB6, respectively. *comB2*, *comB3*, and *comB4* are organized as a separate operon. Thus, for the *H. pylori comB* system, all T4SS core components have been identified except for homologues to VirB1, VirD4, VirB5, and VirB11.

Helicobacter pylori, a highly motile gram-negative bacterial pathogen, is the principal cause of chronic active gastritis and peptic ulcer disease in humans and has been implicated in the development of gastric mucosa-associated lymphoid tissue lymphoma and adenocarcinoma (38, 49). *H. pylori* colonizes a very special habitat at the surface of gastric epithelial cells or in the mucus layer covering the epithelium, which suggests that this bacterium has evolved specialized features for gastric adaptation. A comparison of the two published genome sequences shows considerable diversity in gene content, with about 7% of all putative genes being strain specific (2). The panmictic population structure of *H. pylori* is believed to result from frequent recombination during mixed colonization by unrelated strains (20, 50).

H. pylori is naturally competent for genetic transformation (36). Natural transformation competence in bacteria is a complex process, involving DNA binding, uptake/translocation, and recombination. In general, bacterial competence systems have homology with proteins that are involved in the assembly of type IV pili and type II secretion systems and form a structure that partially spans the cell envelope (see reference 13 for a recent review). In *H. pylori*, DNA uptake is not mediated by a type IV pilus-based apparatus but relies on a transport sys-

tem related to type IV secretion systems (T4SS) designated the *comB* system (26).

The Vir system of *Agrobacterium tumefaciens*, necessary for the transfer of its transferred DNA (T-DNA) into plant cells, is considered the prototype for type IV secretion (10). It is assembled from the products of the *virB* operon (the VirB1 through VirB11 proteins) and the *virD4* gene, which form a cell envelope-spanning channel for the translocation of the substrate. Three putative ATPases, VirD4, VirB4, and VirB11, are involved in this process. The transmembrane channel itself, connecting the inner and outer membranes, is thought to be composed of the proteins VirB7 through VirB10; the properties and interactions between these proteins and the substrate have been studied in detail (11, 43). The polytopic inner-membrane protein VirB6 is considered to form a pore or channel at the cytoplasmic membrane, and the pilin subunit, VirB2, assembles as the T pilus in association with VirB5 and the VirB7 lipoprotein. In addition to the *virB* system, *A. tumefaciens* also carries two other T4SS, the *trb* (32) and the *avhB* (14) systems. The *trb* system encodes functions required for the conjugal transfer of the Ti plasmid between *Agrobacterium* spp., whereas the *avhB* T4SS mediates the conjugal transfer of the pAtC58 cryptic plasmid between cells of *A. tumefaciens*.

The *H. pylori* ComB proteins are named after their orthologues from the *A. tumefaciens* VirB system. As known so far, they consist of five open reading frames (ORFs): *comB4*, encoding a putative ATPase, and *comB7* through *comB10*, supposed to be structural components of the membrane-spanning

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics ^a	Reference or source
<i>H. pylori</i> strains		
P1	<i>H. pylori</i> wild type, naturally competent	26
P12	<i>H. pylori</i> wild type, naturally competent	25
J99	Wild-type, genome-sequenced strain	2
26695	Wild-type, genome-sequenced strain	52
P149	Wild-type, Mongolian gerbil-adapted strain	29
P224	P12; deletion of <i>hp0015</i> (<i>comB2</i>), <i>cat</i> insertion	This study
P225	P12; deletion of <i>hp0016</i> (<i>comB3</i>), <i>cat</i> insertion	This study
P226	P1; deletion of <i>hp0015-hp0017</i> , <i>aphA-3</i> insertion	This study
P227	P1; deletion of <i>hp0037</i> (<i>comB6</i>), <i>cat</i> insertion	This study
P228	P1; deletion of region spanning <i>hp0037</i> (<i>comB6</i>)- <i>hp0042</i> (<i>comB10</i>)	This study
P229	P1Δ <i>hp0015-hp0017</i> complemented with pHel2 plasmid carrying <i>comB2-comB4</i> (pAK20)	This study
P230	P1Δ <i>hp0015-hp0017</i> complemented with pHel2 plasmid carrying <i>comB3-comB4</i> (pAK21)	This study
P231	P1Δ <i>hp0015-hp0017</i> complemented with pHel2 plasmid carrying <i>comB4</i> (pAK22)	This study
P232	P1Δ <i>hp0037-hp0042</i> complemented with pHel2 plasmid carrying <i>comB6-comB10</i> (pAK24)	This study
P233	P1Δ <i>hp0037-hp0042</i> complemented with pHel2 plasmid carrying <i>comB7-comB10</i> (pDHO46)	This study
P234	P149[<i>hp1421::TnHK9</i>] Tn insertion at codon 20	29
<i>E. coli</i> strains		
CC118	Δ(<i>ara-leu</i>)7697 Δ <i>lacX74</i> Δ <i>phoA20</i> <i>galE galK thi rpsE rpoB argE</i> (Am) <i>recA1</i>	31
β2155	<i>thrB1004 pro thi strA hsdS lacZΔM15</i> (F' <i>lacZΔM15 lacI^r traD36 proA⁺ proB⁺</i>) Δ <i>dapA::erm</i> (Erm ^r) <i>pir::RP4</i> [:: <i>kan</i> (Km ^r) from SM10]	17
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^rZΔM15</i>] Tn10 (Tet ^r)	Stratagene
Plasmids		
pRB67	pMin2 plasmid carrying <i>phoA</i> without start codon	This study
pAK03	pBA with region <i>hp0014-hp0018</i> , primers AK04-AK05	This study
pAK04	pBA with <i>hp0015::cat</i> and flanking regions	This study
pAK05	pBA with <i>hp0016::cat</i> and flanking regions	This study
pAK07	pBA with region <i>hp0036-hp0038</i> , AK02-AK03	This study
pAK08	pBA with <i>hp0037::cat</i> and flanking regions	This study
pAK09a	pRB67 with <i>hp0037</i> fragment AK12-AK13 (34 aa)	This study
pAK09b	pRB67 with <i>hp0037</i> fragment AK12-AK14 (62 aa)	This study
pAK09c	pRB67 with <i>hp0037</i> fragment AK12-AK15 (115 aa)	This study
pAK09d	pRB67 with <i>hp0037</i> fragment AK12-AK16 (198 aa)	This study
pAK09e	pRB67 with <i>hp0037</i> fragment AK12-AK23 (236 aa)	This study
pAK09f	pRB67 with <i>hp0037</i> fragment AK12-AK18 (296 aa)	This study
pAK19	pBluescriptIISK <i>comB2-comB4</i> deletion plasmid, <i>aphA-3</i>	This study
pAK20	pDH80, <i>comB2-comB4</i>	This study
pAK21	pDH80, <i>comB3-comB4</i>	This study
pAK22	pDH80, <i>comB4</i>	This study
pAK23	pMin1, <i>comB6-comB10</i> deletion plasmid, <i>aphA-3</i>	This study
pAK24	pDH80, <i>comB6-comB10</i>	This study
pDHO36	pMin1, <i>comB7-comB10</i> deletion plasmid, <i>aphA-3</i>	17
pDHO46	pDH80, <i>comB7-comB10</i>	25

^a aa, amino acids.

secretion pore (24, 25). ComB8 through ComB10 are tightly associated with the membrane, probably forming a bridge between the inner and outer membranes (24). ComB8, ComB9, and ComB10 are essential for transformation competence, whereas ComB7 is not but seems to stabilize the ComB protein complex (25). In addition to the *comB* system, which seems to be universally present in all *H. pylori* strains, a subgroup of *H. pylori* strains is equipped with a second T4SS, the *cag* system, which is dedicated to the translocation of the effector protein CagA into eukaryotic cells. The *cag* and the *com* systems are functionally independent (25, 27). Certain *H. pylori* strains carry a third T4SS, named *tf33* (30).

A number of additional proteins not classified as ComB proteins seem to be involved at different steps in the transformation competence process, such as the cytoplasmic proteins RecA (42) and DprA (3, 46), the inner-membrane protein

ComE3 (53), and the secreted protein ComH (45). The goal of this study was to identify additional components of the T4SS-based ComB DNA uptake machinery. We therefore started a computer-based in silico search for typical type IV-homologous proteins missing in the *comB* system. By this approach we identified putative homologues of VirB2, VirB3, and VirB6, which we named ComB2, ComB3, and ComB6, respectively, and we verified their function in the process of natural transformation competence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. For routine culture, *H. pylori* strains (Table 1) were grown on GC agar plates (Difco) supplemented with vitamin mix (1%), horse serum (8%), vancomycin (10 mg/liter), trimethoprim (5 mg/liter), and nystatin (1 mg/liter) (serum plates). Inoculated plates were incubated for 24 to 48 h under microaerobic conditions (85% N₂, 10% CO₂, 5% O₂) at 37°C.

