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Protein Subassemblies of the *Helicobacter pylori* Cag Type IV Secretion System Revealed by Localization and Interaction Studies▽†

Stefan Kutter,[1] Renate Buhrdorf,[1]‡ Jürgen Haas,[2,3] Wulf Schneider-Brachert,[4] Rainer Haas,[1] and Wolfgang Fischer[1]*

Abteilung Bakteriologie[1] and Abteilung Virologie,[2] Max von Pettenkofer Institut für Hygiene und Medizinische Mikrobiologie, Ludwig Maximilians Universität, 80336 München, Germany; Division of Pathway Medicine, University of Edinburgh, Edinburgh, United Kingdom[3]; and Institut für Medizinische Mikrobiologie und Hygiene, Universität Regensburg, 93042 Regensburg, Germany[4]

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Type IV secretion systems are possibly the most versatile protein transport systems in gram-negative bacteria, with substrates ranging from small proteins to large nucleoprotein complexes. In many cases, such as the *cag* pathogenicity island of *Helicobacter pylori*, genes encoding components of a type IV secretion system have been identified due to their sequence similarities to prototypical systems such as the VirB system of *Agrobacterium tumefaciens*. The Cag type IV secretion system contains at least 14 essential apparatus components and several substrate translocation and auxiliary factors, but the functions of most components cannot be inferred from their sequences due to the lack of similarities. In this study, we have performed a comprehensive sequence analysis of all essential or auxiliary Cag components, and we have used antisera raised against a subset of components to determine their subcellular localization. The results suggest that the Cag system contains functional analogues to all VirB components except VirB5. Moreover, we have characterized mutual stabilization effects and performed a comprehensive yeast two-hybrid screening for potential protein-protein interactions. Immunoprecipitation studies resulted in identification of a secretion apparatus subassembly at the outer membrane. Combining these data, we provide a first low-resolution model of the Cag type IV secretion apparatus.

Type IV secretion systems of gram-negative bacteria have evolved to transport DNA in the form of nucleoprotein complexes, for example, as bacterial conjugation systems, DNA uptake systems, or the virulence-associated VirB system of *Agrobacterium tumefaciens*, but they also transport proteins and are important virulence determinants in many pathogenic bacteria (11). In *Helicobacter pylori*, which is the principal cause of chronic active gastritis and peptic ulcer disease and which is also involved in the development of gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer (46, 59), the Cag type IV secretion system is responsible for induction of a pronounced proinflammatory response and for translocation of the effector protein CagA into various host cells (23). The exact function of CagA protein translocation in vivo is not known, but *H. pylori* strains containing the *cag* pathogenicity island, which encodes the Cag type IV secretion apparatus, are associated with an enhanced risk of developing peptic ulcers or adenocarcinoma (7). Moreover, the CagA protein itself contributes to the development of a corpus-dominant gastritis in Mongolian gerbils and therefore to a highly increased risk of gastric cancer (51). Consequently, the Cag type IV secretion system is considered a major virulence determinant of *H. pylori*.

The VirB system of *A. tumefaciens* is considered a prototypical type IV secretion system (14). The VirB secretion apparatus consists of (i) a core complex comprising the VirB6, VirB7, VirB8, VirB9, VirB10, and probably VirB3 proteins that may form a translocation channel across both bacterial membranes; (ii) two components (VirB2 and VirB5) that build up a periplasmic or extracellular pilus structure together with VirB7; (iii) two inner-membrane-associated ATPases (VirB4 and VirB11) providing the energy for apparatus assembly and/or substrate transport; (iv) a peptidoglycan hydrolase (VirB1) which facilitates transport channel assembly; and (v) the coupling protein VirD4, which is required as a secretion signal receptor and possibly as a DNA substrate translocator. The assembly of this secretion apparatus is thought to occur in distinct steps and to involve subassemblies, which have been defined by stabilization networks, biochemical studies, and yeast two-hybrid interaction studies (reviewed in reference 14).

Type IV secretion systems in other gram-negative bacteria have often been identified due to the presence of genes with *virB* sequence similarities. However, some of these similarities are very limited, and individual type IV secretion systems might have adapted to their particular functions by acquiring additional factors. We have previously shown that the Cag type IV secretion apparatus consists of at least 14 essential components (24), only four of which display significant sequence similarities to components of the *A. tumefaciens* VirB system. Furthermore, seven additional components are necessary for CagA translocation or have auxiliary functions. Despite the

* Corresponding author. Mailing address: Max von Pettenkofer Institut, Pettenkoferstr. 9a, D-80336 München, Germany. Phone: 49 89 51605277. Fax: 49 89 51605223. E-mail: fischer@mvp.uni-muenchen.de.

‡ Present address: 4SC AG, Am Klopferspitz 19a, 82152 Martinsried, Germany.

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lack of sequence similarities, further components with analogous functions to the VirB components are likely to exist. It is currently unknown how the Cag secretion apparatus is assembled, although a recent study detected multiple interactions among a subset of Cag secretion apparatus components using a yeast two-hybrid approach (10).

In this study, we provide evidence for the presence of further VirB-analogous proteins among *cag*-encoded proteins, and we also present data about the localization and mutual interactions between Cag secretion system components. Integrating localization and interaction data, we propose a first structural model of the Cag type IV secretion apparatus. Although these data reveal further similarities of the Cag type IV secretion system to the prototypical type IV secretion systems, the data also underscore the evolutionary distance and individual adaptations of these systems.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. pylori* strains 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) and incubated for 16 to 60 h in a microaerobic atmosphere (85% N_2, 10% CO_2, and 5% O_2) at 37°C. *Escherichia coli* strains Top10 (Invitrogen) and DH5α (BRL) were grown on Luria-Bertani agar plates or in Luria-Bertani liquid medium (54) supplemented with ampicillin (100 mg/liter), gentamicin (10 mg/liter), or kanamycin (40 mg/liter), as appropriate.

DNA manipulations. Standard cloning and DNA analysis procedures were performed according to Sambrook and Russell (54). Plasmid DNA was purified from *E. coli* by the boiling procedure, and *E. coli* cells for electroporation were prepared according to the protocol recommended for the Gene Pulser (Bio-Rad). Amplification of DNA fragments by PCR was performed as described previously (28).

Production of antisera and immunoblotting. The generation of antisera against CagY/HP527 (proteins encoded by hp527) and CagT/HP532 has been described previously (52). The antiserum against Cagα (HP525) was generated by rabbit immunization with purified Cagα protein (36), which was kindly provided by E. Lanka (Max Planck Institute for Molecular Genetics, Berlin, Germany). For the production of antisera against CagX, CagL, and CagM, plasmids pRB18, pJP75, and pQE30-cagM, respectively, were used. Plasmid pRB18 was constructed by amplification of the *cagX* (jhp477) gene of strain J99 (2) without the N-terminal signal sequence using primers RB1 (5′-CGGAATTCTGCAGG TAGGGTGAAAGTGGTG-3′) and RB2 (5′-CCGCTCGAGTTTATCTCTGA CAAGAGG-3′) and cloning into the EcoRI and XhoI sites of the pEV40 expression vector (47). Plasmid pJP75 was constructed by amplification of the *cagL* (jhp487) gene lacking its N-terminal signal sequence using primers JP51 (5′-CGGAATTCTGCAGAAGATATAACAAGCGGC-3′) and JP52 (5′-ACC GCTCGAGTCATTTAACAATGATCTT-3′) and cloning into the same vector. Plasmid pQE30-cagM was generated by cloning the *cagM* (hp537) gene of strain 26695 into a pQE30 expression vector (Qiagen, Hilden, Germany).

