Molecular Microbiology (2001) **41**(2), 379–391

Natural transformation competence in *Helicobacter pylori* is mediated by the basic components of a type IV secretion system

Dirk Hofreuter, Stefan Odenbreit and Rainer Haas*
Max von Pettenkofer Institut für Hygiene und Medizinische Mikrobiologie, Pettenkoferstr. 9a, D-80336 München, Germany.

Summary

***Helicobacter pylori* (*Hp*), a Gram-negative bacterial pathogen and aetiologic agent of gastroduodenal disease in humans, is naturally competent for genetic transformation. Natural competence in bacteria is usually correlated with the presence of type IV pili or type IV pilin-like proteins, which are absent in *Hp*. Instead, we recently identified the *comB* operon in *Hp*, carrying four genes tentatively designated as *orf2*, *comB1*, *comB2* and *comB3*. We show here that all ComB proteins and the 37-amino-acid Orf2 peptide display significant primary sequence and structural homology/identity to the basic components of a type IV secretion apparatus. ComB1, ComB2 and ComB3, now renamed ComB8, ComB9 and ComB10, correspond to the *Agrobacterium tumefaciens* VirB8, VirB9 and VirB10 proteins respectively. The peptide Orf2 carries a lipoprotein motif and a second cysteine residue homologous to VirB7, and was thus designated ComB7. The putative ATPase ComB4, encoded by the open reading frame *hp0017 of* strain 26695, corresponds to *virB4* of the *A. tumefaciens* type IV secretion system. A *Hp comB4* transposon insertion mutant was totally defective in natural transformation. By complementation of a *Hp* Δ*comB* deletion mutant, we demonstrate that each of the proteins from ComB8 to ComB10 is absolutely essential for the development of natural transformation competence. The putative lipoprotein ComB7 is not essential, but apparently stabilizes the apparatus and modulates the transformation efficiency. Thus, pathogenic type I *Hp* strains contain two functional independent type IV transport systems, one for protein translocation encoded by the *cag* pathogenicity island and one for uptake of DNA by natural transformation. The latter system indicates a possible novel mechanism for natural DNA transformation in bacteria.**

Accepted 26 April, 2001. *For correspondence. E-mail haas@m3401.mpk.med.uni-muenchen.de; Tel. (+49) 89 5160 5255; Fax (+49) 89 5160 5223.

Introduction

Helicobacter pylori (*Hp*) is one of the most successful bacterial pathogens, infecting about 50% of the world population. The persistent chronic infection causes type B gastritis and, in a certain percentage of infected persons, peptic ulceration. More severe, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma are also associated with the infection. On the genetic level, *Hp* is one of the most diverse bacterial species known so far, and this is probably the result of a high degree of horizontal gene transfer and free recombination within the species (Suerbaum *et al*., 1998). This genetic diversity is postulated to result from the adaptation of *Hp* to its individual host during years and even decades of infection. Two major types of strains exist: type I strains, which express a functional vacuolating cytotoxin (VacA) and carry the *cag* pathogenicity island (*cag*-PAI); and type II strains, which lack both characteristics (Xiang *et al*., 1995).

Hp displays natural competence for genetic transformation *in vitro* (Nedenskov-Sorensen *et al*., 1990), a feature that might be the basis for horizontal gene transfer and the subsequent generation of a high degree of genetic diversity. To understand the mechanism of natural transformation in this species, we screened systematically for the genes involved. Transposon mutagenesis with the mini-Tn*blaM* transposon Tn*Max9* (Hofreuter *et al*., 1998) yielded a mutation in the *comB* locus, which consists of the genes *comB1* to *comB3* preceded by a short open reading frame (ORF), designated *orf2*. In addition to the *comB* operon, the ORF HP0333 was identified (Ando *et al*., 1999; Smeets *et al*., 2000a), a homologue of the *Haemophilus influenzae dprA* gene (Karudapuram and Barcak, 1997). By the generation of a corresponding knock-out mutant strain (Ando *et al*., 1999; Smeets *et al*., 2000a), *hp0333* was also shown to be necessary for transformation. Smeets *et al*. (2000b) identified a novel competence gene, *comH*, by screening a *Hp* mutant library. The ComH protein carries a putative signal sequence and has no known orthologues in other bacteria.

Many well-studied Gram-negative and Gram-positive bacteria expressing natural transformation competence produce type IV pili or type IV pilin-like molecules involved in competence (Dubnau, 1999). Analysis of the two available complete *Hp* genome sequences (Alm *et al*., 1999) revealed no orthologues to the genes encoding these proteins. The *Hp* ComB2 and ComB3 proteins, however, reveal homology to the VirB proteins of well-described type IV secretion systems. These systems, which are found in a growing number of bacterial species, are involved in DNA export, bacterial conjugation and protein secretion (Christie and Vogel, 2000). *Hp* carries a corresponding system on the *cag* pathogenicity island (Covacci *et al*., 1999), which is involved in injection of the bacterial protein CagA into eukaryotic cells, where it is phosphorylated ($CagA^{P\text{-}tyr}$) (Segal et al., 1999a; Asahi *et al*., 2000; Backert *et al*., 2000; Odenbreit *et al*., 2000; Stein *et al*., 2000). Type IV systems may contain up to three different ATPases, termed VirB4, VirB11 and VirD4 in *A. tumefaciens*, which are all essential for the function of the secretion system.

The *comB* locus is organized in a transcriptional unit, and we have shown by transposon mutagenesis that at least ComB3 is essential for transformation competence (Hofreuter *et al*., 1998). Here, we now demonstrate that the *comB* locus, together with a VirB4-homologous ATPase, constitute a second type IV transport system involved in natural transformation competence of *Hp*.

Results

Homology of the ComB proteins with basic components of a type IV secretion apparatus

Using the BLASTP algorithm, we have already described protein sequence homology for ComB2 and ComB3 to VirB9 and VirB10 of *A. tumefaciens* respectively (Hofreuter *et al*., 1998; 2000). Our recent searches of the database identified significant sequence homology of ComB1 with members of the VirB8 protein family of several bacterial pathogens, including *Legionella pneumophila*, *Brucella suis*, *Bartonella henselae*, *Wolbachia* sp. and *Rickettsia prowazekii* (Table 1). All these bacteria contain type IV secretion systems (Covacci *et al*., 1999). The amino acid sequence of the peptide encoded by *orf2*, located upstream of *comB1*, carries a putative lipoprotein signal sequence (lipobox), analogous to the VirB7 protein of *A. tumefaciens*. A direct comparison between the Orf2 peptide and the VirB7 protein revealed both sequence and structural homologies between these proteins (Fig. 1A). In addition to the lipobox, a second cysteine residue (C27) present in Orf2 is also present in VirB7 (C24) (Fig. 1A). In VirB7, this latter cysteine forms a disulphide bridge with the cysteine C262 of VirB9, which stabilizes VirB proteins and is important for the structure of the type IV secretion system in *A. tumefaciens* (Fernandez *et al*., 1996a; Spudich *et al*., 1996; Baron *et al*., 1997; Das *et al*., 1997).

Table 1. Homology of ComB proteins with components of a type IV secretion system.

ComB protein	versus	Identity (%) (BESTFIT)	Strain/plasmid	Reference
ComB8	ComB1	35.6	*Campylobacter* (pVir)	Bacon *et al*. (2000)
	VirB8 homologue	24.7	*Wolbachia* sp.	Masui *et al*. (2000)
	VirB8 homologue	24.1	*Brucella suis*	O'Callaghan *et al*. (1999)
	LvhB8	24.0	*Legionella pneumophila*	Segal *et al*. (1999b)
	VirB8 homologue	22.6	*Bartonella henselae*	Padmalayam *et al*. (2000)
	CagW (HP0529)	21.0	*Helicobacter pylori*	Censini *et al*. (1996)
	VirB8 homologue	20.2	*Rickettsia prowazekii*	Andersson *et al*. (1998)
ComB9	ComB2	37.7	*Campylobacter* (pVir)	Bacon *et al*. (2000)
	LvhB9	24.7	*Legionella pneumophila*	Segal *et al*. (1999b)
	VirB9	24.5	*Agrobacterium tumefaciens*	Kuldau *et al*. (1990)
	VirB9 homologue	24.3	*Rhizobium etli*	Bittinger *et al*. (2000)
	CagX (HP0528)	23.5	*Helicobacter pylori*	Censini *et al*. (1996)
	VirB9	23.1	*Wolbachia* sp.	Masui *et al*. (2000)
	VirB9 homologue	21.3	*Bartonella henselae*	Padmalayam *et al*. (2000)
ComB10	ComB3	35.0	*Campylobacter (pVir)*	Bacon *et al*. (2000)
	CagY (HP0527)	29.1[a]	*Helicobacter pylori*	Censini *et al*. (1996)
	LvhB10	28.9	*Legionella pneumophila*	Segal *et al*. (1999b)
	VirB10 homologue	27.5	*Bartonella henselae*	Padmalayam *et al*. (2000)
	VirB10 homologue	27.2	*Rhizobium etli*	Bittinger *et al*. (2000)
	TrbI	25.1	pRP4/RK2	Pansegrau *et al*. (1994)
	VirB10 homologue	25.0	*Wolbachia* sp.	Masui *et al*. (2000)
	TrbI	24.6	pR751	Thorsted *et al*. (1998)
	VirB10 homologue	24.0	*Brucella abortus*	Sieira *et al*. (2000)

a. Amino acids 1200–1723.