Escherichia coli strains HB101 (8) and DH5 α (Bethesda Research Laboratories) were grown on Luria-Bertani (LB) agar plates or in LB liquid medium (40) supplemented with ampicillin (100 mg/liter), chloramphenicol (30 mg/liter), or kanamycin (40 mg/liter), as appropriate. For conjugation experiments from *E. coli* into *H. pylori*, strain β 2155 (17) was used grown on the same media supplemented with diaminopimelic acid (0.2 mM). For quantification of *phoA* activity, *E. coli* CC118 (35) was used.

DNA manipulations and plasmid and strain constructions. Cloning and DNA analysis procedures were performed according to Sambrook et al. (40). Chromosomal DNA from *H. pylori* was isolated with the QIAamp tissue kit (QIAGEN, Hilden, Germany). Plasmid DNA was purified from *E. coli* either by the boiling procedure or with the QIAprep Spin Miniprep kit (QIAGEN, Hilden, Germany).

The Δ *comB2-comB4* (*hp0015-hp0017*) mutant P226 was generated by transformation of plasmid pAK19 into *H. pylori* strain P1. To establish plasmid pAK19, two gene fragments, one upstream of *hp0015* and another downstream of *hp0017*, were amplified with primers AK37-AK38 and AK39-AK40 and cloned into the vector pBluescriptIISK. Next, an *aphA-3* resistance gene cassette was inserted between those two gene fragments. The plasmid was introduced into *H. pylori* strain P1 by natural transformation and integrated into the chromosome via homologous recombination, which was verified by PCR. For genetic complementation of mutant P226, plasmids pAK20, pAK21, and pAK22 were introduced by electroporation. The plasmids contained different parts of the deleted genes *hp0015* to *hp0017*, which were cloned into pDH80 (23) with primers AK49, AK50, AK51, and AK52. pAK20 carried the whole deleted region *hp0015* to *hp0017*, pAK21 contained genes *hp0016* and *hp0017*, and pAK22 harbored *hp0017* only.

For construction of the single-gene-deletion mutants P224 (Δ *hp0015*) and P225 (Δ *hp0016*), a gene fragment containing *hp0015-hp0016* and about 500 bp upstream and downstream was amplified with primers AK04 and AK05 and cloned into vector pBA, creating plasmid pAK03. Using primers AK06, AK07, AK08, and AK09, the vector backbone except *hp0015* and *hp0016* was PCR amplified. A *cat* cassette without a terminator was introduced to replace gene *hp0015* or *hp0016*, resulting in plasmids pAK04 and pAK05, respectively. The plasmids were transferred into strain P12 by natural transformation. Correct chromosomal integration was verified by PCR.

As described for the deletion mutants P224 and P225, the single-gene mutant P227 (Δ *hp0037*) was also created by cloning a PCR fragment with primers AK02 and AK03 containing *hp0037* together with flanking regions in vector pBA (pAK07). After amplifying the vector with flanking regions of *hp0037* with primers AK19 and AK20, a *cat* cassette without a terminator was introduced instead of *hp0037*, resulting in plasmid pAK08, which was subsequently transformed in *H. pylori* P12.

The creation of the Δ *comB6-comB10* mutant P228 was achieved by PCR amplification of a gene fragment upstream of *hp0037* with primers AK59 and AK65. This fragment was introduced by using EcoRI restriction sites into EcoRI-digested pDHO36 (25), resulting in pAK23. Successful integration in the *H. pylori* chromosome by homologous recombination resulted in the replacement of genes *hp0037* to *hp0042* by an *aphA-3* resistance cassette. Genetic complementation of deletion mutant P228 was performed starting from shuttle plasmid pDHO46. Using an internal NdeI restriction site in *hp0037*, the amplified N-terminal part of this gene was integrated with primers AK57 and AK58, resulting in plasmid pAK24. Shuttle plasmid pAK24 contained genes *hp0037* to *hp0042*. The plasmid was introduced into mutant strain P228 via electroporation. The deletion mutant P228 was also complemented with unmodified pDHO46 in an analogous way to obtain P233. The mutant gene *hp1421* used in this study was an insertion mutant obtained by using TnHK9, as previously described (29).

Electroporation, natural transformation competence, and conjugation of *H. pylori*. Transformation of *H. pylori* strains was performed with plasmid or chromosomal DNA according to a procedure described previously (22). Bacteria were harvested from serum plates and suspended to an optical density at 550 nm (OD₅₅₀) of 0.1 in brucella broth containing 10% fetal calf serum. DNA was added (1 μ g), and incubation was extended for 5 h under microaerophilic conditions before the suspension was plated on selective serum plates.

Conjugation of the shuttle vector pHel2 carrying the *comB* genes from *E. coli* to *H. pylori* was performed essentially as described previously (23). Growth of the *dapA* mutant β 2155 is strictly dependent on exogenously supplied diaminopimelic acid; hence, the removal of diaminopimelic acid provides an efficient counterselection against this donor.

For electroporation of *H. pylori*, bacterial cells were harvested from serum plates and suspended in 1 ml of cold phosphate-buffered saline (PBS) solution. For each electroporation, bacteria were diluted to an OD₅₅₀ of 1 and 1 ml bacterial suspension was washed twice with PBS and suspended first in 500 μ l

and then in 40 μ l electroporation buffer (44). Forty microliters of *H. pylori* competent cells was mixed with 1 to 2 μ l DNA in prechilled 0.2-cm electroporation cuvettes. Electroporation was performed at 2.5 kV, 200 Ω , and 25 μ F by a Gene Pulser (Bio-Rad, Munich, Germany). After electroporation, 1 ml of brucella broth–10% fetal calf serum was added to each sample. The aliquots were incubated for 4 h in a CO₂ incubator before being plated on selective agar plates.

Quantification of PhoA and green fluorescent protein (GFP) fluorescence. Alkaline phosphatase activities of fusion proteins were determined by partial gene sequences of *comB6* amplified with primers AK12, AK13, AK14, AK15, AK16, AK18, and AK23, which were cloned in front of a *phoA* gene without a start codon in the modified pMin2 vector pRB67 (R. Buhrdorf et al., submitted). The plasmids (pAK09a to pAK09f [Table 1]) were transformed into *E. coli* strain CC118, and the alkaline phosphatase activities were measured as described previously (34).

For analysis of the GFP activities of the ComB6 fusion proteins, the same PCR products that were described for the alkaline phosphatase assay were cloned into the pBluescriptIIKS vector in front of a *gfp* gene without a start codon. The fusion constructs were under the control of the *lac* promoter. Constitutively expressed *gfp* fusions were found to be toxic in *E. coli* DH5 α ; therefore, the plasmids were transferred into *E. coli* XL1-Blue, and the *lac* promoter was repressed by the addition of 2% glucose in the medium. For the activity assay, the cells were grown in 2 ml LB medium supplemented with 2% glucose with ampicillin, up to an OD₅₅₀ of about 0.8. The cells were then washed with PBS and resuspended in fresh LB medium with ampicillin and 100 μ M isopropyl- β -D-thiogalactoside instead of glucose. After growth for another 2 to 4 h, the cells were harvested. One hundred microliters of a suspension in PBS with an OD₅₅₀ of 1.0 together with dilutions was applied to a 96-well plate, and the GFP activities were measured with Fuji FLA 3000 (Raytest, Straubenhardt, Germany).

For both activity assays, at least three different measurements were performed. To exclude negative results due to sequencing errors, the gene constructs of low-activity fusion proteins were sequenced.

RNA extraction, cDNA synthesis, and RT-PCR. Total RNA was extracted from *H. pylori* (wild-type [wt] and mutant strains) using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. The RNA yield, purity, and integrity were determined by measuring the absorbance ratio at 260/280 nm (ratio, >1.8) and checked with a 1% agarose-formaldehyde gel. The RNA samples were digested with DNase I (Roche Diagnostics, Mannheim, Germany). For cDNA synthesis, 800 ng of each RNA sample was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) and gene-specific primers (Table 2). The absence of genomic DNA was verified by PCR using untreated RNA as a template. Reverse transcription (RT)-PCR was carried out using PowerScript polymerase (PAN Biotech GmbH) and the oligonucleotide primers listed in Table 2.