His_6-tagged fusion proteins were overproduced in *E. coli* 2136 or *E. coli* DH5α from plasmids pRB18, pJP75, and pQE30-537 and purified from inclusion bodies according to Strebel et al. (58). The purified fusion proteins were used to raise corresponding rabbit polyclonal antisera. Two rabbit antisera against CagC/HP546 (anti-$CagC_1$ and anti-$CagC_2$) were raised using synthetic peptides corresponding to amino acids 66 to 79 and amino acids 106 to 115, respectively, of the protein from strain 26695 (Eurogentec, Liège, Belgium). Antisera against RecA and AlpB have been described previously (22, 44). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as described previously (57). For the development of Western blots, nitrocellulose or polyvinylidene difluoride filters were blocked with 3% bovine serum albumin in 50 mM Tris-HCl (pH 7.5)–150 mM NaCl and incubated with the corresponding antiserum at a dilution of 1:3,000. Protein A-conjugated alkaline phosphatase (Sigma, Deisenhofen, Germany) was used to visualize bound antibody. Quantitative values were obtained by densitometric analysis of at least three independent immunoblots.

Bacterial cell subfractionation. *H. pylori* cells were grown on solid or in liquid medium for 24 to 48 h and then harvested, washed, and resuspended in preparation buffer (10 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin). Bacteria were lysed by passage through a French pressure cell press, and the lysate was centrifuged for 10 min at 7,000 × *g* to remove unbroken cells and cell debris. The supernatant was collected and separated by ultracentrifugation (45 min at 230,000 × *g*) into soluble (cytoplasmic and periplasmic) and total membrane fractions. Proteins in the soluble fractions were concentrated by chloroform-methanol precipitation (67), and the membrane fractions were washed with preparation buffer and resuspended in SDS-PAGE sample solution. For differential extraction of membranes, the pellet containing the total membranes was resuspended in preparation buffer, and the membranes were extracted by addition of either *N*-lauroyl sarcosine or zwittergent 3–14 to a final concentration of 1% (wt/vol), incubated on ice for 30 min, and subjected to ultracentrifugation (45 min at 230,000 × *g*). The pellet of the *N*-lauroyl sarcosine extraction was considered an outer membrane fraction, and the pellet of the zwittergent 3–14 extraction was considered a cytoplasmic membrane fraction.

TABLE 1. Genes or gene fragments that were used for the yeast two-hybrid screen[a]

Gene	Protein designation	Region[b]
hp522	Cagδ/Cag3	23–481
hp523	Cagγ/Cag4	1–169
hp524	$Cag\beta_a/Cag5_a$	95–146
hp524	$Cag\beta_b/Cag5_b$	162–748
hp525	Cagα/VirB11	1–330
hp527	$CagY_a$	1–345
hp527	$CagY_b$	363–1799
hp527	$CagY_c$	1815–1927
hp528	CagX	29–522
hp529	CagW	26–535
hp530	CagV	59–252
hp531	CagU	1–218
hp532	CagT	21–280
hp537	CagM	18–376
hp538	CagN	25–306
hp539	CagL	21–237
hp541	CagH	52–370
hp543	CagF	1–268
hp544	CagE	82–983
hp545	CagD	31–207
hp546	CagC	1–115
hp547	CagA	1–1186

[a] Genes and gene fragments were cloned in parallel into bait and prey vectors pDEST-GBKT7 and pDEST-GADT7, respectively.
[b] Amino acid ranges encoded by the cloned fragments are indicated.

Yeast two-hybrid assay. To generate yeast two-hybrid bait and prey libraries comprising *cag* pathogenicity island genes, 22 full-length open reading frames (excluding N-terminal signal sequences), and 10 partial open reading frames (Table 1) were amplified from chromosomal DNA of strain 26695 by nested PCR (64). The internal forward and reverse primers contained internal parts of *attB1* and *attB2* recombination sites (5′-AAAAAGCAGGCTCCGCCATG-3′ and 5′-AGAAAGCTGGGTCTA-3′, respectively) together with the corresponding gene-specific sequences. External forward and reverse primers (5′-GGGGACA AGTTTGTACAAAAAAGCAGGCT-3′ and 5′-GGGGACCACTTTGTACAA GAAAGCTGGGT-3′, respectively) contained external *attB1* and *attB2* sequences. PCR products obtained with these primers were cloned, using BP clonase, into the entry vector pDONR207 (Invitrogen) and subsequently into the destination vectors pDEST-GADT7 (prey vector) and pDEST-GBKT7 (bait vector) using LR clonase. Bait and prey plasmids were transformed into the haploid *Saccharomyces cerevisiae* strains Y187 and AH109. Mating and selection of diploid yeast cells were performed as described previously (64). Briefly, yeast strains containing the different prey plasmids were cultivated in a 96-well plate in duplicate and spotted on a synthetic dextrose (SD) agar plate using a 384-pin replicator, resulting in an array with eight colonies for each construct. This master plate was replicated once for each bait construct. Yeast strains carrying the bait plasmids were cultivated in OmniTrays (Nunc) and transferred on top of one prey master plate replica each. Yeasts on these plates were then allowed to mate, and mating was selected for by transferring on SD medium lacking tryptophan (Trp^-) and leucine (Leu^-), thus generating all possible combinations of bait and prey plasmids. After growth on SD-Trp^--Leu^- medium, yeast colonies

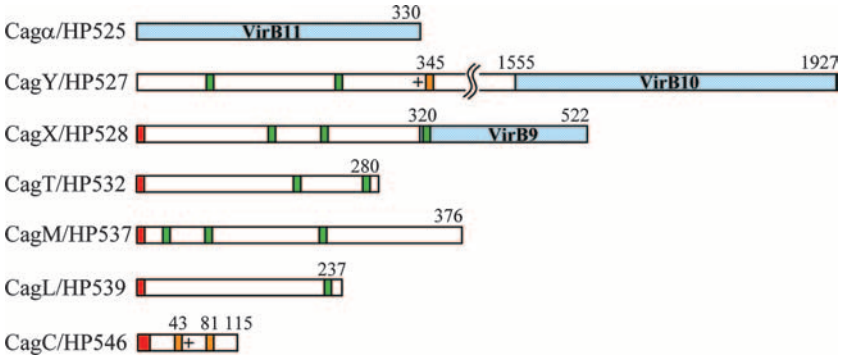

FIG. 1. Graphical representation of potential membrane-spanning segments and sequence similarity regions of Cag secretion apparatus components examined in this study. The proteins are drawn to scale as bars, with the number of amino acid residues deduced from the published genome sequence of strain 26695 (63) indicated at their C-terminal ends. The regions of similarity to the corresponding *A. tumefaciens* VirB proteins are shown in blue. Potential transmembrane helices predicted by the PHDhtm or TMPred algorithms are marked as orange boxes with the position of the first transmembrane amino acid indicated above; red boxes at the N termini indicate Sec-dependent signal sequences. A surplus of positively charged amino acids, which predicts the cytoplasmic orientation of a transmembrane helix according to the positive-inside rule, is indicated by a plus sign. Coiled coils predicted by the COILS algorithm are indicated by green boxes.

were transferred to SD-Trp$^-$-Leu$^-$-His$^-$ medium in order to select for interactions. Growth after 3 to 6 days indicated bait-prey interactions. Additionally, the stringency of this screen was enhanced by selection on SD-Trp$^-$-Leu$^-$-His$^-$ medium containing the competitive inhibitor 3-aminotriazole (5 mM).