Fig. 1. A. Amino acid sequence comparison of the VirB7 lipoprotein of *A. tumefaciens* (Atu-VirB7) and the corresponding ComB7 homologues of *Hp* (Hpy-ComB7) and *C. jejuni* (Cje-ComB7). Sequences are aligned according to their putative conserved cysteine residues (C1, grey box) used for cleavage of the lipoprotein signal sequence (bold sequence). A second cysteine (C2, shaded black), which forms a disulphide bridge with VirB9 in the *A. tumefaciens* type IV secretion system, is conserved in the ComB7 proteins. The sequence upstream of the second cysteine is conserved between *Hp* and *C. jejuni* (boxed sequence). The Cje-ComB7 amino acid sequence was deduced from AF226280 (nucleotides 153–278; Bacon *et al.*, 2000). A putative ribosome binding site (AAGGAG; nt 140–146) is located upstream of Cje-*comB7*.
B. Alignment of the amino acid sequences of *A. tumefaciens* VirB9 (Atu-VirB9) and its homologue in *Hp* (Hpy-ComB9) and *C. jejuni* (Cje-ComB2). The cysteine in VirB9 (C262) identified as forming a disulphide bridge with VirB7 (C24) is shaded grey.

These comparisons suggest that C316 in the C-terminus of ComB2 may be a functional homologue of the C262 in VirB9 (Fig. 1B). On the DNA level, the arrangement of *orf2*/*comB1* shows striking similarities to the organization of *virB7*/*virB8*; both pairs of genes show a similar overlap. Thus, the sequence comparisons suggest that *orf2* might be a further component of the *comB* operon. For a better correlation with the corresponding *A. tumefaciens virB* genes, we propose to rename the tentatively designated *comB* genes according to the nomenclature of the *virB* genes. Thus, *comB1*, *comB2* and *comB3* are now termed *comB8*, *comB9* and *comB10*, and *orf2* is referred to as *comB7*.

Production of ComB proteins in independent Hp *strains*

The complete genome sequence of *Hp* strain 26695 (Tomb *et al.*, 1997) reveals a different genetic organization of the *comB* operon compared with strains J99 (Alm *et al.*, 1999) and P1 (Hofreuter *et al.*, 1998). In contrast to the latter two strains, the *comB9* and *comB10* genes in strain 26695 are split into two genes, *hp0039/hp0040* and *hp0041/hp0042* respectively. To test the occurrence of the *comB*-expressed proteins in *Hp*, 11 independent *Hp* isolates, including type I and type II strains, were screened in immunoblots with anti-ComB antisera. The blots show that all strains produced proteins of the size predicted from the sequences of genes *comB8* to *comB10* (Fig. 2). Furthermore, strain 26695 synthesized proteins ComB8 to ComB10, indicating that *hp0039/40* represents a single gene corresponding to *comB9* and *hp0041/42* corresponding to *comB10*. The most plausible explanation for this difference between the published gene structure and the phenotypic expression data presented here is a sequencing error in the sequence of the 26695 genes. Indeed, deletion of a single C nucleotide at position 222 of *hp0039* would restore a single reading frame, as found in other strains (Hofreuter *et al.*, 1998; Alm *et al.*, 1999). For *hp0041/42*, the insertion of TTAAA (position 313–317 of *hp0041*) causes an out of frame situation. The addition of a single nucleotide would also restore the reading frame and combine *hp0041/42* to a single ORF. Thus, *comB* gene structure seems to be well conserved in different strains.

Genetic complementation of a ΔcomB *mutant strain*

Tn*Max* transposon insertion into each individual *comB* gene caused a severe reduction in, or a complete loss of, the natural transformation competence in *Hp* (Hofreuter *et al.*, 1998). An immunoblot using specific antisera raised

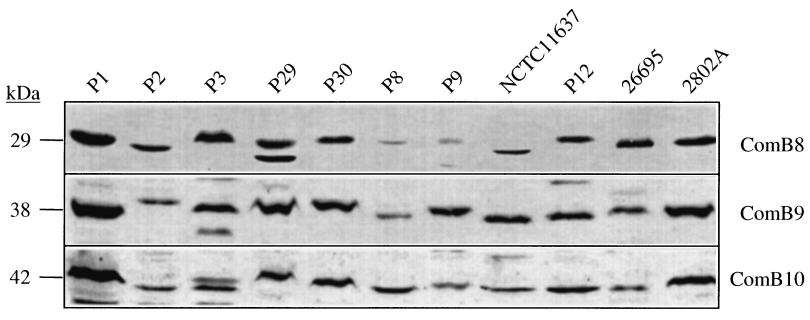

Fig. 2. Production of ComB8, ComB9 and ComB10 in individual *Hp* strains (Table 2). Equal quantities of total-cell lysates of independent *Hp* strains grown on serum plates were separated by 10% SDS–PAGE and analysed by Western blotting using rabbit anti-ComB antisera. The proteins, as recognized by the corresponding antisera and the size of the protein bands, are given at the right and left margins respectively. The occurrence of the reactive protein doublets in certain strains probably corresponds to a processing of the ComB proteins or a cross-reactivity of the serum with other (ComB-like) proteins in the corresponding strains.

against individual ComB proteins (AK250/ComB8, AK251/ComB9 and AK252/ComB10) revealed that genes located downstream of an individual Tn*Max* insertion were not expressed as a result of polar effects (Hofreuter *et al.*, 1998). Thus, ComB10 can be considered as essential for competence development, but the contribution of the other ComB proteins was uncertain.

To analyse the role of the ComB proteins, we chose to delete the complete *comB* operon from the *Hp* chromosome and to reintroduce individual *comB* genes sequentially *in trans*, using the pHel shuttle vector (Heuermann and Haas, 1998). The *comB* deletion mutant was constructed in strain P1 by replacing the *comB* operon with the *aphA-3* resistance gene. Plasmid pDHO34, which carries the flanking sequences of the *comB* operon separated by the *aphA-3* resistance cassette, was transferred into the *Hp* chromosome by transformation-mediated homologous recombination, resulting in *Hp* mutant P147 (Fig. 3A, Table 2). The correct deletion of the complete operon was verified by polymerase chain reaction (PCR) using primers from the *comB* flanking region, by the complete loss of ComB protein production in the P147 mutant (Fig. 4) and by Southern hybridization (data not shown). As natural transformation competence was abolished in the deletion mutant, we used conjugation to introduce the shuttle vector carrying the various *comB* genes into the *Hp* mutant strain (see *Experimental procedures* for details). The *comB* genes were expressed under the control of the *Hp flaA* promoter (Fig. 3A), and production of the individual ComB proteins was monitored in the immunoblot assay by the ComB-specific antisera (Fig. 4).

Expression of the genes was tested first in the *Escherichia coli* host strain β2155 by immunoblotting (data not shown). ComB8 and ComB10 were produced at the expected size. When ComB9 was produced without ComB8 and ComB10 (pDHO51), several ComB9 degradation products were observed. Furthermore, the presence of *comB10* alone (pDHO52) (Fig. 3A) resulted in the production of the corresponding recombinant protein, but the presence of additional genes upstream of *comB10*, in plasmids pDHO53, pDHO54 and pDHO46 (Fig. 3A), abrogated *comB10* expression. This might result from the overlapping of the stop and start codons of the genes in the operon (Fig. 3B), in which the start codons might not be recognized by *E. coli* ribosomes.