RESULTS

In silico identification of novel components of the ComB transformation competence system. Increasing amounts of published sequence data about T4SS during the last few years have revealed that the organization of these operons could differ significantly, but it seems that the two gene clusters consisting of *virB2* to *virB4* and *virB6* to *virB10* often maintain a unit. The *comB* transformation competence system consists of the genes *comB4* and *comB7* to *comB10*. To identify further components of the *comB* DNA uptake machinery, we analyzed the regions upstream and downstream of the *comB* operon by intensive database comparison of individual ORFs. The criteria used for this analysis were (i) their position relative to the already known basic T4SS genes *comB4* and *comB7* to *comB10*, (ii) a similar secondary structure prediction of the putative ORFs, and (iii) sequence identity of the predicted ORFs to their corresponding VirB/Trb/AvhB counterparts of *A. tumefaciens*. This search resulted in the identification of three putative *virB*-related ORFs encoding proteins homologous to VirB2 (HP0015), VirB3 (HP0016), and VirB6 (HP0037). The putative *comB2* (*hp0015*) and *comB3* (*hp0016*)

TABLE 2. Oligonucleotide sequences used in this study

Use	Primer	Sequence
cDNA synthesis and PCR	CH80	5'-TCAAAAAGTGATAGAACTAGCG-3'
	CH82	5'-GATTAGCGTGTCTTTGG-3'
PCR	AK02	5'-CGGGCTCGAGATCCACTCATTAGCGGAG-3'
	AK03	5'-GAAGATCTCGCCCAATGAGCGAACG-3'
	AK04	5'-GCCGCTCGAGGGGGTGTGTAACAATTC-3'
	AK05	5'-GGGGATCCCCATTAATGTATTCCGC-3'
	AK06	5'-GCCGCTCGAGCAAAAACCCCTTCTGTTAA-3'
	AK07	5'-GCGGATCCAGCGAATTGGTTTATGGG-3'
	AK08	5'-GCCGCTCGAGTAAAAAATCCCATAAAC-3'
	AK09	5'-GCGGATCCGTTATAGAGCGTAGAATG-3'
	AK12	5'-ACCGCTCGAGCTTTAAGAAGGAGATACATATGAAAAATGACGCTTATG-3'
	AK13	5'-GCGGATCCCGCATGCAAAGTGTAGAG-3'
	AK14	5'-GCGGATCCAGCGCTGAAGAAATCTG-3'
	AK15	5'-GCGGATCCAGAAAAGTTACTCAAGCT-3'
	AK16	5'-GCGGATCCTAGGCATATAACCACAAC-3'
	AK18	5'-GCGGATCCTGGGGTCGTGATGTATTG-3'
	AK19	5'-GCCGCTCGAGCAATACTTCAACGACTT-3'
	AK20	5'-GCGGATCCAGGAATTTAATGAGAATT-3'
	AK23	5'-GCGGATCCTAAGTCTTGTTTTCTTG-3'
	AK37	5'-CCGCTCGAGTATCGCTTTAGGCTATGCTA-3'
	AK38	5'-GCATCGATCAAAAACCTTCTGTTAATT-3'
	AK39	5'-GGAATTCGCACTCCTATTCAGATGGCT-3'
	AK40	5'-ACGAGCTCCGGCTCTTTACCATCTCAA-3'
	AK49	5'-ATGCCATATGTCCGCTCATTTTTTAAA-3'
	AK50	5'-ATGCCTCGAGAGCCATCTGAATAGGAGTGC-3'
	AK51	5'-ATGCCATATGATTATCCTGTCCAGCGAG-3'
	AK52	5'-CGGCCATATGTTAGAAAAGCTTTTAAAG-3'
	AK57	5'-CAGCATATGTAAAGTCCGTTGAAGTATTG-3'
	AK58	5'-TCTTGCATATGAGCGTCTA-3'
	AK59	5'-GGAATTCGAAGAACATCATAAGCGTTT-3'
	AK65	5'-CAGAATTCCAATACTTCAACGACTTT-3'
	CH77	5'-CCAAAAGAACACGCTAATC-3'
	CH78	5'-ATGGAGAAAAAATCACTGG-3'
	CH79	5'-TTCTTCACGCTCGCTG-3'
	CH81	5'-TAGTAGGCATGTGCGTTTC-3'
CH83	5'-GCAAGAGTTTTTAGGTTTTG-3'	

genes are located directly upstream of *comB4* (*hp0017*) (Fig. 1), whereas the putative *comB6* gene (*hp0037*) is positioned directly upstream of the *comB7*-to-*comB10* operon (*hp0038*-*hp0042*) (Fig. 1B). Using ConPred II, which is a prediction server of transmembrane (TM) topology based on a consensus approach by combining the results of several proposed methods (4), three TM regions were predicted for HP0015, with the first TM domain having the property of a Sec-dependent signal peptide. Therefore, HP0015 has a secondary structure similar to VirB2. ConPred II predicted one central TM domain for HP0016 (Fig. 1), a characteristic shared with VirB3. For HP0037, several TM domains, including a Sec-dependent signal peptide, are predicted, similar to VirB6, suggesting that it is as well a polytopic inner-membrane protein. Direct amino acid sequence comparison revealed limited primary sequence conservation between AvhB2/ComB2 (38.6% similarity and 27.2% identity over 104 amino acids) and VirB3/ComB3 (34.7% similarity and 19.5% identity over 46 amino acids) (Fig. 1). The best primary amino acid sequence homology of HP0037 was found with *A. tumefaciens* TrbL (34.1% similarity and 21.2% identity over 108 amino acids), which is in the range of similarity and identity usually observed between T4SS-homologous proteins in different bacterial species. In summary, with this bioinformatics approach we were able to identify

three additional putative components of the *comB* DNA uptake machinery.

Identification and functional characterization of *hp0015* and *hp0016* as essential genes for *H. pylori* natural transformation competence. Next, we were interested in verifying whether or not HP0015 or HP0016 is essential for transformation competence in *H. pylori*. Therefore, in *H. pylori* strain P12 precise chromosomal deletions of *hp0015* and *hp0016* were generated separately by homologous recombination with plasmids pAK04 and pAK05, respectively (Fig. 2A; Table 1). The ORFs were replaced by a promoterless and terminatorless chloramphenicol acetyltransferase gene (*cat*), which usually does not cause a polar effect on downstream genes. The correct deletions were verified by PCR (data not shown). To test the individual P12Δ*hp0015* (P224) and P12Δ*hp0016* (P225) mutant strains for their natural transformation competence, they were transformed with (i) plasmid pDH29 carrying an *H. pylori* *recA* gene interrupted by an erythromycin resistance gene cassette (*erm*) or (ii) chromosomal DNA carrying a streptomycin resistance mutation (*str*) (21). Each of the deletions resulted in complete abrogation of natural transformation competence for plasmid as well as chromosomal DNA in the corresponding mutant strains (transformation frequency, $<10^{-9}$) (Table 3; Fig. 2A).