β-Galactosidase assay. All diploid yeast cells that grew on SD-Trp$^-$-Leu$^-$-His$^-$ medium were analyzed further for β-galactosidase activity using a yeast β-galactosidase assay kit (Pierce). Briefly, yeast cells were grown for 16 to 24 h in SD-Trp$^-$-Leu$^-$ medium to optical densities of 0.6 to 0.8 at 660 nm. A total of 100 μl of each culture was transferred to microplate wells, and 100 μl of a working solution containing yeast protein extraction reagent in β-galactosidase assay buffer was added. After incubation at 37°C for 20 to 40 min, absorbances at 420 nm were determined. The β-galactosidase activity was calculated using the following formula: $E = 1{,}000 \times A_{420}/t \times V \times A_{660}$, where E is the enzyme activity, t is the time of incubation, and V is the assay volume. Activities shown represent mean values of at least three independent experiments.

Immunoprecipitation. Bacteria grown on agar plates were suspended in phosphate-buffered saline and washed twice. An amount of 5×10^{10} bacteria was resuspended in radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin), and the cells were lysed by sonication. Unbroken cells were removed by centrifugation for 10 min at 10,000 × *g*. To remove nonspecifically interacting proteins, the lysates were incubated with prewashed protein G-agarose (Roche Diagnostics) for 2 h at 4°C and centrifuged. To the supernatants, 5 μl of the appropriate antiserum was added, and samples were incubated for 3 h at 4°C. Then, 50 μl of prewashed protein G-agarose was added, and samples were incubated at 4°C for an additional 2 h. After three washing steps with RIPA buffer, proteins were eluted with 100 mM glycine, pH 2.7, or by boiling in SDS-PAGE sample solution.

Protein sequence analysis. Transmembrane topology predictions of *cag*-encoded proteins were performed using the PHDhtm program (53) of the Predict Protein server (http://cubic.bioc.columbia.edu/predictprotein) or, alternatively, with the TMPred (http://www.ch.embnet.org/index.html) or TMHMM (37) algorithms. Signal sequences were predicted using the SignalP or the PSORT programs (42, 43). Coiled coils were predicted using the COILS algorithm (41).

RESULTS

Secondary structure and topology prediction of essential and accessory Cag secretion apparatus components. We have previously defined a set of 17 *cag* pathogenicity island genes that are required both for full induction of an interleukin-8 response in epithelial cells and for translocation and subsequent tyrosine phosphorylation of the CagA protein (24). Some of the gene products show sequence similarities to components of the prototypical *A. tumefaciens* VirB type IV secretion system (1, 13), but for the majority of Cag proteins, no information about their localization and putative functions is available. Therefore, we analyzed their amino acid sequences with respect to characteristic features of membrane proteins (see Fig. S1 and other results in the supplemental material for a complete description). N-terminal, Sec-dependent signal sequences are present in nine components including CagX, CagT, CagM, CagL, and CagC (Fig. 1), and the CagT signal sequence is a typical bacterial lipoprotein signal sequence. Potential transmembrane helices anchoring the proteins in the cytoplasmic membrane were identified in eight components including CagY and CagC (Fig. 1). According to the positive-inside rule (65), the N terminus of CagY is predicted to reside in the cytoplasm, whereas the N terminus of CagC (after processing of its N-terminal signal sequence) is predicted to be located in the periplasmic space.

Thus, according to the computer predictions, all essential or accessory components of the secretion apparatus, except Cagγ and Cagα, are likely to integrate into the bacterial inner membrane or to be exported to the periplasm, which is consistent with their putative roles as components of a multiprotein complex spanning both bacterial membranes.

Identification of further VirB-like components in the Cag system. Since the sequence similarities of Cag type IV secretion system components to the VirB proteins are generally weak and often do not include significant portions of the proteins (see supplemental material), we asked whether additional VirB-like functions may be encoded on the *cag* pathogenicity island. Such functions have been proposed for Cagγ, which contains sequence motifs that are common to VirB1 family members and has a corresponding lytic transglycosylase activity (32, 70), and for CagC, which is weakly similar to VirB2 and partially exposed on the *H. pylori* surface (3, 34).

The predicted transmembrane regions of CagC are also typical for VirB2-like pilins, and a closer inspection of the CagC amino acid sequence reveals the presence of weakly conserved motifs (Fig. 2A). In case of VirB2 and the VirB2-like protein TrbC of the plasmid RP4 conjugation system, these motifs are used to perform an intramolecular cyclization reaction, during which a C-terminal peptide is cleaved off (19, 34). We raised

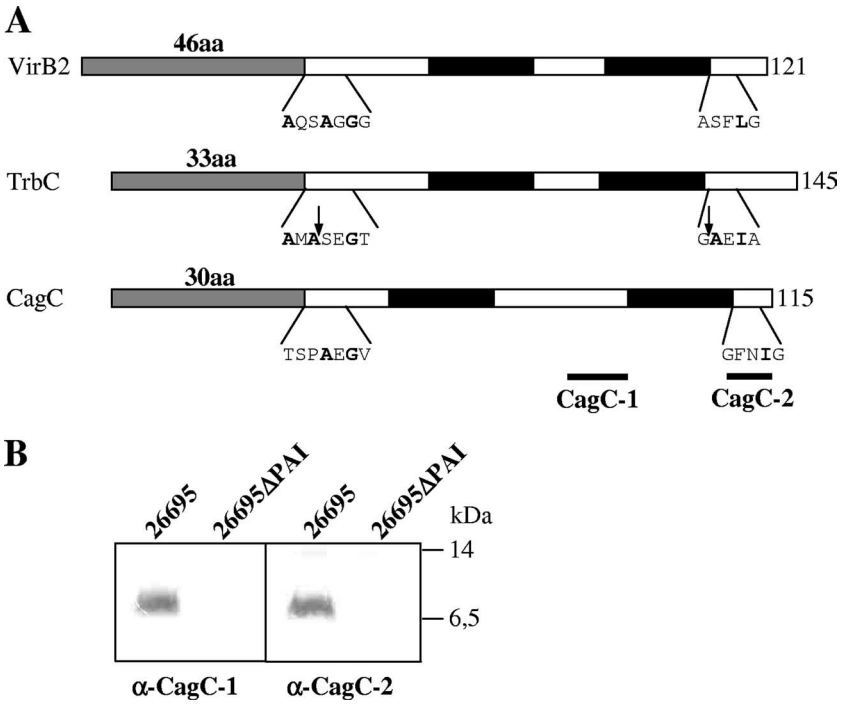

FIG. 2. Sequence analysis and characterization of the CagC protein. (A) VirB2 and VirB2-like proteins such as TrbC of plasmid RP4 are characterized by unusually long N-terminal signal sequences (dark gray boxes with the respective numbers of amino acids indicated above) and the presence of two transmembrane helices (black boxes). Conserved motifs have been identified at the signal peptidase cleavage site and a C-terminal processing site that is used for protein cyclization (indicated by arrows in the TrbC protein). A similar arrangement of transmembrane helices and weakly conserved motifs is also found in the CagC protein. Black bars below the CagC protein indicate regions from which peptides were derived for the generation of CagC antisera. (B) Immunoblot showing CagC production in the wild-type strain 26695 in comparison to the ΔPAI mutant lacking the *cag* pathogenicity island (PAI). Two different antisera recognizing an internal and a C-terminal peptide of CagC were used. α, anti; aa, amino acids.