The respective shuttle plasmids were subsequently introduced into a *Hp* Δ*comB* mutant strain, and individual transconjugants were tested for expression of the ComB proteins. In contrast to *E. coli* β2155, *Hp* was able to express the partial or complete operon from the shuttle vector as well as the individually cloned genes (Fig. 4). With the exception of the plasmid containing *comB8–9–10* (pDHO54), all constructs caused strong overproduction of the ComB proteins, probably because of the strong *flaA* promoter (Fig. 4). In plasmid pDHO54, the lower expression might be explained by an alternative intrinsic promoter activity or by a different, inefficient

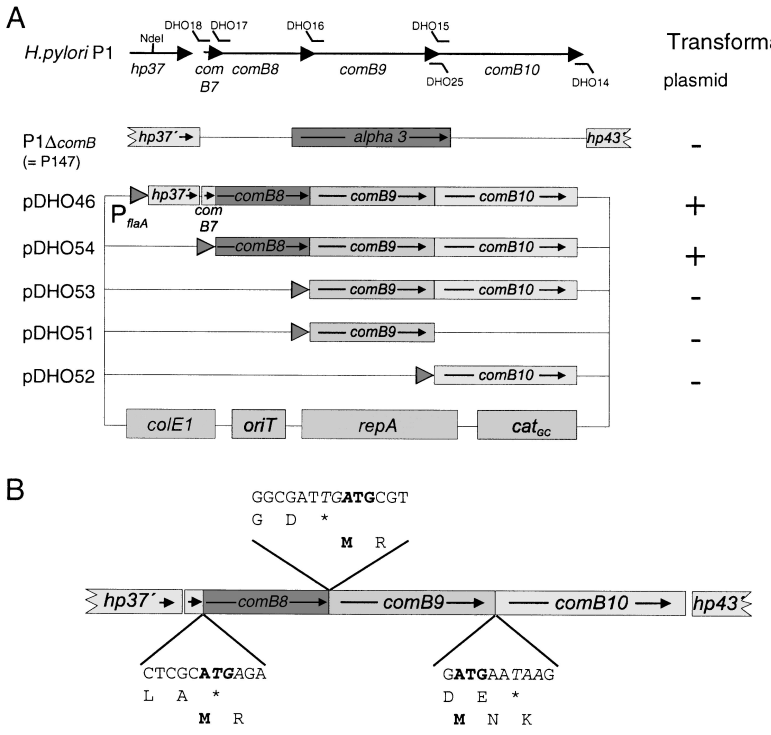

Fig. 3. Construction of a Δ*comB* deletion mutant and its genetic complementation with individual *comB* genes.
A. Schematic overview of the construction of the Δ*comB* deletion mutant P147 and structure of the various plasmids (pDHO46 and pDHO51–pDHO54), based on the shuttle vector pHel2, used for complementation of the deletion mutant. The transformability of Δ*comB* mutants complemented with defined plasmid constructs is indicated at the right border (+, transformation regained; –, no transformation observed). The backbone of the shuttle plasmid is shown at the bottom. *ColE1*, origin of replication; *oriT*, origin of transfer, *repA* gene encoding replication initiation protein for *Hp* plasmid. cat_{GC}, chloramphenicol acetyltransferase gene; *aphA-3*, kanamycin resistance cassette; P_{flaA}, promoter sequence of the *Hp flaA* gene. The location of the primers used for PCR amplification of the *comB* genes is shown on top.
B. Arrangement of the *comB* genes in the operon. The overlap of start (bold) and stop codons (italics) of the open reading frames and the corresponding amino acids in the *comB* operon are highlighted. *stop codon.

Table 2. Bacterial strains and plasmids used in this study.

Strain, plasmid	Genotype or relevant characteristics	Reference or source
E. coli		
HB101	F^- *hsdS20* (r_B^- m_B^-) *recA13 ara-14*	Boyer and Roulland-Dussoix (1969)
β2155	*thrB1004 pro thi strA hsdS lacZ*Δ*M15* (F′ *lacZ*Δ*M15 lacI*^{q} *traD36 proA*$^+$ *proB*$^+$) Δ*dapA*::*erm* (Erm^r) *pir*::RP4 [::*kan* (Km^r) from SM10]	Dehio and Meyer (1997)
E181	HB101 carrying λCH616 prophage	Odenbreit *et al.* (1996)
E145	*polA*1, *su*$^-$, *tsx*, *endA*1, *rnsA*100, *rpsL* λCH616 *rif*	Haas *et al.* (1993b); Haas *et al.* (1993a)
Hp strains		
P1	*Hp* wt strain	Haas *et al.* (1993a)
P2	*Hp* wt clinical isolate	
P3	*Hp* wt clinical isolate	
P8	*Hp* wt clinical isolate	
P9	*Hp* wt clinical isolate	
P12	*Hp* wt strain	Schmitt and Haas (1994)
P24	*Hp* wt clinical isolate	
P29	*Hp* wt strain	Schmitt and Haas (1994)
P30	*Hp* wt clinical isolate	
26695	*Hp* wt strain	Tomb *et al.* (1997)
J99	*Hp* wt strain	Alm *et al.* (1999)
2802 A	*Hp* wt strain	Heep *et al.* (2000)
P147	*Hp* strain P1, Δ*com*B	This study
P12ΔPAI	*Hp* strain P12; deletion of complete *cag*-PAI	Odenbreit *et al.* (2001)
Plasmids		
pMin1	ori_{colE1}, *tet*, *oriT*, t_{fd}, t_{trpA}	Kahrs *et al.* (1995)
pTn*Max5*	cat_{GC}, *res*, ori_{fd}, *tnpR*, *tnpA*	Kahrs *et al.* (1995)
pDH29	pBluescript-II KS carrying *Hp recA*::*ermC*	Schmitt *et al.* (1995)
pDH80	pHel3 carrying *gfp* under control of *flaA* promoter	Heuermann and Haas (1998)
pDHO34	*comB* deletion plasmid, *aphA-3, tet*, pMin1	This study
pDHO51	pHel3 carrying *comB9* gene, Km^r	This study
pDHO52	pHel3 carrying *comB10* gene, Km^r	This study
pDHO53	pHel3 carrying *comB9* and *comB10* gene, Km^r	This study
pDHO54	pHel3 carrying *comB8–comB10* genes, Km^r	This study
pDHO55	pHel3 carrying *comB7–comB10* genes, Km^r	This study
pDHO72	pMin1 carrying *hp0017* gene	This study
pEG21	pCR2.1 TOPO with *rpsL* gene for Sm^R	Fischer *et al.* (1999)
pRK2013	ori_{colE1}, RP4 *tra* genes (mobilizator functions)	Figurski and Helinski (1979)
pHel3	*E. coli–Hp* shuttle vector, *aphA-3*, *oriT*, ori_{colE1}	Heuermann and Haas (1998)

Shine–Dalgarno (SD) sequence used by the RNA polymerase.

All ComB proteins are essential for the natural transformation competence of Hp

To test the individually complemented Δ*comB* deletion mutant for a functional restoration of natural transformation competence, the different complemented mutants were transformed with (i) a plasmid (pDH29) carrying a *Hp recA* gene interrupted by an erythromycin gene cassette; and (ii) chromosomal DNA isolated from different streptomycin-resistant *Hp* mutant strains. *Hp* strains P1 and P1Δ*comB* were used as positive and negative controls in all experiments respectively. Interestingly, ComB9 and ComB10 alone or in combination were sufficient to re-establish transformation competence (Fig. 5A). The minimal requirement for complementation was the presence of the three ComB proteins, ComB8, ComB9 and ComB10. Addition of the *comB7* coding sequence resulted in a further increase in transformation rates with the plasmid construct pDH29 (Fig. 5A). In contrast, transformation with chromosomal *Hp* DNA was reduced (Fig. 5A). Thus, ComB7 appears to have a different effect on transformation by plasmid-derived cloned *Hp* genes isolated from *E. coli* and on *Hp* chromosomal DNA, and apparently modulates the transformation efficiency.

Further components of a transformation-associated second type IV transport system in Hp

In addition to the membrane-spanning structural components, a functional type IV secretion system is characterized by the presence of two or three ATPases, located at the cytoplasmic side of the inner membrane. Such ATPases are thought to provide energy for the correct assembly of the secretion apparatus or the delivery

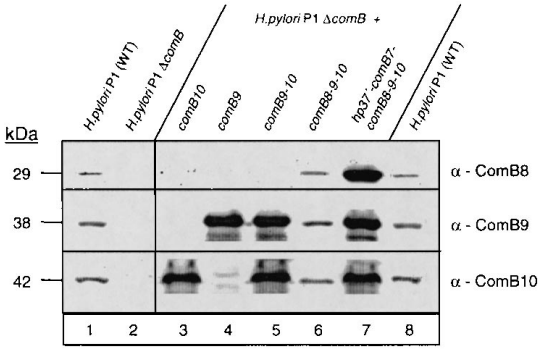

Fig. 4. Expression of individual *comB* genes in the Δ*comB* deletion mutant after genetic complementation. Equal amounts of protein from total lysates of *Hp* were separated by 10% SDS–PAGE, blotted and reacted with polyclonal rabbit antisera AK250 (α-ComB8), AK251 (α-ComB9) and AK252 (α-ComB10). The genotypes of the *Hp* strains are denoted above the lanes. Lanes 1 and 8, *Hp* P1 wt; lane 2, *Hp* P1Δ*comB* (P147); lane 3, P147[pDHO52]; lane 4, P147[pDHO51]; lane 5, P147[pDHO53]; lane 6, P147[pDHO54]; lane 7, P147[pDHO46]. Size markers and the corresponding antisera are denoted.