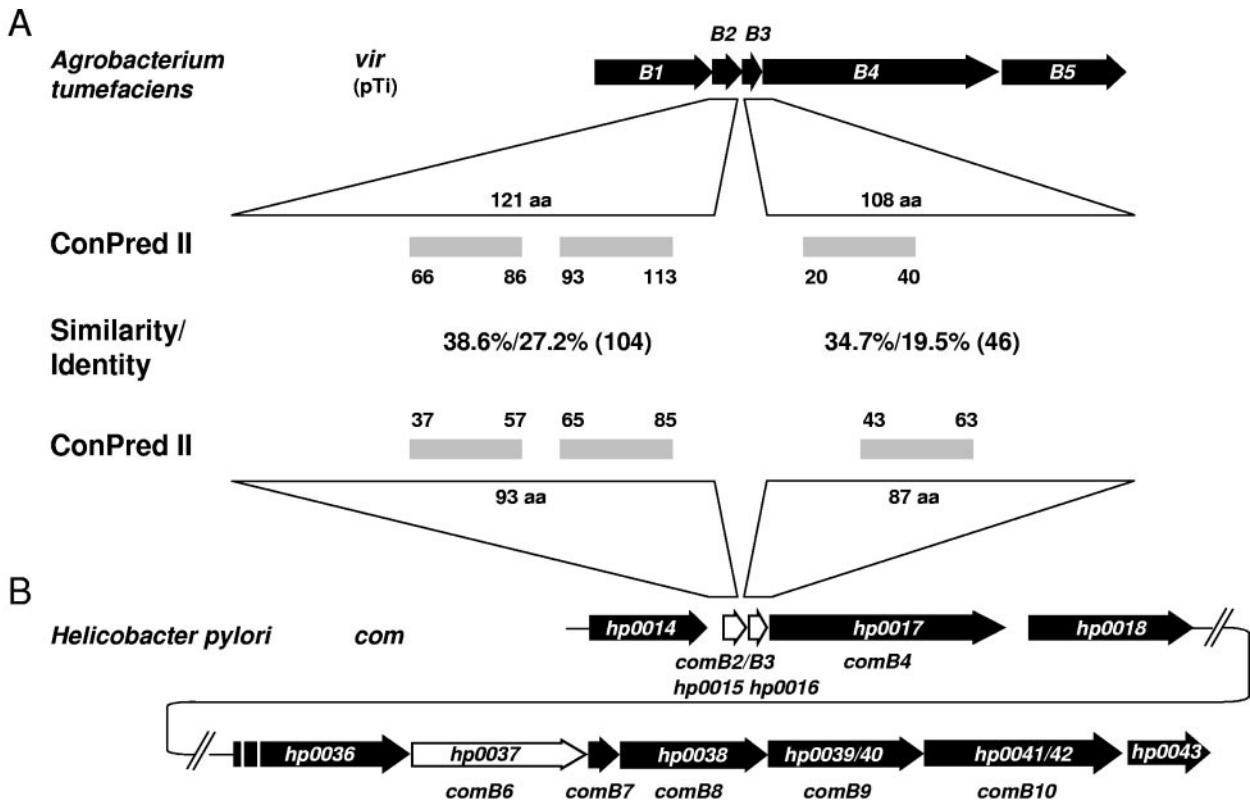


FIG. 1. Identification of novel type IV-related genes of the *comB* gene cluster. (A) Schematic representation of the *A. tumefaciens* *virB* locus with the number and location of putative TM domains in the VirB2 and VirB3 proteins, as predicted by ConPred II. (B) Overview of the complete *H. pylori* *comB* gene cluster and depiction of the *comB* region *hp0014* to *hp0018*, with a comparison of the ComB2 (HP0015) and ComB3 (HP0016) TM domains, as predicted by ConPred II. Amino acid (aa) similarities and identities of the VirB and ComB homologues are shown between the corresponding ORFs.

Genes *hp0015*, *hp0016*, and *hp0017* are organized as an operon. The genetic organizations of *hp0015-hp0017* and *jhp0013-jhp0015* in *H. pylori* strains 26695 and J99, respectively, are very similar. The stop and start codons of *hp0015-hp0016* (*jhp0013-jhp0014*) partially overlap, and the stop codon of *hp0016* (*jhp0014*) and start codon of *hp0017* (*jhp0015*) are separated by one nucleotide only. This indicated that the corresponding region might be organized as an operon. To test this hypothesis experimentally, we used an approach to verify a common transcript of *hp0015* to *hp0017* by RT-PCR. Therefore, RNAs of the wild-type *H. pylori* strain 26695 and 26695 Δ *hp0015* were prepared, and cDNA was generated with primers CH80 and CH82 (see Materials and Methods and Fig. 2). CH80-generated cDNA, 26695 chromosomal DNA, and RNA without the RT step were used as templates for RT-PCR using primer pair AK05-CH81 (Fig. 3). For chromosomal DNA and cDNA, but not for RNA templates, PCR fragments of the expected size were obtained (Fig. 3, lanes 1 through 3). To verify whether or not *hp0014* is also cotranscribed with the *comB* operon, the same templates were used with primer pair AK05-CH77 (Fig. 2; Table 2), which did not result in a PCR product (Fig. 3, lanes 4 through 6). In contrast, when a cDNA was generated from the sequence upstream of *hp0015* (primer pair CH82-CH83), an RT-PCR product comprising the *hp0014* gene was obtained (Fig. 3, lanes 7 through 9), demonstrating the presence of a separate transcriptional unit upstream of

hp0015. Finally, we also demonstrated that insertion of the *cat* resistance gene into *hp0015* (in strain 26695 Δ *hp0015::cat*) did not cause a polar effect (primer pair CH78-CH79), since a common transcript comprising the *cat* and the *hp0016* genes was obtained (Fig. 3, lanes 9 through 12). Taken together, these data show that *hp0015-hp0017* is organized as an operon.

Genetic complementation of the Δ *hp0015* and Δ *hp0016* mutant strains in *H. pylori* 26695. The deletion mutants showed a very drastic effect on transformation competence. The RT-PCR data for the wt and mutant strains already ruled out a polar effect of the *cat* gene replacement mutations on the expression of *comB4*, which is well described to be essential for transformation competence (25). To verify these data independently and to rule out any secondary mutation in another locus essential for natural competence, we sought to complement strain P1 Δ *hp0015-hp0017* (strain P226) in *trans*, using the pHel2 shuttle vector (23). For complementation, *hp0015* to *hp0017*, *hp0016-hp0017*, or *hp0017* alone was used (strains P229, P230, and P231, respectively [Table 1]) (Fig. 2B). The complementing genes were cloned into the shuttle vector pHel2 under the control of the *H. pylori* *flaA* promoter. Since none of the constructs resulted in a detectable transformation frequency except for complementation with the complete set of genes (*hp0015-hp0016-hp0017*), we conclude that both *comB2* (*hp0015*) and *comB3* (*hp0016*) are essential for transformation competence.