polyclonal rabbit antisera against two peptides ($CagC_1$ and $CagC_2$) derived from the CagC sequence (Fig. 2A) and examined bacterial cell lysates by immunoblotting (Fig. 2B). Both antisera recognized a protein with an apparent molecular size of 8 to 9 kDa, which is consistent with the presence of a mature form lacking the N-terminal signal sequence. The fact that anti-$CagC_2$, which was raised against a peptide corresponding to the 10 C-terminal amino acids of CagC, recognized the same product as anti-$CagC_1$, which was raised against an internal CagC epitope (Fig. 2B), suggests that CagC is not subject to processing similar to that for TrbC or VirB2, although this does not exclude an intramolecular cyclization reaction.

By a closer inspection of the N-terminal CagE amino acid sequence, we could furthermore detect weak similarities to VirB3 and especially to the CmgB3/B4 protein of *Campylobacter* species (5), suggesting that CagE might represent a protein fusion of a VirB3-like and a VirB4-like component (see Fig. S2 in the supplemental material). Finally, the CagW protein contains sequence motifs and a predicted membrane topology that are very similar to VirB6 (see supplemental material). Taken together, these data suggest that the Cag system contains components analogous to all VirB proteins except VirB5.

Localization of Cag components in subcellular fractions. To obtain experimental evidence for the predicted localization of Cag secretion apparatus components, we used antisera raised against a subset of *cag* gene products. The generation and characterization of antisera against CagL, CagT, CagY, and Cagα have been described previously (24, 52, 55). Here, we raised additional rabbit polyclonal antisera against CagC (see above), CagM, and CagX (see Materials and Methods for details). All antisera recognized proteins of the expected sizes in lysates of wild-type strains but failed to do so in lysates of the corresponding isogenic mutant strains (Fig. 3A and data not shown).

To examine whether these Cag proteins are associated with the bacterial membranes, we separated *H. pylori* cells into soluble and membrane fractions. Immunoblot analysis of these fractions with an antiserum against the integral cytoplasmic membrane protein ComB10 (30) showed that the soluble fraction did not contain membrane proteins (Fig. 3A). Since no antiserum against an exclusively cytoplasmic *H. pylori* protein was available, we probed the fractions with an antiserum against RecA, which was shown previously to be both soluble in the cytoplasm and peripherally associated with the cytoplasmic membrane (22). Immunoblot analysis with the Cag antisera showed that the CagC, CagM, CagT, CagX, and CagY proteins are present in a membrane fraction of *H. pylori* strain 26695, whereas CagL was found almost only in the soluble fraction, which contains cytoplasmic and periplasmic proteins (Fig. 3A). Cagα was localized in both the soluble and membrane fractions, which is similar to data obtained with VirB11 (36, 50).

For a closer examination of the localization of membrane-

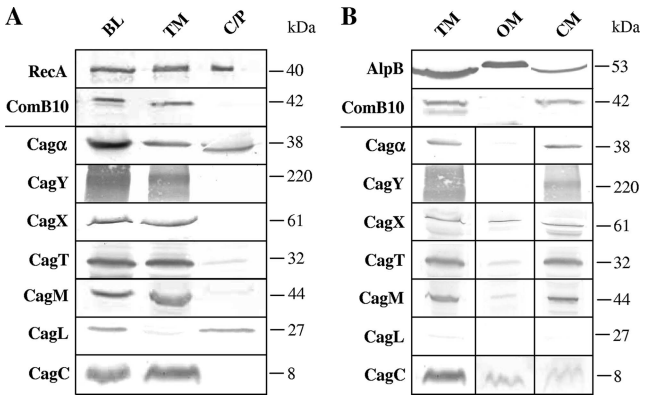

FIG. 3. Immunoblot analysis of the localization of Cag secretion apparatus proteins. (A) *H. pylori* bacterial cell lysates (BL) were separated by ultracentrifugation into a total membrane fraction (TM) and a soluble fraction containing cytoplasmic and periplasmic proteins (C/P). The fractions were probed with antisera raised against the indicated proteins. As controls, antisera against the integral cytoplasmic membrane protein ComB10 and the cytoplasmic protein RecA were used. (B) Differential extraction of an *H. pylori* total membrane (TM) fraction. For an evaluation of the extraction procedure, antisera against the outer membrane protein AlpB and against ComB10 were used. The distribution of Cag secretion apparatus proteins in the outer membrane (OM) and cytoplasmic membrane (CM) fractions was determined by immunoblotting the fractions with the indicated antisera.

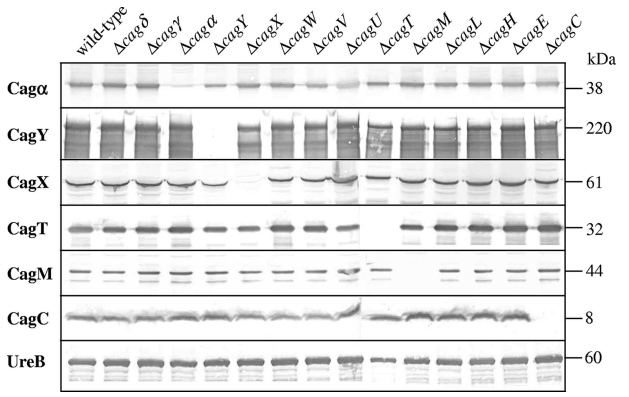

FIG. 4. Stabilization effects among Cag secretion apparatus proteins. The production of the indicated proteins was determined in adjusted cell lysates of isogenic *H. pylori* mutants in genes encoding essential secretion apparatus proteins. Production of the urease B subunit (UreB) was determined as a loading control. Representative immunoblots are shown. Pronounced stabilization effects, which were also confirmed by densitometry, occur for the CagX protein (in the *cagY* mutant) and for the CagT protein (in the *cagM*, *cagX*, and *cagY* mutants). The weak production of Cagα in the *cagY* mutant and of CagT in the *cagU* mutant is possibly due to a polar effect.