of the effector molecules, which might be DNA or protein. In the prototype *A. tumefaciens* system, these ATPases are VirB4, VirB11 and VirD4. In addition to the type IV system ATPase genes located on the *cag*-PAI, a second set of such genes is present in *Hp* 26695 corresponding to the ORFs *hp0017*, *hp0441* and *hp0459* (*virB4* homologues) and *hp1421* (*virB11* homologue). The ORFs *hp0441* and *hp0459* are located on the so-called plasticity region of *Hp* strain 26695 and are absent in the *Hp* J99 genome. As *Hp* J99 is naturally transformable, these latter genes cannot be essential for this process. We generated a *Hp* 26695 knock-out mutant in *hp0017* by transposon shuttle mutagenesis using pTn*Max5* (Kahrs *et al.*, 1995). The corresponding mutant was characterized by PCR amplification, and the correct insertion of the mini-Tn in the *hp0017* ORF was verified by partial DNA sequencing of the PCR fragment (data not shown). Interestingly, the knock-out mutant was completely defective in natural transformation competence ($< 10^{-9}$) (Fig. 5C), indicating that the *virB4* homologue *hp0017*, now designated *comB4*, is part of the transformation-associated type IV system of *Hp*.

The comB *and* cag *type IV transport systems are functionally independent*

An obvious question following on from these findings was whether the two type IV systems in *Hp* share some components, which would indicate that one system is dependent on the other. To address this question, we tested first whether a deletion of the complete *cag*-PAI in *Hp* P12 affected the transformation frequency of this strain. The ΔPAI mutant strain was, however, at least as efficient in transformation with plasmid and chromosomal DNA as the wild-type strain (Fig. 5B). Furthermore, the P1 and P12 Δ*comB* deletion mutants, as well as the *Hp* 26695 *comB4*$^-$ mutant, which were completely defective in natural transformation, were not affected in their ability either to translocate the bacterial protein CagA into gastric epithelial cells or to induce interleukin (IL)-8 in AGS cells (data not shown). This indicates that the *cag*-dependent type IV secretion system is functional and that both type IV systems act independently from each other in *Hp*.

Localization of ComB proteins in the bacterial envelope

Detailed computer analysis predicted distinct types of

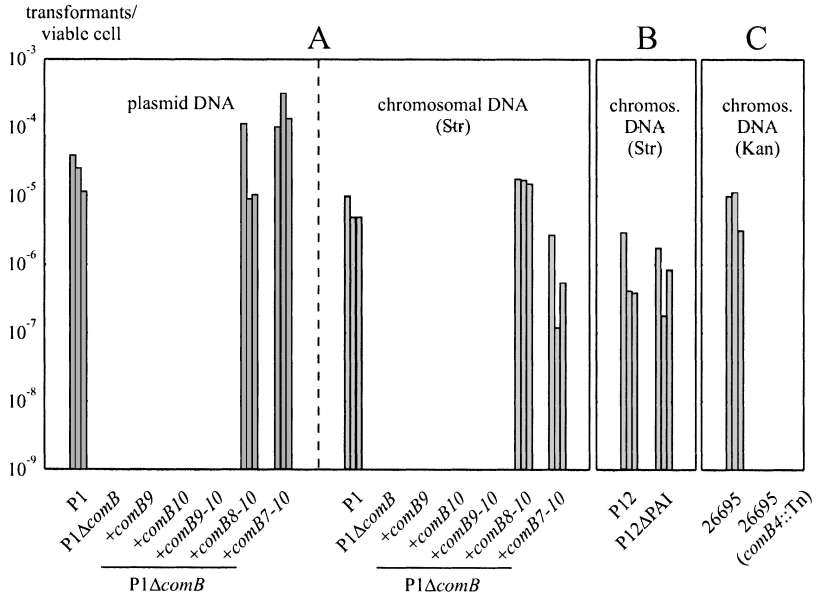

Fig. 5. Transformation rates of the various *Hp* mutant strains and their genetic complementation with genes expressed *in trans* from a shuttle plasmid.
A. Either plasmid pDH29 (1 μg) or chromosomal DNA (1 μg) from a streptomycin-resistant *Hp* P1 derivative was used in the transformation experiments. The P1 wt, P1Δ*comB* mutant and the various complementation steps of P1Δ*comB* are shown below the bars.
B. Transformation rates of the P12 wt and the P12ΔPAI mutant strains obtained with chromosomal DNA of a streptomycin-resistant P12 strain.
C. Determination of the transformation rates of *Hp* 26695 wt and a 26695 *comB4* transposon insertion mutant with chromosomal DNA of a 26695 mutant strain carrying a deletion in the *cagA* gene (*aphA-3*). Transformation frequencies $< 10^{-9}$ are considered as transformation negative. Each bar represents an individual transformation experiment. The transformation rates are represented as the number of transformants per viable *Hp* cell (see *Experimental procedures* for details).

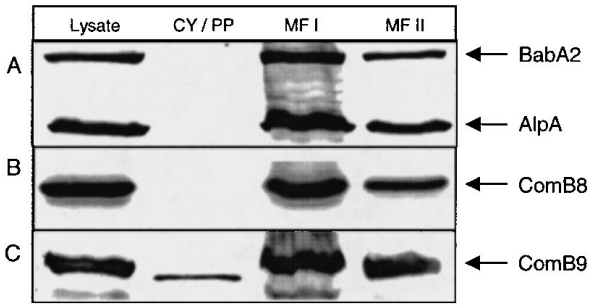

Fig. 6. Membrane association of ComB8 and ComB9.
A–C. A total bacterial lysate of P147[pDHO46] (Lysate) was fractionated in a soluble, cytoplasmic and periplasmic (CY/PP) fraction (lane CY/PP) and the total membrane fraction. Separation of inner and outer membrane was performed according to the N-laurylsarcosine method, resulting in membrane fraction I (lane MFI, corresponding to the CM) and membrane fraction II (lane MFII, corresponding to the OM). The membrane separation was not complete, as seen by the distribution of the outer membrane proteins AlpA and BabA2 between both membranes (A). Equal amounts of protein were loaded on the different lanes.

membrane associations of the individual ComB proteins. According to these analyses, ComB8, like VirB8, carries a transmembrane domain, but no N-terminal signal sequence. ComB9 has both an N-terminal signal sequence and a 20-amino-acid amphipathic helical domain that might interact with the membrane. ComB10 has an N-terminal signal sequence and an α-helical coiled-coil domain (Fig. 7A). To support the computer analysis, *Hp* strain P1 Δ*comB*[pDHO46], which overproduces the ComB proteins (Figs 3A and 4), was used to determine the intracellular location of the ComB proteins. The presence of the individual proteins was tested in the immunoblot assay. ComB8 and ComB10 (not shown) were found exclusively in the membrane fractions, whereas ComB9 was distributed between the soluble and membrane fractions, although the majority was membrane associated (Fig. 6). A clear separation between inner and outer membrane localization by the N-laurylsarcosine method (Filip *et al*., 1973) was, however, not feasible, supporting the experience of other investigators and indicating that