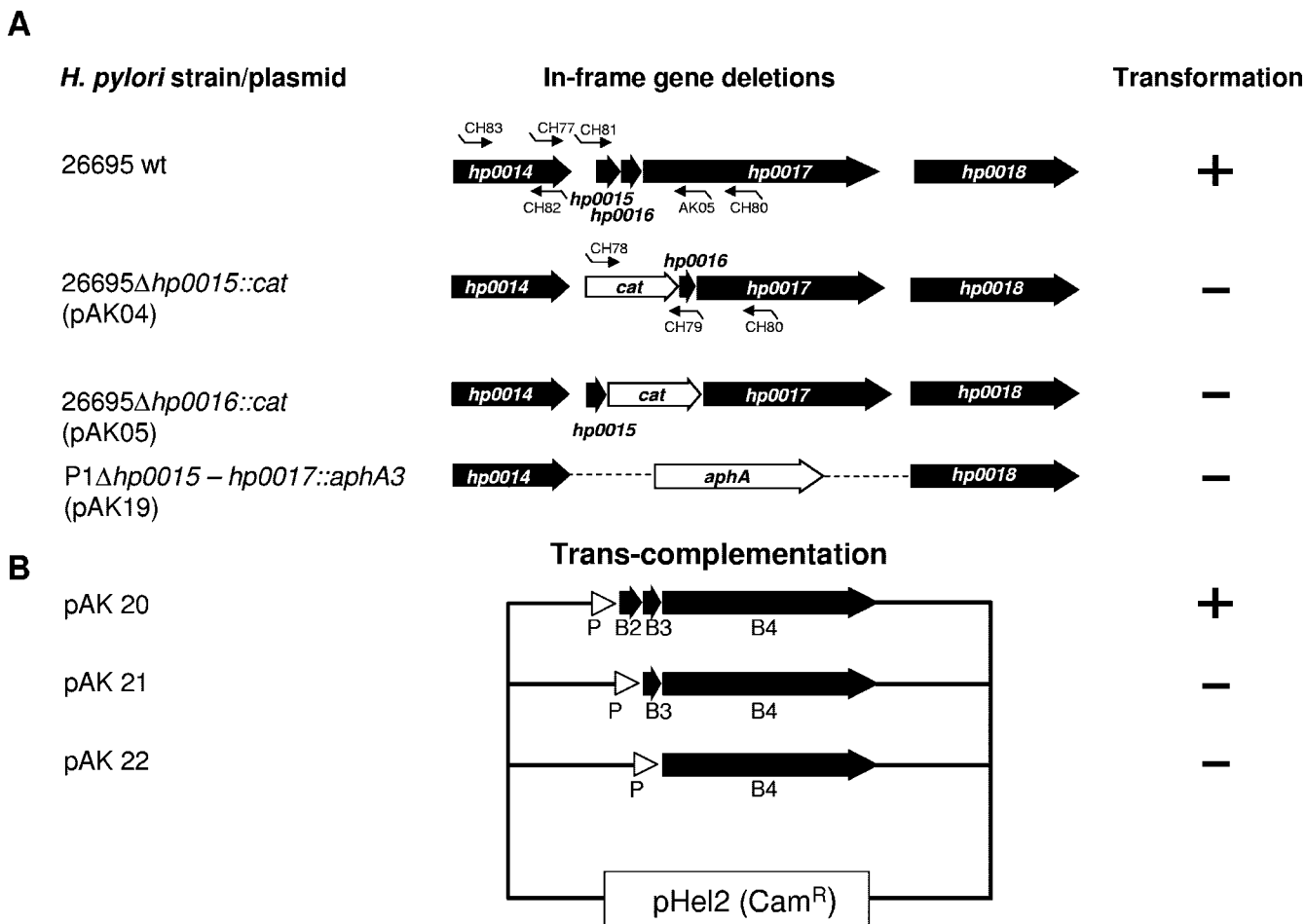


FIG. 2. Construction of precise deletions of *comB2* (*hp0015*) or *comB3* (*hp0016*) and genetic complementation. (A) Precise deletions of the *hp0015*, *hp0016*, or *hp0015* to *hp0017* genes were constructed by replacing the corresponding gene sequences with a chloramphenicol acetyltransferase (*cat*) or kanamycin (*aphA-3*) resistance gene cassette. (B) Complementation of the corresponding genes was obtained by cloning the *comB4*, *comB3-comB4*, or *comB2* to *comB4* gene(s) in the shuttle vector pHel2 behind the *H. pylori flaA* promoter (P) of plasmid pDH80 (pAK20 to pAK22) and transfer of the shuttle plasmid into P1Δ*hp0015*-*hp0017*::*aphA-3*. The competence for genetic transformation of the corresponding strains is indicated (+ or -). For quantitative data of transformation experiments, see Table 3.

HP0037 is an essential component of the ComB type IV transformation competence system. Our computer predictions identified the putative protein encoded by *hp0037*, which is located directly upstream of *comB7*, as a polytopic membrane protein like VirB6 from *A. tumefaciens* (Fig. 4A). Depending on the prediction program used, HP0037 possesses five to seven putative TM regions, very similar in their distribution to those of VirB6. To test whether HP0037 is an essential component of the *comB* transformation competence system, *hp0037* was precisely deleted and replaced by the terminatorless *cat* resistance gene. Plasmid pAK08 was used to generate a gene knockout for *hp0037* in *H. pylori* P1 by transformation and homologous recombination, resulting in P1Δ*comB6* (P227) (see Materials and Methods). The mutant strain completely lost its natural transformation competence for plasmid or chromosomal DNA (Table 3). Interestingly, transfer of plasmid or chromosomal DNA into the *H. pylori* mutant strain by electroporation was still possible, supporting our assumption that ComB6 is involved only in the process of DNA binding or uptake in a bacterial membrane compartment, rather than

having an additional function via its cytoplasmic domain in a later step of transformation competence in the cytoplasm.

Production of the ComB8 to ComB10 proteins was reduced in the P1Δ*hp0037* mutant compared to the P1 wt strain (data not shown). To confirm that the DNA uptake defect was dependent on *comB6*, rather than being a polar effect of the mutation on expression of *comB7* to *comB10*, we performed a genetic complementation experiment. First, a mutant strain was generated in which the chromosomal region spanning *comB6* to *comB10* was deleted and replaced by a kanamycin (*aphA-3*) resistance cassette (P1Δ*comB6-comB10*) (data not shown). The corresponding region was then inserted into *H. pylori* P228 by using the shuttle vector pHel2, either including (pAK24, strain P232) or excluding (pDH046, strain P233) *hp0037* (Fig. 4B). The complemented strain, but not the deletion mutant strain, produced the ComB8, ComB9, and ComB10 proteins, as detected by immunoblot analysis (data not shown). Though lower than that of the wt, competence for genetic transformation was restored in the complemented strain carrying *hp0037* but not in the strain without *comB6*.

TABLE 3. Transformation frequencies of *H. pylori* wt and mutant strains

<i>H. pylori</i> strain and mutation	Transformation frequency with:	
	Chromosomal <i>H. pylori</i> DNA (<i>str</i>) ^a	Plasmid DNA (pDH29) (<i>erm</i>) ^b
P1	$(1.3 \pm 0.5) \times 10^{-5}$	$(1.1 \pm 0.3) \times 10^{-5}$
P12	$(1.3 \pm 0.6) \times 10^{-6}$	$(2.0 \pm 0.9) \times 10^{-5}$
P12 Δ <i>comB2</i>	$<10^{-9}$	$<10^{-9}$
P12 Δ <i>comB3</i>	$<10^{-9}$	$<10^{-9}$
P1 Δ <i>comB2-comB4</i>	$<10^{-9}$	$<10^{-9}$
P1 Δ <i>comB2-comB4</i> [pAK20]	$(5.2 \pm 1.1) \times 10^{-6}$	$(3.4 \pm 1.2) \times 10^{-6}$
P1 Δ <i>comB2-comB4</i> [pAK21]	$<10^{-9}$	$<10^{-9}$
P1 Δ <i>comB2-comB4</i> [pAK22]	$<10^{-9}$	$<10^{-9}$
P1 Δ <i>comB6</i>	$<10^{-9}$	$<10^{-9}$
P1 Δ <i>comB6-comB10</i>	$<10^{-9}$	$<10^{-9}$
P1 Δ <i>comB6-comB10</i> [pAK24]	$(0.4 \pm 0.1) \times 10^{-7}$	$(1.9 \pm 0.7) \times 10^{-7}$
P1 Δ <i>comB6-comB10</i> [pDHO46]	$<10^{-9}$	$<10^{-9}$
P149	ND	$(5.4 \pm 1.9) \times 10^{-4}$
P149[<i>hp1421::TnHK9</i>]	ND	$(3.8 \pm 1.4) \times 10^{-4}$

^a Chromosomal DNA from strain P12 (*str*) or P1 (*str*) was used for transformation. *str*, streptomycin resistance gene cassette. ND, not done.

^b Plasmid pDH29 was used for transformation. It consists of pBluescript plasmid with the *H. pylori recA* gene interrupted by an *erm* (erythromycin resistance) cassette (25).

These results clearly identified *comB6* as an essential gene for the process of natural transformation competence.

Topology mapping of HP0037 identifies the protein as a structural VirB6 homologue. The HP0037 protein was predicted by several computer algorithms to be a typical transmembrane protein (Fig. 5A). Recent studies indicate that the topology of a membrane protein can be predicted reliably when several topology prediction methods show a consensus (18). We used the topology prediction programs TMpred, HMMTOP 2.0, TopPred, and TMHMM. In order to verify these predictions experimentally, we chose a reporter technology by fusing HP0037 N-terminal fragments to alkaline phosphatase (PhoA), which is active only in the periplasm, or to

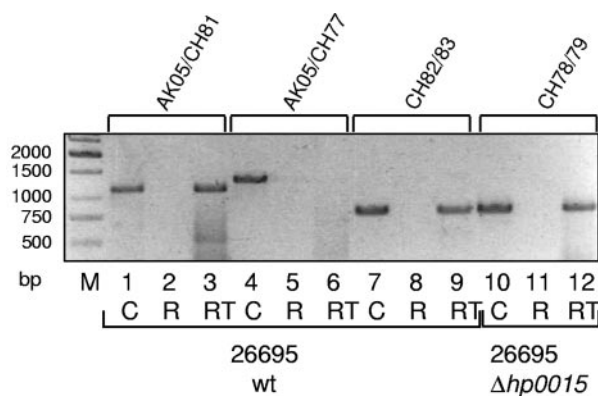


FIG. 3. RT-PCR analysis of *comB* mRNA expression in *H. pylori* 26695 wt and Δ *comB* mutant strains. Lanes 1 through 9, *H. pylori* 26695 wt; lanes 10 through 12, 26695 Δ *hp0015*. RT-PCR was performed with *H. pylori* 26695 wt cDNA (RT) (see Materials and Methods) using primer pairs AK05/CH81 (lane 3), AK05/CH77 (lane 6), and CH82/CH83 (lane 9) and with *H. pylori* 26695 Δ *hp0015* cDNA using primer pair CH78/CH79 (lane 12). As positive controls, analogous PCRs with chromosomal DNA (C) of *H. pylori* 26695 wt (lanes 1, 4, and 7) and *H. pylori* 26695 Δ *hp0015* (lane 10) were performed. No amplification products were detected in the negative controls (lanes 2, 5, 8, and 11), where RNA only, without cDNA synthesis (R), served as a template for PCR. Lane M, DNA size marker.