associated proteins, we performed a separation of inner and outer membranes of *H. pylori*. As previously described, membrane separation of *H. pylori* cells is not easily achieved by standard methods used for other gram-negative bacteria (18, 21). Here, we applied a differential extraction procedure of a total membrane fraction with either *N*-lauroyl sarcosine or zwittergent 3–14, detergents that are supposed to selectively solubilize inner or outer membrane proteins, respectively. To control the extraction specificities of these two detergents, we performed immunoblotting using antisera against ComB10 and against the AlpB protein, which is a member of the Hop family of *H. pylori* outer membrane proteins (44). As shown in Fig. 3B, membranes extracted with *N*-lauroyl sarcosine (outer membranes) contain the majority of AlpB but almost no contamination with ComB10. In contrast, membranes extracted with zwittergent 3–14 (cytoplasmic membranes) contained the major amount of ComB10 and also some contamination with the AlpB protein. A comparison of both extraction procedures thus allows for the assignment of an inner membrane localization of a given protein as well. Accordingly, immunoblot analysis of the fractions with the different Cag antisera revealed an inner membrane association of Cagα (in its membrane-bound form) and CagY. For CagC, CagM, CagT, and CagX, a portion of the proteins was also detected in the outer membrane fractions, particularly in case of the VirB9 analogue CagX (Fig. 3B). This observation suggests that these proteins are at least partially associated with the outer membrane.

Stability of Cag components. The formation of a multiprotein complex such as a secretion apparatus requires multiple interactions between the components involved. Therefore, the absence of individual components may result in destabilization of other subunits that would usually interact with the missing component. Such stabilization effects are well known for the VirB complex of *A. tumefaciens*, where, for instance, a deletion of the *virB7* gene results in reduced amounts of VirB9, VirB10, and VirB11 (20). To examine similar stabilization phenomena in the Cag system, we analyzed the production of Cag proteins in the background of deletion mutants in all genes encoding essential secretion apparatus components (Fig. 4). Densitometric analysis indicated that the Cagα, CagY, CagM, and CagC proteins were produced in comparable amounts in all mutants (data not shown). A reduced amount of Cagα production was observed in not only the *cagY* mutant (Fig. 4) but also a *cagZ* mutant (data not shown), suggesting that this phenotype might be due to a polar effect of the deletions on expression of the *cag*α gene. A similar downstream effect might also account for the reduction of CagT in the *cagU* mutant, which was therefore not considered further.

CagX is produced in comparable amounts as in the wild type in all mutants except the Δ*cagY* mutant, where a densitometric evaluation indicated a CagX production of about 44% ± 4% in comparison to the wild-type strain (Fig. 4 and data not shown). This observation indicates a stabilization of CagX by CagY and therefore an interaction between these proteins. Likewise, the CagT protein was produced in significantly lower amounts in the *cagM*, *cagX*, and *cagY* mutants (densitometry values of 60% ± 11%, 64% ± 3%, and 59% ± 4%, respectively, in comparison to wild type) (data not shown), again suggesting that interactions among these proteins occur. Conversely, the CagM, CagX, and CagY proteins are not differently produced in the *cagT* mutant, indicating that the presence of these proteins may assist CagT in its proper integration into the secretion apparatus complex.

Identification of protein-protein interactions among Cag secretion system components. Apart from interactions between apparatus components that result in mutual stabilization, multiple, additional protein-protein interactions would be expected. To identify such interactions, we performed a comprehensive yeast two-hybrid screen containing all components of

TABLE 2. Protein-protein interactions among Cag secretion apparatus components identified in the yeast two-hybrid screen

Bait protein	Prey protein	β-Galactosidase activity[a]	Previously described interaction(s)[b]
$Cag\beta_a$	$CagY_c$	++	−
Cagα	Cagα	++	+[c,d]
$CagY_a$	CagF	++	+[c,e]
$CagY_b$	CagN	+	−
$CagY_b$	$CagY_c$	++	−
$CagY_c$	$CagY_b$	+	−
CagX	CagM	+++	+[c]
CagV	CagN	++	−
CagU	CagA	+	−
CagT	$Cag\beta_a$	++	−
CagM	CagX	+++	+[c]
CagM	CagM	+++	+[c]
CagN	$CagY_b$	+	−
CagL	$Cag\beta_a$	+	−
CagH	$Cag\beta_a$	+	−
CagF	$CagY_a$	+++	−[f]
CagF	CagA	+++	+[g]
CagF	CagX	+	−
CagA	CagF	++	+[g]
CagA	$CagY_c$	+	−
CagA	CagA	+	−

[a] +++, β-galactosidase activity higher than with the positive control (>35 Miller units); ++, β-galactosidase activity similar to positive control (28 to 35 Miller units); +, β-galactosidase activity lower than positive control but significantly higher than negative control (21 to 28 Miller units).

[b] +, yes; −, no.

[c] Identified as yeast two-hybrid interaction (10).

[d] Identified as a hexamer in the X-ray structure (68).

[e] The region of CagY that was used by Busler et al. (10) extends from amino acids 1 to 500; the region used in this study from amino acids 1 to 345.

[f] In the study of Busler et al. (10), a yeast two-hybrid interaction between $CagY_a$ and CagF was found only in one direction.

[g] Identified by coimmunoprecipitation and pull-down experiments (15, 45).

the Cag type IV secretion apparatus. For that purpose, we inserted full-length or partial *cag* genes (Table 1) by recombinatorial cloning into yeast two-hybrid bait and prey vectors (see Materials and Methods for details). Because of the characteristic domain structure of CagY (4, 40), the *cagY* gene was split into three parts, corresponding to the 5′ repeat region (amino acids 1 to 344; designated $CagY_a$), the middle repeat region including 5′ and 3′ conserved regions (amino acids 363 to 1799; $CagY_b$), and the region with similarity to VirB10 (amino acids 1815 to 1927; $CagY_c$). From the *cag*β gene, a region encoding a putative periplasmic loop (amino acids 91 to 146; designated $Cag\beta_a$) and the region encoding the large cytoplasmic domain (amino acids 162 to 748; $Cag\beta_b$) were cloned separately. From the *cagE* and *cagH* genes, we cloned only the regions encoding the predicted cytoplasmic domains, and from *cagV* we cloned only the region encoding the periplasmic domain. All other genes were cloned as full-length constructs but without N-terminal signal sequences, as appropriate.

To screen for protein-protein interactions, we generated diploid yeast cells containing all possible combinations of bait and prey plasmids and screened these diploid cells for growth on a triple-selective medium (see Materials and Methods for details), which was obtained for 21 different combinations of bait and prey plasmids (Table 2). Growth after 2 to 3 days in the presence of the competitive inhibitor 3-aminotriazole indicated strong interactions, and growth at later time points or in the absence of 3-aminotriazole indicated weaker interactions. To validate this classification in an independent assay, each of the diploid yeasts that grew on triple-selective medium was tested for the expression of the *lacZ* reporter gene (see Materials and Methods for details). As controls, diploid yeast cells containing the positive or negative control plasmids were used. All of the diploid yeast cells that were able to grow on triple-selective medium produced higher levels of β-galactosidase activity than the yeast cells containing the negative control plasmids (Fig. 5). Interactions were scored as strong, medium, or weak interactions according to their β-galactosidase activity (Table 2). They included interactions identified previously by other techniques, such as the homotypic interaction of Cagα with itself (68) or the heterotypic interaction of CagA with CagF (15, 45), thus validating the method; they also included several interactions that were found recently in a similar yeast two-hybrid approach (10). For instance, interactions between $CagY_a$ and CagF, between CagM and CagX, and of CagM with itself were also detected in that study (Table 2). Furthermore, we were able to identify a range of novel protein-protein interactions. For example, the nonessential apparatus component CagN was found to interact with the essential apparatus components CagV and CagY. Interactions were also identified between the periplasmic domain of Cagβ and the apparatus components CagY and CagL (Table 2).