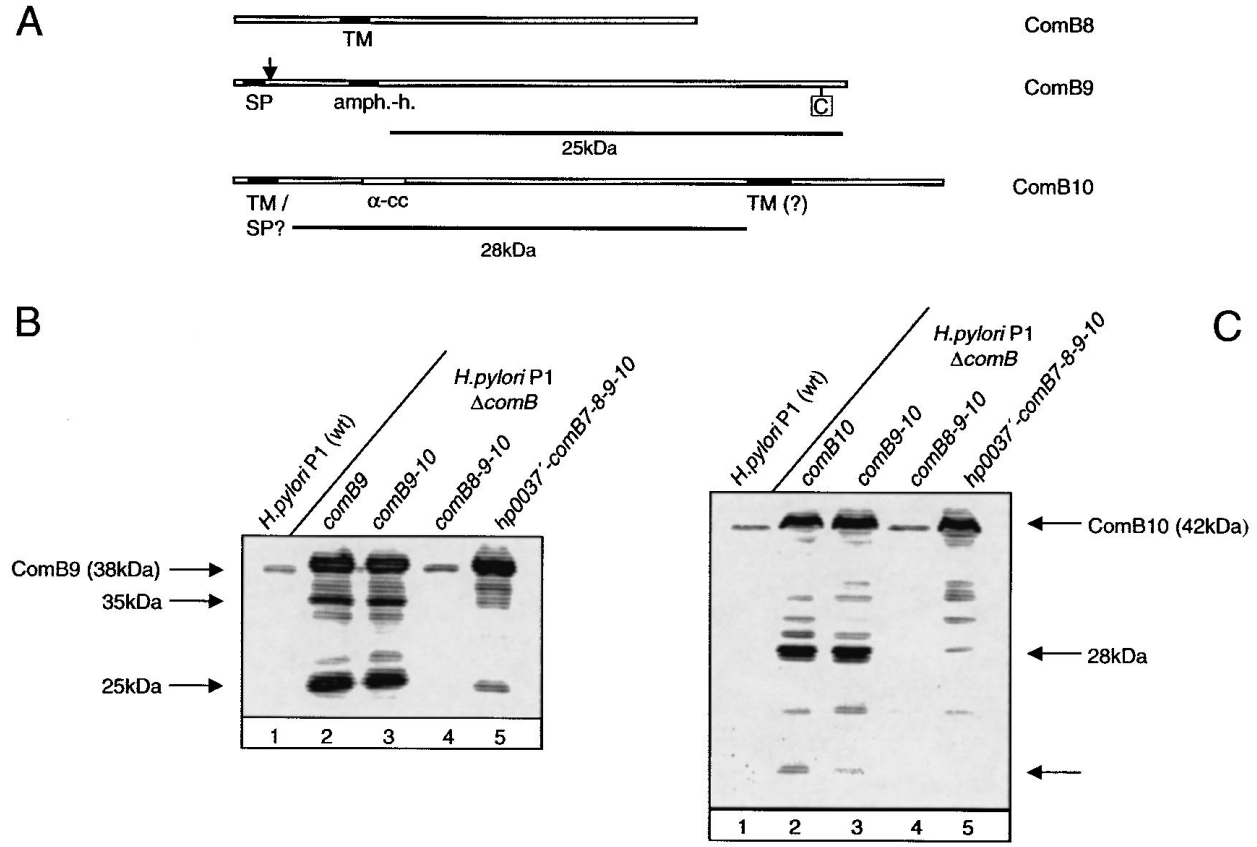

Fig. 7. Structural elements of ComB proteins and the protective effect of ComB7/ComB8 on proteolytic degradation of ComB9 and ComB10.
A. Schematic overview of the location of secondary structure elements of ComB9 and ComB10. SP, signal peptide; α-CC, α-helical coiled-coil domain; TM, transmembrane domain; amph.-h., amphipathic helix, putative membrane-interacting domain; C, cysteine.
B and C. Total-cell lysates of *Hp* P1 wt and complemented P1Δ*comB* mutants were separated by 10% SDS–PAGE and blotted. The genotypes of the corresponding strains are shown above the lanes. The α-ComB9 antiserum (B) and α-ComB10 antiserum (C) both react with the full-length ComB proteins. A major degradation product of 25 kDa (ComB9) or 28 kDa (ComB10) is produced much more strongly when the ComB7/8 proteins are not present.

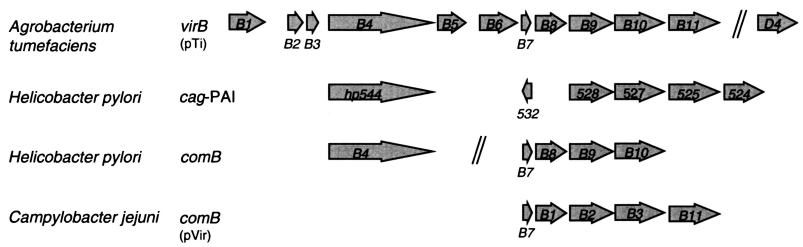

Fig. 8. Organization of two *Hp* type IV systems (*cag*-PAI and *comB*) compared with a plasmid-encoded system in *C. jejuni* involved in natural transformation, adherence and invasion (Bacon *et al*., 2000) and the *A. tumefaciens* prototype type IV system.

the membrane structure of *Hp* is different from that of many other bacteria (Doig and Trust, 1994). According to their structural features and the results from membrane fractionations, ComB8 and ComB10 were considered as integral proteins of the inner membrane.

Putative interacting domains of ComB proteins: stabilization of ComB9 and ComB10 by ComB7/8

Production of the ComB proteins from the shuttle plasmid in the chromosomal Δ*comB* deletion mutant resulted in a strong overproduction of ComB8, ComB9 and ComB10, compared with the wild-type strain (Fig. 4). This overproduction gave rise to a specific processing of ComB9 into major 35 kDa and 25 kDa products and of ComB10 into a 28 kDa fragment (Fig. 7B and C). Interestingly, this rather specific degradation was strongly reduced when ComB7/8 were overproduced together with the other two ComB proteins, indicating that ComB7 or ComB8 might have a stabilizing effect on ComB9 and ComB10. A schematic representation of the structural features of ComB9 and ComB10 revealed that the C-terminal domain of ComB9, which follows the putative transmembrane domain, is 26 kDa. Furthermore, the N-terminal domain between the leader sequence and a putative transmembrane domain in ComB10 corresponds to a 28 kDa fragment, which is in accordance with the size of the corresponding processed fragments (Fig. 7A). Topology studies indicate that both these domains have a periplasmic location. Thus, the data are best explained by stabilization of ComB9 and ComB10 by ComB7/8. When ComB7/8 are absent, the periplasmic domains of ComB9 (25 kDa) or ComB10 (28 kDa) might be rapidly accessible and cleaved by a periplasmic protease.

Discussion

The process of natural transformation is well studied in model organisms such as *Bacillus subtilis*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria gonorrhoeae*. It can be divided into at least five steps: (i) binding of DNA to the bacterial surface; (ii) traversing the outer membrane and the periplasm in Gram-negative bacteria or the murein sacculus in Gram-positive bacteria; (iii) degradation of one DNA strand; (iv) translocation of the remaining DNA single strand through the cytoplasmic membrane into the cytoplasm; and (v) stable integration of the DNA into the chromosome by homologous recombination. From the localization of the ComB proteins, they could be theoretically involved in steps ii–iv, although we do not have a detailed knowledge of the specific function of all ComB proteins.

In most of the naturally competent microorganisms studied thus far, type IV pili, or pilin-like proteins, play a major role in transformation. Such proteins are believed to form a channel spanning the Gram-negative inner and outer membrane or traversing the peptidoglycan layer in Gram-positive bacteria (Dubnau, 1999). After the analysis of two complete *Hp* genomes, we did not detect either any homologues of type IV pilin sequences or a corresponding pilin peptidase (Tomb *et al*., 1997; Alm *et al*., 1999). Instead, we report here that the ComB proteins (ComB4, ComB7–ComB10) are components of a basic type IV secretion apparatus, homologous to the VirB proteins (VirB4, VirB7–VirB10) of *A. tumefaciens* and to the corresponding proteins of other type IV secretion systems (Christie and Vogel, 2000).

Using shuttle vector-based complementation of a Δ*comB* deletion mutant, we clearly demonstrate that each of the ComB proteins is important for the function of the transformation system. Conjugation of plasmids between *Hp* strains has not been shown clearly, but a conjugation-like mechanism has been reported (Kuipers *et al*., 1998). It is notable that the bacterial conjugation of the complementing shuttle plasmid from *E. coli* into *Hp* was not affected by the Δ*comB* deletion, despite the fact that bacterial conjugation is also mediated by type IV transporter systems.

Furthermore, *Hp* carries an additional type IV transporter encoded by the *cag*-PAI, which is involved in translocation of the bacterial protein CagA into human cells. As demonstrated by independent genetic deletion of each locus, these systems act autonomously, which is in agreement with the findings of Israel *et al*. (2000), who also showed that natural transformation is not affected by the *cag*-PAI. Whether the expression of the *comB* genes correlates with the transformation rate in particular *Hp* strains has to be clarified. It has been shown that the modification status of the transforming DNA and the expression of restriction and modification enzymes in the

particular strain determine the natural transformation efficiency. Furthermore, plasmid DNA is restricted to a greater extent than chromosomal DNA during *Hp* transformation (Ando *et al*., 2000; Donahue *et al*., 2000).

The ComB proteins are associated with the inner and/or outer membrane, as shown by fractionation and computer-assisted structural predictions. In addition, the putative ATPase ComB4 with significant sequence homology to the VirB4 ATPase of *A. tumefaciens* is encoded by the *hp0017* gene, located close to the *comB* operon. ComB4 carries a typical ATP/GTP binding site motif A (P-loop). A knock-out mutant in this gene was completely defective in natural transformation, indicating that ComB4 is also part of the transformation-mediating type IV transporter complex. Interestingly, the yeast two-hybrid protein–protein interaction map of *Hp* reveals an interaction of HP0017 with the ComB10 protein, supporting the functional interplay between both components (Rain *et al*., 2001). In *A. tumefaciens*, the VirB4 ATPase is a cytoplasmic membrane protein exposed at the periplasmic surface (Dang and Christie, 1997).