GFP, which is active only in the cytoplasm (18, 34). Recombinant *E. coli* strains producing the fusion proteins displayed a fairly consistent pattern of reporter protein activities. These proteins could be demonstrated to be stable fusion proteins with corresponding immunoblots (data not shown). *E. coli* strains producing fusion proteins with junction sites at positions 34, 115, and 236 of HP0037 exhibited intermediate to high levels of PhoA but low levels of GFP fluorescence activity, suggestive of a periplasmic location of the corresponding junction sites (Fig. 5B). Conversely, *E. coli* strains producing fusion proteins with junction sites at positions 62, 198, and 296 each displayed relatively high levels of GFP fluorescence but low levels of PhoA activity, suggestive of a cytoplasmic location (Fig. 5). One critical position was the GFP fusion at position 236, which was found always with rather high levels of activity. Since the predictions at this position were identical among all prediction programs and the hydrophobicity plot also indicated a clear distribution of TM sequences (Fig. 5A), we assume that the GFP fusion becomes mislocated (in the cytoplasm) in this case. The C terminus of the protein is located in the cytoplasm, as suggested by the low-level PhoA activity and high-level GFP fluorescence of the corresponding fusions (Fig. 5B). The topology prediction for the N terminus of HP0037 was less definite: not all programs predicted a TM domain for the first 25 amino acids, and in addition the SIGCLEAVE program but not SIGNALP predicted a Sec-dependent signal peptide. As we were able to generate an active PhoA fusion protein at position 34, we conclude that this is strong evidence for an N-terminal Sec-dependent secretion signal. Therefore, our topology model, which is supported by the computer predictions and the reporter gene assays, confirms five TM regions for ComB6, with the N terminus located in the periplasm and an extended periplasmic loop between TM domains 2 and 3, as well as a long C-terminal region in the cytoplasm (Fig. 6). Thus, the overall topology of HP0037 is reminiscent of the structure of VirB6 of *A. tumefaciens* (28), which, together with its essential function and its conserved location, justifies the designation ComB6 for this protein.

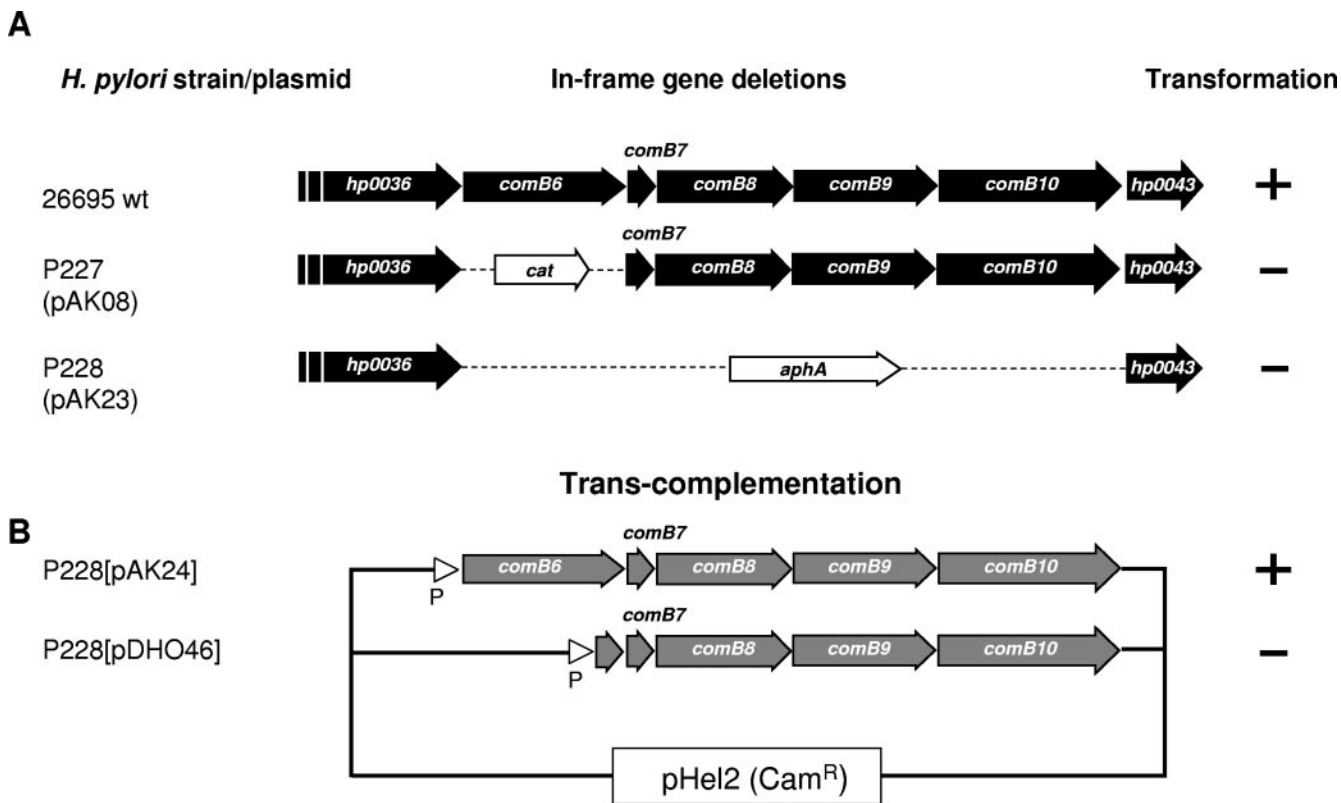


FIG. 4. Essential role of *comB6* for transformation competence as revealed by deletion and complementation studies. (A) Precise deletions of *comB6* (*hp0037*) or the region comprising *comB6* to *comB10* (*hp0037* to *hp0042*) were constructed by replacing the corresponding gene sequences with a *cat* or a kanamycin (*aphA-3*) resistance gene cassette, respectively. (B) Complementation of the corresponding genes was obtained by cloning the *comB6* to *comB10* or the *comB7* to *comB10* genes in the shuttle vector pHel2 behind the *H. pylori* *flaA* promoter (plasmid pDH80) to result in pAK24 or pDHO46 (25), respectively. The shuttle plasmids were transferred into strain P228 ($P1\Delta comB6-comB10::aphA-3$). The competence for genetic transformation of the corresponding strains is indicated at right (+ or -). For quantitative data of transformation experiments, see Table 3.

HP1421, a putative VirB11-homologous ATPase, is not essential for the function of the ComB T4SS. Bacterial T4SS involved in DNA transfer (conjugation systems, T-DNA transfer system) or protein translocation use several inner-membrane-associated ATPases for substrate transfer, such as VirB4, VirB11, and VirD4 homologues. For the ComB system, only one such ATPase homologue has been identified, ComB4, a functional homologue of VirB4, which is essential for the function of the T4SS (25). In *A. tumefaciens*, VirB11 interacts with VirB9 and VirB10 and is involved in the assembly of the transport system. Two genes in the *H. pylori* 26695 genome, *hp0525* and *hp1421*, encode putative VirB11 homologues. The product of *hp0525* has been identified as the VirB11-homologous ATPase of the *cag* pathogenicity island-encoded T4SS, and our earlier results showed that HP0525 is not functionally involved in DNA transformation competence (25). The *H. pylori* *virB11* homologue might also be part of the *comB* system. We therefore analyzed *hp1421*, the second putative *virB11*-homologous gene in *H. pylori*, which might be involved in natural transformation competence, as suggested earlier (47). Therefore, a set of independent transposon insertion mutants was generated with the in vitro transposon pTnHK9 (29), a derivative of EZ::Tn (Epicenter, Madison, WI). A functional inactivation of the *hp1421* gene was generated in *H.*

pylori strain P149 and verified by PCR and DNA sequencing of the transposon insertion site (P234 [Table 1]). The transformation efficiency of the mutant strain was not significantly lower than that of the corresponding wt strain (Table 3), suggesting that the putative HP1421 ATPase is not functionally involved in the natural transformation competence of *H. pylori*.