Identification of a Cag type IV secretion apparatus subassembly. To obtain independent evidence for protein-protein interactions identified with the yeast two-hybrid screen and/or suggested by the stabilization data, we performed immunoprecipitation experiments with antisera against Cag secretion apparatus components. Proteins were immunoprecipitated from whole-cell extracts of the wild-type *H. pylori* strains 26695 and P12 or of the corresponding isogenic mutants. In each case, the lysates were precleared with protein G-agarose to remove nonspecifically interacting proteins. The CagL antiserum AK271 was not capable of immunoprecipitating CagL, but the other antisera (anti-CagX, anti-CagY, anti-CagM, and anti-CagT) were able to precipitate their cognate proteins (Fig. 6A to C and data not shown).

Next, we used immunoblot analysis to identify coprecipitating proteins. In the CagX immunoprecipitation from wild-type bacterial cells, a coprecipitation of CagM, CagY, and CagT was observed. In contrast, although these proteins were also present in the starting extracts of the *cagX* mutant, none of them was precipitated in the *cagX* mutant, demonstrating the specificity of the coprecipitation experiment (Fig. 6A). Similarly, CagX, CagM, and CagT were found to coprecipitate with CagY in immunoprecipitations with the CagY antiserum (Fig. 6B). However, CagM was also nonspecifically precipitated by the CagY antiserum from the *cagY* mutant, whereas CagX and CagT were not (Fig. 6B), again indicating specific interactions between CagX, CagT, and CagY. Finally, CagX, CagY, and CagT were coprecipitated with CagM from wild-type cells but not from the *cagM* mutant (Fig. 6C). Immunoprecipitations with the anti-CagT antiserum resulted in coprecipitation of CagY, CagX, and CagM, and these proteins were also precipitated from the *cagT* mutant; therefore, these results had to be considered as unspecific (data not shown). We did not find

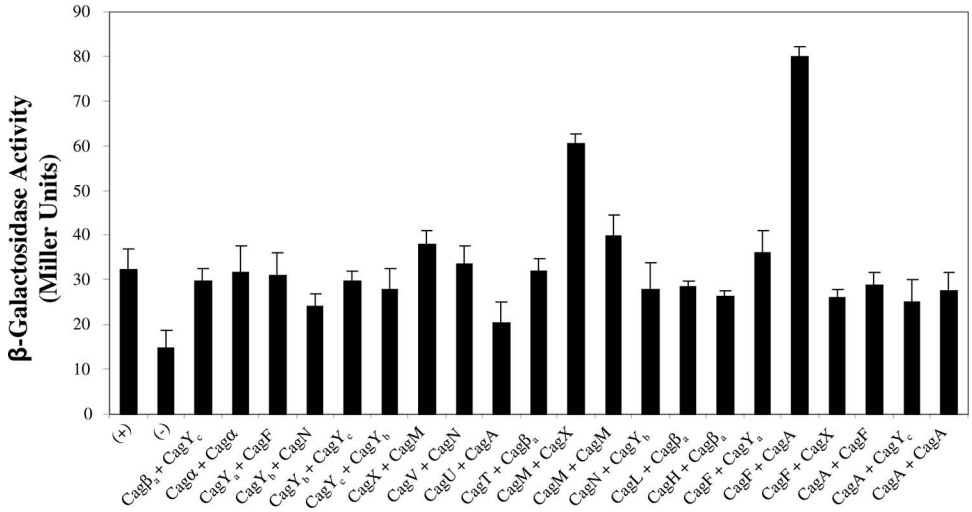

FIG. 5. Protein-protein interactions among Cag proteins identified by a yeast two-hybrid screen. Diploid yeast cells containing the indicated plasmid pairs (given as bait + prey plasmid), all of which were selected for growth on triple-selective medium (SD medium lacking tryptophan, leucine, and histidine), and yeast cells containing positive control (+) or negative control (−) plasmids were assayed for β-galactosidase activity, as described in Materials and Methods. The values shown are mean values of three independent experiments including standard deviations. The activities shown were classified into three categories, as described in Table 2.

coprecipitation of either Cagα, CagL, or CagC in these immunoprecipitations (data not shown). Taken together, these results clearly indicate that CagX, CagM, CagT, and CagY form a complex in *H. pylori*.

To discriminate whether the mutual interactions between CagY, CagX, CagT, and CagM are direct or indirect, we repeated the individual coimmunoprecipitation experiments in isogenic mutant strains lacking one of the other components. According to the yeast two-hybrid data, the interaction between CagX and CagM should be direct. Consistently, CagM coprecipitated with CagX both from the *cagT* and the *cagY* mutants (Fig. 7A, left panel). Likewise, CagX coprecipitated with CagM from the *cagT* and *cagY* mutants, indicating that the interaction between CagX and CagM is independent of CagT and CagY (Fig. 7A, right panel). The weak CagM coprecipitation in the *cagY* mutant is probably due to the fact that CagX is produced in smaller amounts in this mutant (Fig. 6B). Likewise, CagY coprecipitated in a CagX immunoprecipitation, and CagX coprecipitated in a CagY immunoprecipitation, independently of the presence of CagT or CagM (Fig. 7B), indicating that the interaction between CagX and CagY is probably direct. In contrast, CagY did not coprecipitate with CagM in the *cagX* mutant, although it did in the *cagT* mutant (Fig. 7C), suggesting that CagM does not interact directly with CagY but only via the CagM-CagX and CagX-CagY interactions demonstrated above. The coprecipitation of CagM with CagY in the *cagX* and *cagT* mutants could not be evaluated, since CagM was precipitated nonspecifically with the CagY antiserum (data not shown). Finally, we determined whether CagT could be coprecipitated with CagM, CagX, or CagY from the corresponding mutants. In a CagM immunoprecipitation, CagT coprecipitated irrespective of the presence of CagX or CagY (Fig. 7D), suggesting that CagT interacts directly with CagM. In contrast, CagT coprecipitation with CagX was dependent on CagM but independent of CagY (Fig. 7E), suggesting that the CagT-CagX interaction is mediated by CagM. Furthermore, CagT did not coprecipitate with CagY from the *cagX* or *cagM* mutants (Fig. 7F), which is consistent with the observations that CagT interacts directly with CagM (Fig. 7D) and that CagM interacts with CagY via CagX (Fig. 7C). Thus, the results of our immunoprecipitation experiments