The comparison of several type IV secretion systems in unrelated bacteria with different functions suggests that the proteins homologous to VirB7–VirB10 and the ATPases VirB4 and VirB11 constitute the basic components of a type IV secretion system (Fig. 8). The additional genes that were found to be essential for *Hp* transformation, such as the *dprA* gene or the *comH* gene, might fulfil additional functions, for example binding of the DNA on the cell surface or enzymatic processing, which might be essential in addition to the transport process. The mechanism of DNA translocation might be comparable to the transport of T-DNA from the cytoplasm of *A. tumefaciens* into the plant cell by the VirB protein-mediated type IV secretion apparatus or bacterial plasmid conjugation systems, but in the opposite direction. During conjugation, DNA usually enters the cytoplasm as a single strand that is not a target for restriction endonucleases. Whether *Hp* plasmid or chromosomal DNA passes the ComB channel as single- or double-stranded DNA is unknown.

The following findings support our model that the ComB proteins are part of a DNA uptake apparatus: (i) transposon insertion mutations in any one of the *comB* genes disturbs the transformation competence; (ii) the complementation studies prove that each individual ComB protein is essential for competence development; and (iii) the ComB8, ComB9 and ComB10 proteins are membrane associated and apparently interact to stabilize each other.

ComB7 is a putative lipoprotein with sequence and structural homology to VirB7 of *A. tumefaciens*. A *virB7* knock-out mutant of *A. tumefaciens* causes a clear reduction in the amount of VirB8, VirB9 VirB10 and VirB11 (Fernandez *et al*., 1996b). In the complemented *Hp* Δ*comB* deletion mutant, we also observed a lower amount of ComB8–ComB10, compared with the same proteins co-expressed with ComB7 (Fig. 4). It is not clear, however, whether this corresponds to a lower stability of the ComB proteins or to a lower expression in the first compared with the last construct. Recent data from the *A. tumefaciens* secretion apparatus indicate that VirB8, VirB9 and VirB10 interact and that VirB8 is the initiator protein for the assembly process (Das and Xie, 2000; Kumar *et al*., 2000). Furthermore, in a *virB7* deletion mutant, VirB8 was shown to be involved in stabilization of VirB9 and VirB10, similar to the stabilizing effect shown here for ComB7/8 on ComB9 and ComB10.

Previous studies have demonstrated that a subset of VirB proteins, including VirB3, VirB4 and VirB7–10, have a positive effect on the ability of *A. tumefaciens* to act as a recipient for the conjugation of the plasmid RSF1010 (Bohne *et al*., 1998). Furthermore, A. *tumefaciens* VirB4 mutants with defects in the Walker A nucleotide-binding motif are non-functional for T-DNA export (Berger and Christie, 1993). It was suggested that the oligomeric structure of VirB4, but not its capacity to bind ATP, is important for the assembly of VirB proteins as a DNA uptake system. Thus, in *A. tumefaciens*, VirB4 dimers contribute structural information for the assembly of a *trans*-envelope channel competent for bidirectional DNA transfer, whereas an ATP-dependent activity is required for configuring this channel as a dedicated export machine (Dang *et al*., 1999). The ComB4 protein is essential for the ComB type IV transporter, but it is still unclear whether the ATPase is necessary to constitute the structure of the channel or to energize the DNA transport. In *Hp*, the secretion system for effector proteins (*cag*-PAI) and the DNA import system (ComB system) are provided by two independent type IV systems.

Together with the recently described *comB* system found on a *Campylobacter jejuni* plasmid (Bacon *et al*., 2000), the *comB* system of *Hp* is the first functional type IV system involved in DNA uptake via natural transformation (Fig. 8). This indicates that, through the process of evolution and natural selection, a second, general mechanism for DNA uptake in bacteria may have developed as an alternative to the well-known type IV pilus-based systems. Given the wealth of information from genome sequencing projects and subsequent functional genomics studies, it is probably only a matter of time before additional type IV DNA uptake systems will be discovered in other bacteria.

Experimental procedures

Bacterial strains and culture conditions

Hp strains were grown on GC agar plates (Difco)

supplemented with horse serum (8%), vancomycin (10 mg l^{-1}), trimethoprim (5 mg l^{-1}) and nystatin (1 mg l^{-1}) (serum plates) and incubated for 2–3 days in a microaerobic atmosphere (85% N_2, 10% CO_2, 5% O_2) at 37°C. For the selection of *Hp* mutant strains, serum plates supplemented with chloramphenicol (6 mg l^{-1}) were used. Transformation frequencies of *Hp* were determined on serum plates containing streptomycin (250 mg l^{-1}), kanamycin (8 mg l^{-1}) or erythromycin (10 mg l^{-1}). *E. coli* strains HB101 (Boyer and Roulland-Dussoix, 1969) and DH5α (BRL) were grown on Luria–Bertani (LB) agar plates or in LB liquid medium (Sambrook *et al.*, 1989) supplemented with ampicillin (100 mg l^{-1}), chloramphenicol (30 mg l^{-1}) or tetracycline (15 mg l^{-1}), as appropriate. *E. coli* strain β2155 was grown in LB broth supplemented with 1 mM diaminopimelic acid (DAP) and appropriate antibiotics.

DNA manipulations and plasmid constructions

Cloning and DNA analysis procedures were performed according to the methods of Sambrook *et al*. (1989). Isolation of *Hp* chromosomal DNA was performed as described by Leying *et al*. (1992) or using the QIAamp tissue kit (Qiagen). Plasmid DNA was purified from *E. coli* by the boiling procedure, and *E. coli* cells were made competent for electroporation according to the protocol recommended for the Gene Pulser (Bio-Rad). The Δ*comB* deletion mutant P147 was generated by transformation of *Hp* strain P1 with plasmid pDHO34. Plasmid pDHO34 was constructed by cloning the *aphA-3* cassette between the truncated *hp0037* and *hp0043* genes in vector pMin1. O*rf1* was amplified from chromosomal DNA of *Hp* strain P1 by PCR using primers DHO8 and DHO9; oligonucleotides DHO10 and DHO11 were used for the amplification of *hp0043′*. For complementation of the Δ*comB* mutant P147, different parts of the *comB* locus were amplified by PCR and subcloned into plasmid pDH80 (Heuermann and Haas, 1998). The plasmids pDHO52 (*comB*10) and pDHO51 (*comB*9) were generated by amplification of the corresponding gene fragments pairing oligonucleotides DHO15/DHO14 and DHO16/DHO25 and using chromosomal DNA of *Hp* P1 as template. The genes *comB*9–*comB*10 were amplified with primers DHO16/DHO14; *comB*8 to *comB*10 were amplified with oligonucleotides DHO17/DHO14, resulting in plasmids pDHO53 and pDHO54 respectively. Generally, the *comB* genes were first cloned into the *Bam*HI–*Xho*I sites of pBluescript-II KS+ and then transferred as *Xba*I–*Xho*I fragments into the corresponding cloning sites of pDH80 carrying the *flaA* promoter of *Hp*. For the generation of plasmid pDHO46 (*hp0037′-comB*7–8–9–10), the *comB* locus was amplified with oligonucleotides DHO8 and DHO14, and the resulting PCR product was cloned as an *Nde*I–*Xho*I fragment into the compatible sites of pDH80. The sequences of all oligonucleotides used in this study will be made available upon request.

Transposon shuttle mutagenesis of comB4

The *comB4* gene (*hp0017*) was cloned by PCR amplification of the corresponding reading frame from chromosomal DNA of *Hp* 26695 using primers DHO83 and DHO84, and the fragment was cloned into the minimal vector pMin1 (Table 2). For random insertional mutagenesis with the Tn*Max5* transposon, the plasmid pDHO72 was transformed into *E. coli* E181 harbouring plasmid pTn*Max5*. High-frequency transposition of the mini-transposon was induced by growing the bacteria overnight on LB agar containing tetracycline and chloramphenicol to select for the maintenance of both plasmids and 100 μM IPTG. After transposition, total plasmids were transferred into *E. coli* E145 via conjugation. Single transconjugants were analysed, and the insertion sites of the mini-transposons were mapped by restriction digests and DNA sequencing. The interrupted gene was inserted into the chromosome of *Hp* 26695 by natural transformation of the plasmid after homologous recombination. The correct insertion of the transposon in the chromosome was verified by PCR using primers DHO83 and DHO84.