DISCUSSION

Gram-positive and gram-negative bacteria use related proteins and molecular machines to import DNA. Components of these competence systems have homology with proteins that are involved in the assembly of type IV pili and type II secretion systems and form a structure that partially spans the cell envelope. The only exceptions known so far are the *comB* systems of *H. pylori* (26) and *Campylobacter jejuni* (5), which both use components of a T4SS for natural transformation competence. The *comB* apparatus as described previously hitherto consisted of the proteins ComB4 and ComB7 to ComB10 (24, 25). In this paper we report the identification and functional characterization of three additional basic components of T4SS for the *comB* system, ComB2 (HP0015), ComB3 (HP0016), and ComB6 (HP0037).

The genes *comB2-comB3* and *comB6* were first identified by

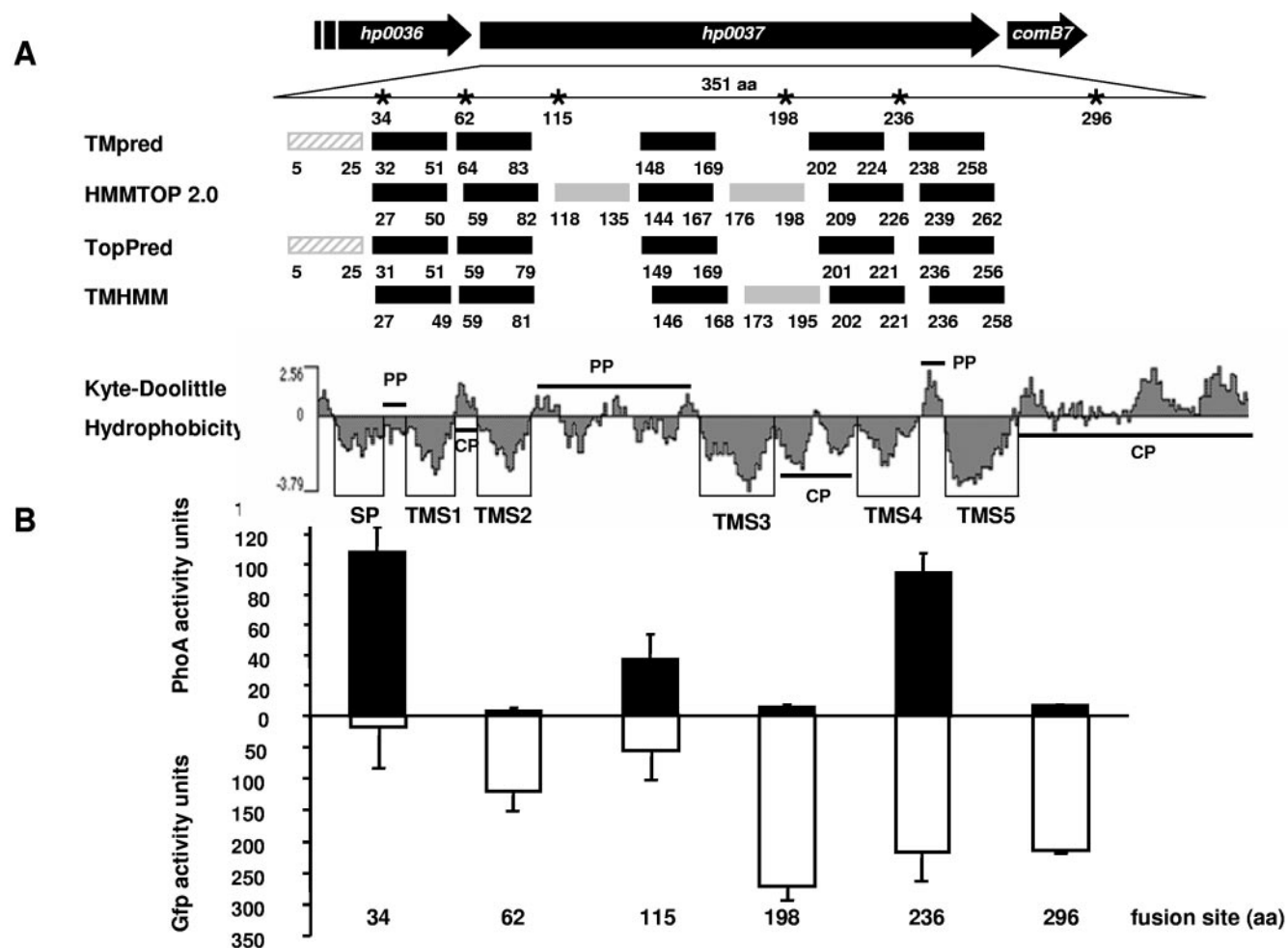


FIG. 5. Topology analyses of the ComB6 subunit of the *H. pylori* transformation competence-mediating type IV transport system. (A) The computer programs listed at left predicted the transmembrane segments shown as solid rectangles with residue numbers relative to the N terminus. Solid rectangles identify transmembrane segments supported by results of reporter protein fusion studies; shaded rectangles are not supported by experimental data. *, amino acid (aa) positions of the PhoA or GFP fusions represented in panel B. Kyte-Doolittle hydrophobicity blot of HP0037: SP, signal peptide; TMS1 to TMS5, transmembrane segments 1 to 5; CP, cytoplasmic location; PP, periplasmic location. (B) Reporter protein activity levels of *E. coli* CC118 (*phoA*) or XL1-Blue (*gfp*) expressing six defined fragments of *comB6* translationally fused to *phoA* (above the *x* axis) or *gfp* (below). Numbers at the bottom refer to the fusion site of the corresponding ComB fusion protein tested. Reporter protein activities represent the average of at least three experiments, with standard deviations indicated.

their location in respect to the known T4SS components *comB4* and *comB7* to *comB10*, respectively. The involvement of the genes *hp0015* and *hp0037* in natural transformation competence was identified previously with a transposon mutagenesis screen (12), but polar effects on *comB4* or the *comB7*-to-*comB10* operon had not been ruled out. In our study, the generation of knockout mutant strains in the corresponding genes and subsequent complementation studies verified their essential function for the natural transformation competence of *H. pylori*. In addition, we were able to demonstrate a topology of the corresponding proteins similar to the homologous proteins of the prototype VirB T4SS in *A. tumefaciens*. The basis for these topological studies was a solid secondary structure prediction, performed either by a set of independent secondary structure prediction programs (TM-Pred, HMMTOP 2.0, TopPred, and TMHMM) or by ConPred II, a software package that combines a number of individual programs in a single package (4). Interestingly, despite rather

low sequence identities between VirB2-ComB2 (27.2%) and VirB3-ComB3 (19.5%), the total length of the putative proteins, the elongated signal sequence (for VirB2-ComB2), and the secondary structure prediction were well conserved between these putative paralogous pairs of proteins.

The VirB2 protein is a subunit of a surface-exposed pilus structure of the VirB system. Whether the ComB transformation competence system also uses a surface-located pilus and what the function of such a pilus might be during DNA uptake and transformation are completely unknown. For *H. pylori*, flagella (48) and *cag* pathogenicity island-encoded surface appendages (39, 51), but no typical pili, have been described. Therefore the ComB2 protein might be part of a “stump structure” in the cell envelope (43) that might facilitate DNA uptake but does not form an extended extracellular pilus. Such a scenario is also known from type IV pili in gram-positive as well as gram-negative bacteria. In *Neisseria gonorrhoeae*, the Pile protein is able to form long, extended surface pili; al-