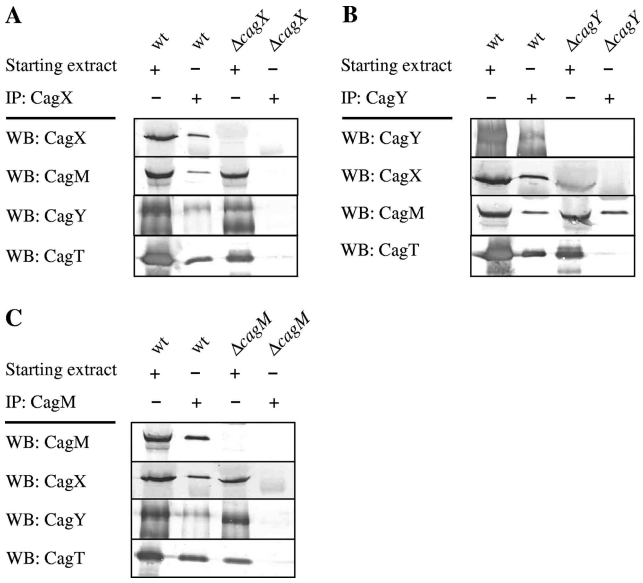

FIG. 6. Identification of a secretion apparatus subcomplex composed of CagT, CagM, CagX, and CagY in *H. pylori* cells. Bacteria were lysed in RIPA buffer, and the lysates (starting extracts) were incubated with protein G-agarose to remove nonspecifically binding proteins and subsequently immunoprecipitated (IP) with antisera against CagX (A), CagY (B), and CagM (C). Coprecipitating proteins were identified by Western blotting (WB) with the indicated antisera. Control immunoprecipitations from the corresponding isogenic mutants show the specificity of the coprecipitations, except for CagM, which coprecipitated nonspecifically in a CagY immunoprecipitation.

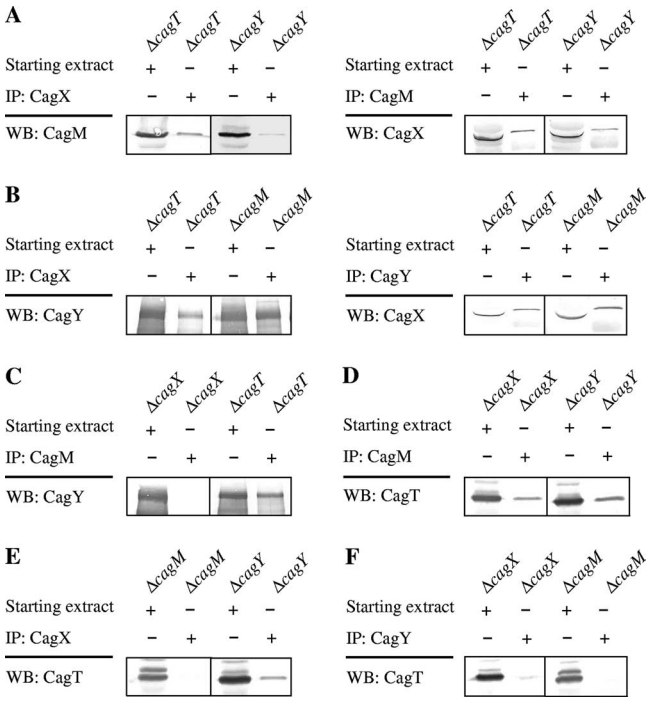

FIG. 7. Determination of direct and indirect interactions. Immunoprecipitations with antisera against CagX, CagY, and CagM were performed as described in the legend of Fig. 6, except that the indicated isogenic mutants were used. (A) The CagX-CagM interaction is independent of CagT and CagY. (B) The CagX-CagY interaction is independent of CagT and CagM. (C) The CagM-CagY interaction is independent of CagT but dependent on CagX. (D) The CagM-CagT interaction is independent of CagX and CagY. (E) The CagX-CagT interaction depends on CagM but is independent of CagY. (F) The CagY-CagT interaction depends on both CagX and CagM. IP, immunoprecipitation; WB, Western blotting.

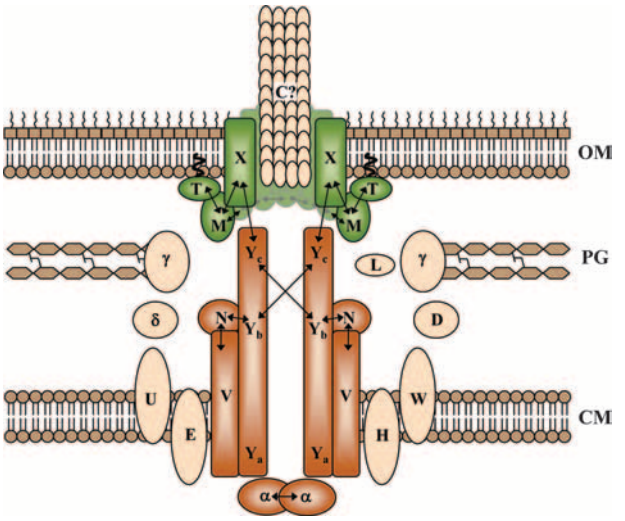

FIG. 8. Schematic model of the composition of the Cag type IV secretion apparatus. All components of the secretion apparatus are shown in their putative localizations as suggested by computer prediction, fractionation, and interaction data. Protein names are abbreviated such that X represents CagX, for example. The putative localization of (parts of) CagY (52) and CagL (38) on extracellular appendages produced by the Cag system is not depicted. Protein-protein interactions that were identified or confirmed in this study are indicated by double arrows. The direct interactions between components at the outer membrane (CagY-CagX, CagX-CagM, and CagM-CagT) were confirmed by coimmunoprecipitation; all other interactions were shown only by yeast two-hybrid data. The putative assembly of a secretin-like heterooligomeric complex at the outer membrane is represented by green boxes; a subassembly at the cytoplasmic membrane is shown by dark orange boxes. Components for which no interactions were identified are shown in light orange. See text for further details. CM, cytoplasmic membrane; PG, peptidoglycan; OM, outer membrane.

strongly suggest that an interaction chain exists from CagY via CagX and CagM to CagT.

DISCUSSION

Type IV secretion systems are macromolecule transporters with a wide variety of substrates and target cells (11), and individual systems have adapted to different requirements such as protein secretion, nucleoprotein secretion, or nucleic acid import. The prototypical VirB/VirD4 secretion system of *A. tumefaciens* contains a set of 11 VirB proteins that are necessary for constitution of the secretion apparatus, but additional factors such as the coupling protein VirD4, the secretion chaperone VirE1, or the periplasmic proteins VirJ and AcvB are required for substrate transfer (11). In other alphaproteobacteria, such as *Bartonella* or *Brucella*, operons related to the *A. tumefaciens virB* operon with the same gene order are found; however, the similarities among some of the gene products, e.g., VirB6, VirB7, and VirB8, are limited even in these related systems. In the Cag system of *H. pylori*, which is a member of the more distantly related epsilonproteobacteria, a simple assignment of functions according to sequence similarities is much more difficult.