Natural transformation and conjugation of Hp

Natural transformation of *Hp* strains was performed with plasmid or chromosomal DNA according to the procedure described by Haas *et al*. (1993a). Chromosomal DNA was isolated from streptomycin-resistant mutants of *Hp* (NCTC11637, P1, P12) to transform *Hp* to streptomycin resistance (0.5–1 μg of DNA). Plasmid pDH29 (*recA*::*ermC*) was used for plasmid transformation (Heuermann and Haas, 1998). Bacteria were harvested from serum plates and suspended to an OD_{550} of 0.1 in brain–heart infusion (BHI) medium containing 10% FCS. DNA was added, and incubation was extended for 4–6 h under microaerophilic conditions (10% CO_2, 37°C), before the suspension was plated on selective serum plates.

Conjugation of the shuttle vector pHel2 carrying the *comB* genes from *E. coli* to *Hp* was essentially performed as described previously (Heuermann and Haas, 1998). *E. coli* β2155 was used as a donor for conjugation. The pHel2 plasmid carrying the *oriT* sequence of plasmid RP4 confers resistance to chloramphenicol (Cm^r) and is mobilized *in trans* by transfer functions of plasmid RK4 integrated in the chromosome of β2155 (Table 2). Growth of the *dapA* mutant β2155 is strictly dependent on exogenously supplied DAP; hence, the removal of DAP provides an efficient counterselection against this donor. The transformation frequency for a given mutant was calculated as the number of chloramphenicol-, kanamycin- or streptomycin-resistant colonies per colony-forming unit (cfu). Three independent experiments were performed.

SDS–PAGE, immunoblots and antisera

For the detection of *comB* gene expression in *Hp* wild-type and isogenic mutant strains, the bacteria were collected from serum plates, suspended in 300 μl of sample solution (Laemmli, 1970) and sonicated. Boiled aliquots were subjected to SDS–PAGE using a mini-gel apparatus (Bio-Rad) and blotted onto nitrocellulose membranes at 1 mA cm^{-2} using a semi-dry blot system (Biotec Fischer). The filters were blocked with 3% bovine serum albumin (BSA) in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated with antisera AK250, AK251 or AK252 (1:1000 dilution). Alkaline phosphatase-coupled protein A was used to visualize bound antibody. For the detection of AlpA and BabA2, rabbit antisera

were generated against the corresponding fusion proteins (Odenbreit *et al*., 1999; unpublished results).

Computer analyses

Protein predictions were performed using the program PREDICTPROTEIN (http://www.embl-heidelberg.de/predictprotein/). Membrane associations were calculated using the programs PEPTIDESTRUCTURE, TMPRED, TMHMM and PREDICTPROTEIN. The P-loop in ComB4 was predicted by the MOTIFS program (http://www.motif.genome.ad.jp/).

Acknowledgements

The authors are grateful to C. Dehio for the *E. coli* strain β2155, and wish to thank B. P. Burns for critical comments on the manuscript. We thank J. Püls for performing the IL-8 assays with the *comB4* mutant. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) to R.H. (HA2697/3-1).

References

Alm, R.A., Ling, L.S., Moir, D.T., King, B.L., Brown, E.D., Doig, P.C., *et al.* (1999) Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* **397**: 176–180.

Andersson, S.G., Zomorodipour, A., Andersson, J.O., Sicheritz-Ponten, T., Alsmark, U.C., Podowski, R.M., *et al.* (1998) The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396**: 133–140.

Ando, T., Israel, D.A., Kusugami, K., and Blaser, M.J. (1999) HP0333, a member of the *dprA* family, is involved in natural transformation in *Helicobacter pylori*. *J Bacteriol* **181**: 5572–5580.

Ando, T., Xu, Q., Torres, M., Kusugami, K., Israel, D.A., and Blaser, M.J. (2000) Restriction–modification system differences in *Helicobacter pylori* are a barrier to interstrain plasmid transfer. *Mol Microbiol* **37**: 1052–1065.

Asahi, M., Azuma, T., Ito, S., Ito, Y., Suto, H., Nagai, Y., *et al.* (2000) *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. *J Exp Med* **191**: 593–602.

Backert, S., Ziska, E., Brinkmann, V.Z.-A.U., Fauconnier, A., Jungblut, P.R., Naumann, M., and Meyer, T.F. (2000) Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cell Microbiol* **2**: 155–164.

Bacon, D.J., Alm, R.A., Burr, D.H., Hu, L., Kopecko, D.J., Ewing, C.P., *et al.* (2000) Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. *Infect Immun* **68**: 4384–4390.

Baron, C., Thorstenson, Y.R., and Zambryski, P.C. (1997) The lipoprotein VirB7 interacts with VirB9 in the membranes of *Agrobacterium tumefaciens*. *J Bacteriol* **179**: 1211–1218.

Berger, B.R., and Christie, P.J. (1993) The *Agrobacterium tumefaciens virB4* gene product is an essential virulence protein requiring an intact nucleoside triphosphate-binding domain. *J Bacteriol* **175**: 1723–1734.

Bittinger, M.A., Gross, J.A., Widom, J., Clardy, J., and Handelsman, J. (2000) *Rhizobium etli* CE3 carries *vir* gene homologs on a self-transmissible plasmid. *Mol Plant–Microbe Interact* **13**: 1019–1021.

Bohne, J., Yim, A., and Binns, A.N. (1998) The Ti plasmid increases the efficiency of *Agrobacterium tumefaciens* as a recipient in *virB*-mediated conjugal transfer of an IncQ plasmid. *Proc Natl Acad Sci USA* **95**: 7057–7062.

Boyer, H.W., and Roulland-Dussoix, D. (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41**: 459–472.

Censini, S., Lange, C., Xiang, Z.Y., Crabtree, J.E., Ghiara, P., Borodovsky, M., *et al.* (1996) Cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci USA* **93**: 14648–14653.

Christie, P.J., and Vogel, J.P. (2000) Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends Microbiol* **8**: 354–360.

Covacci, A., Telford, J.L., Del Giudice, G., Parsonnet, J., and Rappuoli, R. (1999) *Helicobacter pylori* virulence and genetic geography. *Science* **284**: 1328–1333.

Dang, T.A., and Christie, P.J. (1997) The VirB4 ATPase of *Agrobacterium tumefaciens* is a cytoplasmic membrane protein exposed at the periplasmic surface. *J Bacteriol* **179**: 453–462.

Dang, T.A., Zhou, X.R., Graf, B., and Christie, P.J. (1999) Dimerization of the *Agrobacterium tumefaciens* VirB4 ATPase and the effect of ATP-binding cassette mutations on the assembly and function of the T-DNA transporter. *Mol Microbiol* **32**: 1239–1253.

Das, A., and Xie, Y.H. (2000) The *Agrobacterium* T-DNA transport pore proteins VirB8, VirB9, and VirB10 interact with one another. *J Bacteriol* **182**: 758–763.

Das, A., Anderson, L.B., and Xie, Y.H. (1997) Delineation of the interaction domains of *Agrobacterium tumefaciens* VirB7 and VirB9 by use of the yeast two-hybrid assay. *J Bacteriol* **179**: 3404–3409.

Dehio, C., and Meyer, M. (1997) Maintenance of broad-host-range incompatibility group-P and group-Q plasmids and transposition of Tn5 in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*. *J Bacteriol* **179**: 538–540.

Doig, P., and Trust, T.J. (1994) Identification of surface-exposed outer-membrane antigens of *Helicobacter pylori*. *Infect Immun* **62**: 4526–4533.

Donahue, J.P., Israel, D.A., Peek, R.M., Blaser, M.J., and Miller, G.G. (2000) Overcoming the restriction barrier to plasmid transformation of *Helicobacter pylori*. *Mol Microbiol* **37**: 1066–1074.

Dubnau, D. (1999) DNA uptake in bacteria. *Annu Rev Microbiol* **53**: 217–244.

Fernandez, D., Dang, T.A., Spudich, G.M., Zhou, X.R., Berger, B.R., and Christie, P.J. (1996a) The *Agrobacterium tumefaciens virB7* gene product, a proposed component of the T-complex transport apparatus, is a membrane-associated lipoprotein exposed at the periplasmic surface. *J Bacteriol* **178**: 3156–3167.

Fernandez, D., Spudich, G.M., Zhou, X.R., and Christie, P.J. (1996b) The *Agrobacterium tumefaciens* VirB7 lipoprotein is required for stabilization of VirB proteins during assembly

of the T-complex transport apparatus. *J Bacteriol* **178**: 3168–3176.

Figurski, D.H., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci USA* **76**: 1648–1652.

Filip, C., Fletcher, G., Wulff, J.L., and Earhart, C.F. (1973) Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J Bacteriol* **115**: 717–722.

Fischer, W., Schwan, D., Gerland, E., Erlenfeld, G.E., Odenbreit, S., and Haas, R. (1999) A plasmid-based vector system for the cloning and expression of *Helicobacter pylori* genes encoding outer membrane proteins. *Mol Gen Genet* **262**: 501–507.