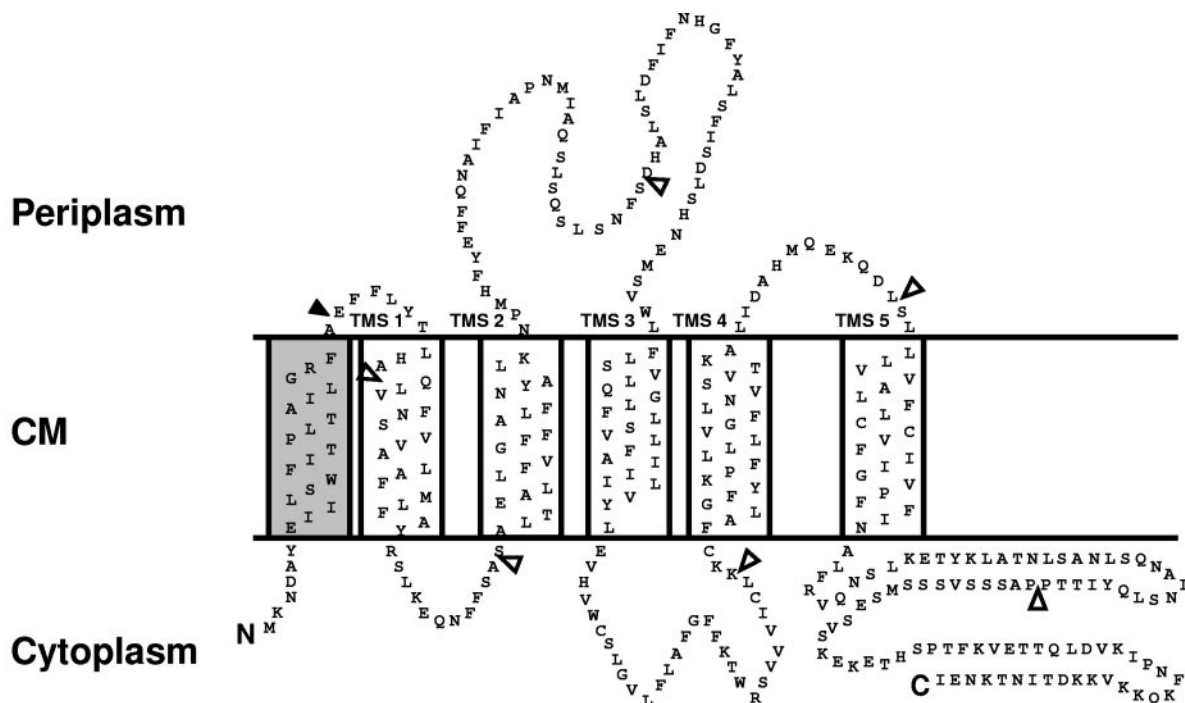


FIG. 6. Proposed membrane topology model of ComB6. The model is derived from predictions by four widely used computer algorithms and the results of PhoA and GFP reporter protein fusion studies. The experimental data provide support for the five transmembrane segments (TMS) shown. The N-terminal signal sequence is shown as an additional TMS (shaded). A long C-terminal segment is located in the cytoplasm. The filled arrowhead represents a postulated signal sequence cleavage site; open arrowheads mark GFP or PhoA fusion sites. CM, cytoplasmic membrane.

though such extended pilus structures are neither necessary nor sufficient for transformation competence, the pilin protein and other pilE homologues are essential (1). VirB2 is expressed as a propilin, which is cleaved at the N terminus by the signal peptidase. In certain systems VirB2 is processed further at the C terminus, which results in the cyclization of the protein (19). We did not find in ComB2 a conserved AEIA motif, characteristic of certain TrbC-like pilins, indicating that ComB2 does not belong to the group of cyclic pilins.

The VirB3 homologues are essential components of T4SS, the precise function of which remains largely unknown. In *A. tumefaciens*, ConPred II predicts one hydrophobic region for VirB3. Originally the protein was suggested to localize to the periplasmic face of the outer membrane. More recent analyses suggest that VirB3 subunits assemble as a functional complex with the VirB4 ATPase at the inner membrane (16). For ComB3 of *H. pylori* we found a similar arrangement of the hydrophobic sequence (Fig. 1), but the localization of the protein and its function for DNA transformation competence are unknown.

The identification of a VirB6-homologous protein (HP0037, ComB6) in the *H. pylori comB* system was unexpected, since HP0037 was annotated as an NADH-ubiquinone oxidoreductase subunit in the *H. pylori* 26695 genome sequence (52). Furthermore, several years ago we reported on an *H. pylori* mutant strain, designated P87, in which a TnMax5 insertion in *comB6* interrupted the gene at its 3' end, resulting in a truncation of the last 29 amino acids of the protein (26). The corresponding strain showed a reduction in its transformation rate by 10 or 20%, depending on the use of chromosomal or

plasmid DNA for transformation, respectively. At that time the gene was considered nonessential for transformation competence (26). The secondary structure prediction of ComB6 reveals now a five-transmembrane-spanning topology with the C-terminal 92 amino acids in the cytoplasm. Thus, the truncated ComB6 protein in strain P87 might still be active, since all membrane-spanning segments and part of the cytoplasmic tail are still present. Now we show that ComB6 is absolutely essential for DNA transformation competence. A precise, complete deletion of the gene resulted in a complete loss of transformation competence of the corresponding mutant strain, and genetic complementation resulted in a restoration of the phenotype, at least in part. A complete restoration might depend on an exact stoichiometry between ComB6 and other components of the apparatus. Due to the lack of an antibody, we were unable to test the level of *comB6* expression directly in our complementation assay, but we can exclude a defect in the production of ComB8, ComB9, and ComB10 from this plasmid in the corresponding *H. pylori* P1 genetic background (25). Thus, our data indicate that ComB6 is an essential component of the T4SS involved in DNA transformation competence.

Recently, Yeh and coworkers identified a protein (HP1361) with sequence homology to *Bacillus subtilis* ComEC (also known as ComE3), which was absolutely essential for DNA transformation competence in *H. pylori* (53). ComEC is a putative channel protein for DNA uptake in *B. subtilis* and other genetically transformable bacteria. Thus, ComEC might have a function in the classical competence systems similar to that of VirB6 in the T4SS. *H. pylori* apparently has both types of proteins, a ComEC homologue and a ComB6 protein, both of

which are considered to be multispansing transmembrane proteins and absolutely essential for transformation competence in these bacteria. HP1361 has been reported to be involved in DNA binding as well as in DNA uptake (53), although a defect in DNA binding might be sufficient to eliminate DNA uptake. Whether both proteins are involved in DNA transfer through the cytoplasmic membrane is not known so far, but since they can apparently not complement each other, they seem to fulfill independent functions. Interestingly, an *H. pylori comEC* knockout mutant was resistant to transformation via electroporation, a method that should be independent of DNA binding or a DNA uptake channel in the bacterial membrane (53). Our *H. pylori comB6* mutant was readily transformable via electroporation, as shown by the complementation experiment, indicating that the corresponding protein is involved in DNA binding or in the mechanism of uptake through the bacterial membrane.

It was suggested that T4SS-mediated T-DNA transfer of *A. tumefaciens* might be a two-step process (15, 37) and that conjugation-mediated uptake of plasmid DNA in *A. tumefaciens* is enhanced by components of the VirB T4SS (7, 33). As *H. pylori* produces neither a type IV prepilin peptidase nor (pseudo)pilin proteins, we speculate that the ComB-mediated apparatus bridging the space between the outer and inner membranes might facilitate DNA uptake via the outer membrane into the periplasm. The transfer of the DNA across the inner membrane into the cytoplasm could be mediated by components of the *comB* T4SS and/or the ComEC homologue HP1361.

With the data presented here, we demonstrate that the *comB* DNA transformation competence system is comprised of a nearly complete set of basic T4SS components. These consist of proteins ComB2 to ComB4 and the core complex ComB6 to ComB10 but lack VirB1 and VirB5 homologues, as well as a VirB11-like ATPase. We cannot exclude the possibility that functional homologues of the latter two VirB proteins with only slight primary sequence homology might be encoded by *H. pylori*. It was shown that the murein hydrolase VirB1 is not essential for substrate translocation by the T4SS in *A. tumefaciens* (6); therefore, it is possible that the ComB apparatus of *H. pylori* could be assembled without the VirB1-mediated local disruption of the peptidoglycan cell wall. VirB5 is a secreted protein that localizes mainly to the T pilus (41). In addition, an adhesionlike function was demonstrated for its homologue, TraC (54). The establishing of cell-to-cell contact is required for T-DNA transfer and bacterial conjugation but not for DNA uptake by natural transformation competence, which could explain the absence of a VirB5 homologue in *H. pylori*. Interestingly, the *pil* T4SS of *Bordetella pertussis*, which is essential for the contact-independent secretion of the pertussis toxin, also lacks a *virB5* homologue (9).

Although we have identified here a further set of basic components of the *H. pylori* DNA uptake system, further studies are necessary to completely understand the composition and function of the *comB* transformation competence system in detail.

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