Haas, R., Meyer, T.F., and van Putten, J.P.M. (1993a) Aflagellated mutants of *Helicobacter pylori* generated by genetic transformation of naturally competent strains using transposon shuttle mutagenesis. *Mol Microbiol* **8**: 753–760.

Haas, R., Kahrs, A.F., Facius, D., Allmeier, H., Schmitt, R., and Meyer, T.F. (1993b) Tn*Max* – a versatile mini-transposon for the analysis of cloned genes and shuttle mutagenesis. *Gene* **130**: 23–31.

Heep, M., Odenbreit, S., Beck, D., Decker, J., Prohaska, E., Rieger, U., and Lehn, N. (2000) Mutations at four distinct regions of the *rpoB* gene can reduce the susceptibility of *Helicobacter pylori* to rifamycins. *Antimicrob Agents Chemother* **44**: 1713–1715.

Heuermann, D., and Haas, R. (1998) A stable shuttle vector system for efficient genetic complementation of *Helicobacter pylori* strains by transformation and conjugation. *Mol Gen Genet* **257**: 519–528.

Hofreuter, D., Odenbreit, S., Henke, G., and Haas, R. (1998) Natural competence for DNA transformation in *Helicobacter pylori*: identification and genetic characterization of the *comB* locus. *Mol Microbiol* **28**: 1027–1038.

Hofreuter, D., Odenbreit, S., Püls, J., Schwan, D., and Haas, R. (2000) Genetic competence in *Helicobacter pylori:* mechanisms and biological implications. *Res Microbiol* **151**: 487–491.

Israel, D.A., Lou, A.S., and Blaser, M.J. (2000) Characteristics of *Helicobacter pylori* natural transformation. *FEMS Microbiol Lett* **186**: 275–280.

Kahrs, A.F., Odenbreit, S., Schmitt, W., Heuermann, D., Meyer, T.F., and Haas, R. (1995) An improved Tn*Max* mini-transposon system suitable for sequencing, shuttle mutagenesis and gene fusions. *Gene* **167**: 53–57.

Karudapuram, S., and Barcak, G.J. (1997) The *Haemophilus influenzae dprABC* genes constitute a competence-inducible operon that requires the product of the *tfoX* (*sxy*) gene for transcriptional activation. *J Bacteriol* **179**: 4815–4820.

Kuipers, E.J., Israel, D.A., Kusters, J.G., and Blaser, M.J. (1998) Evidence for a conjugation-like mechanism of DNA transfer in *Helicobacter pylori*. *J Bacteriol* **180**: 2901–2905.

Kuldau, G.A., De Vos, G., Owen, J., McCaffrey, G., and Zambryski, P. (1990) The *virB* operon of *Agrobacterium tumefaciens* pTiC58 encodes 11 open reading frames. *Mol Gen Genet* **221**: 256–266.

Kumar, R.B., Xie, Y.H., and Das, A. (2000) Subcellular localization of the *Agrobacterium tumefaciens* T-DNA transport pore proteins: VirB8 is essential for the assembly of the transport pore. *Mol Microbiol* **36**: 608–617.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**: 680–685.

Leying, H., Suerbaum, S., Geis, G., and Haas, R. (1992) Characterisation of *flaA*, a *Helicobacter pylori* flagellin gene. *Mol Microbiol* **6**: 2863–2874.

Masui, S., Sasaki, T., and Ishikawa, H. (2000) Genes for the type IV secretion system in an intracellular symbiont, *wolbachia*, a causative agent of various sexual alterations in arthropods. *J Bacteriol* **182**: 6529–6531.

Nedenskov-Sorensen, P., Bukholm, G., and Bovre, K. (1990) Natural competence for genetic transformation in *Campylobacter pylori*. *J Infect Dis* **161**: 365–366.

O'Callaghan, D., Cazevieille, C., Allardet-Servent, A., Boschiroli, M.L., Bourg, G., Foulongne, V., *et al.* (1999) A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. *Mol Microbiol* **33**: 1210–1220.

Odenbreit, S., Wieland, B., and Haas, R. (1996) Cloning and genetic characterization of *Helicobacter pylori* catalase and construction of a catalase-deficient mutant strain. *J Bacteriol* **178**: 6960–6967.

Odenbreit, S., Till, M., Hofreuter, D., Faller, G., and Haas, R. (1999) Genetic and functional characterisation of the *alpAB* gene locus essential for adhesion of *Helicobacter pylori* to human gastric tissue. *Mol Microbiol* **31**: 1537–1548.

Odenbreit, S., Püls, J., Sedlmaier, B., Gerland, E., Fischer, W., and Haas, R. (2000) Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* **287**: 1497–1500.

Odenbreit, S., Gebert, B., Püls, J., Fischer, W., and Haas, R. (2001) Interaction of *Helicobacter pylori* with professional phagocytes: role of the *cag* pathogenicity island and translocation, phosphorylation and specific processing of CagA. *Cell Microbiol* **3**: 21–31.

Padmalayam, I., Karem, K., Baumstark, B., and Massung, R. (2000) The gene encoding the 17-kDa antigen of *Bartonella henselae* is located within a cluster of genes homologous to the *virB* virulence operon. *DNA Cell Biol* **19**: 377–382.

Pansegrau, W., Lanka, E., Barth, P.T., Figurski, D.H., Guiney, D.G., Haas, D., *et al.* (1994) Complete nucleotide sequence of Birmingham IncP alpha plasmids. Compilation and comparative analysis. *J Mol Biol* **239**: 623–663.

Rain, J.C., Selig, L., De Reuse, H., Battaglia, V., Reverdy, C., Simon, S., *et al.* (2001) The protein–protein interaction map of *Helicobacter pylori*. *Nature* **409**: 211–215.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Nolan, C., and Ferguson, M. (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schmitt, W., and Haas, R. (1994) Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. *Mol Microbiol* **12**: 307–319.

Schmitt, W., Odenbreit, S., Heuermann, D., and Haas, R. (1995) Cloning of the *Helicobacter pylori recA* gene and functional characterization of its product. *Mol Gen Genet* **248**: 563–572.

Segal, E.D., Cha, J., Lo, J., Falkow, S., and Tompkins, L.S. (1999a) Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc Natl Acad Sci USA* **96**: 14559–14564.

Segal, G., Russo, J.J., and Shuman, H.A. (1999b) Relationships between a new type IV secretion system and the *icm/dot* virulence system of *Legionella pneumophila*. *Mol Microbiol* **34**: 799–809.

Sieira, R., Comerci, D.J., Sanchez, D.O., and Ugalde, R.A. (2000) A homologue of an operon required for DNA transfer in *Agrobacterium* is required in *Brucella abortus* for virulence and intracellular multiplication. *J Bacteriol* **182**: 4849–4855.

Smeets, L.C., Bijlsma, J.J., Kuipers, E.J., Vandenbroucke-Grauls, C.M., and Kusters, J.G. (2000a) The *dprA* gene is required for natural transformation of *Helicobacter pylori*. *FEMS Immunol Med Microbiol* **27**: 99–102.

Smeets, L.C., Bijlsma, J.J., Boomkens, S.Y., Vandenbroucke-Grauls, C.M., and Kusters, J.G. (2000b) comH, *a novel gene essential for natural transformation of* Helicobacter pylori. *J Bacteriol* **182**: 3948–3954.

Spudich, G.M., Fernandez, D., Zhou, X.R., and Christie, P.J. (1996) Intermolecular disulfide bonds stabilize VirB7 homodimers and VirB7/VirB9 heterodimers during biogenesis of the *Agrobacterium tumefaciens* T-complex transport apparatus. *Proc Natl Acad Sci USA* **93**: 7512–7517.

Stein, M., Rappuoli, R., and Covacci, A. (2000) Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after *cag*-driven host cell translocation. *Proc Natl Acad Sci USA* **97**: 1263–1268.

Suerbaum, S., Smith, J.M., Bapumia, K., Morelli, G., Smith, N.H., Kunstmann, E., *et al.* (1998) Free recombination within *Helicobacter pylori*. *Proc Natl Acad Sci USA* **95**: 12619–12624.

Thorsted, P.B., Macartney, D.P., Akhtar, P., Haines, A.S., Ali, N., Davidson, P., *et al.* (1998) Complete sequence of the IncPbeta plasmid R751: implications for evolution and organisation of the IncP backbone. *J Mol Biol* **282**: 969–990.

Tomb, J.-F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., *et al.* (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**: 539–547.

Xiang, Z.Y., Censini, S., Bayeli, P.F., Telford, J.L., Figura, N., Rappuoli, R., and Covacci, A. (1995) Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect Immun* **63**: 94–98.