The Cag secretion apparatus consists of at least 14 essential and two accessory components, as determined by systematic mutagenesis of genes on the *cag* pathogenicity island (24). The results of this and of previous studies suggest that 10 of these components (Cagγ, CagC, CagE, CagW, CagT, CagV, CagX, CagY, Cagα, and Cagβ) fulfill analogous functions to the VirB1, VirB2, VirB3/4, VirB6, VirB7, VirB8, VirB9, VirB10, VirB11, and VirD4 proteins of *A. tumefaciens*, respectively. In *A. tumefaciens*, the structural proteins VirB3, VirB4, VirB6, VirB7, VirB8, VirB9, and VirB10 are considered to form a multiprotein complex, with VirB4, VirB6, VirB8, VirB10, and possibly also VirB3 localized in the cytoplasmic membrane and VirB7 and VirB9 at the outer membrane (14). Contact between the inner membrane and outer membrane components is probably mediated by VirB10 in an energy-dependent fashion (12). Our data support a similar localization of the corresponding Cag components (Fig. 8). This is also the case for CagC, which fractionates with the cytoplasmic membrane and also associates with the outer membrane, consistent with its putative role as a VirB2-like pilin. The observation that CagC is the most abundant protein encoded on the *cag* pathogenicity island (8) also argues in favor of its role as a pilus subunit. CagC was shown to form extracellular structures (3), but whether it is a component of the characteristic extracellular appendages of the Cag type IV secretion apparatus (52, 60) remains to be shown. We were unable to detect any interactions of CagC with other Cag components, possibly because of its pronounced hydrophobicity.

Our computer predictions further suggest that CagE represents a protein fusion of a VirB3- and a VirB4-analogous domain similar to the CmgB3/B4 protein of *Campylobacter* species (5). Apart from its N-terminal portion, CagE shows a significant similarity to VirB4, and it also contains conserved motifs that have been identified for this protein family (48). In analogy to the ComB system, where ComB4 interacts with ComB10 (49, 61), an interaction of CagE with CagY would be expected and has been found by yeast two-hybrid analysis (10). Given that CagW is probably a VirB6-analogous protein of the Cag system, the only VirB-like component lacking in the Cag type IV secretion system seems to be a VirB5-like protein. A VirB5-like protein is also absent from the Ptl system of *Bordetella pertussis* (66). VirB5 is a minor pilus component in the VirB system (56) with a putative adhesin-like function (69). A corresponding function may be adopted in the Cag system by CagL, which has recently been shown to be a surface component interacting with integrins as host cell receptors (38).

Although most VirB-like components seem to be present in the Cag system, our data suggest a different assembly of the secretion apparatus. For example, the yeast two-hybrid data indicate interactions of periplasmic domains of the VirB8-like protein CagV and the VirB10-like protein CagY with the periplasmic component CagN. However, CagN is not essential for a functional Cag secretion apparatus, indicating that these interactions are not absolutely required. Recently, CagN was reported to be processed close to its C terminus (9), but the functional significance of this processing is currently unclear. Although the corresponding combinations were negative in our yeast two-hybrid screen, it is possible that CagV and CagY also interact directly with each other, analogous to the interaction between VirB8 and VirB10 (16). Interestingly, the middle region of CagY interacted with its C-terminal region in the yeast two-hybrid assay, suggesting an oligomerization of CagY. It is presently unclear whether such an interaction may take place in the periplasm (as depicted in Fig. 8) or at the cell surface, where at least the $CagY_b$ region was detected (52). The C-terminal 500 amino acids of CagY, which overlap with the $CagY_b$ region and comprise the whole $CagY_c$ region, were also shown to interact in a yeast two-hybrid experiment with CagX (10). The fact that we did not find a yeast two-hybrid interaction between $CagY_c$ and CagX may indicate that a part of $CagY_b$ is also required for this interaction.

The essential components Cagδ, CagU, CagM, CagL, and CagH and the accessory components CagN and CagD are specific for the Cag secretion apparatus. CagU and CagH were also predicted as integral cytoplasmic membrane proteins (Fig. 8). We found weak interactions of these proteins in the yeast two-hybrid screen, but whether they are part of a Cag secretion apparatus core complex or have different functions, is presently unclear. In general, we detected few interactions between cytoplasmic membrane-associated components of the secretion apparatus, possibly due to the presence of transmembrane helices in some of the constructs used. In a recent yeast two-hybrid study (10), interactions were also identified with CagE, CagV, and Cagβ constructs which still contained their transmembrane domains. This difference may reflect a higher stringency in our approach, resulting in the identification of stronger interactions only, or may simply be based on the different bait and prey vectors used. We did not detect any interactions involving the periplasmic proteins Cagδ and CagD, whereas Busler et al. (10) identified several interactions of Cagδ, for example, with CagV and CagM.

An important finding of this study is the identification of a Cag protein subcomplex located at the outer membrane. We demonstrate stabilizing functions between CagM, CagX, CagY, and the lipoprotein CagT. Whereas CagX fractionates similarly to the outer membrane marker AlpB, we found only small amounts of CagM and CagT in the outer membrane fraction. This might be due to the fact that CagM probably interacts indirectly with the outer membrane via CagX and/or CagT and that lipoproteins such as CagT often show an atypical detergent extraction behavior. The yeast two-hybrid data show interactions of CagM with itself and with the putative outer membrane component CagX. By our immunoprecipitation experiments, we provide a detailed characterization of the mutual interactions between CagY, CagX, CagM, and CagT. Taken together, stabilization effects, localization, and protein-protein interaction data suggest that a complex of CagX, CagT, and CagM exists at the periplasmic face of the outer membrane and that this complex interacts via CagX with CagY as the putative bridging component between the cytoplasmic and outer membrane (Fig. 8). Since all of these proteins (except CagY) are predicted to contain coiled-coil regions (Fig. 1), it is tempting to speculate that their interactions are mediated by these domains. Alternatively, these coiled coils might be used for interactions with the effector protein CagA, as suggested for type III secretion systems (17).

In support of the notion that CagT is a VirB7-like protein (1), we show here that CagT associates with the outer membrane, and it has previously been shown to be, at least partially, exposed on the bacterial surface (52, 60). However, VirB7 and VirB7-like proteins are small lipoproteins of 45 to 70 amino acids that stabilize, and interact directly with, their cognate VirB9-like factors, whereas CagT is a much larger protein and does not stabilize CagX. Our data rather suggest that the interaction between CagX and CagT is indirect, via CagM, but it should be noted that conserved motifs, which contribute to the interaction surface between VirB9- and VirB7-like proteins (6), are also present in the CagX sequence (data not shown). With its size of 280 amino acids, CagT seems to be a member of a second class of lipoproteins involved in type IV secretion systems. For example, plasmid conjugation of RP4 requires the 160-amino-acid outer membrane lipoprotein TrbH (26), and F-like conjugation systems also contain essential outer membrane lipoproteins such as TraV, with a size of 171 amino acids (29, 39). Interestingly, the MagB type IV secretion system of *Actinobacillus actinomycetemcomitans* contains both proteins with similarity to VirB7 and to CagT (25), and the ComB and Tfs3 type IV secretion systems of *H. pylori* contain VirB7-like, but no CagT-like, proteins (31, 35).

Since parts of the CagX protein are exposed on the bacterial surface (60), it seems reasonable to speculate that CagX forms a secretin-like pore in the outer membrane, as also suggested for VirB9 (6, 33). This pore may allow the passage of the pilus-like surface appendages and/or translocated substrates across the outer membrane. In type II and type III protein secretion systems, type IV pilus biogenesis systems, and filamentous phage secretion systems, secretins typically form homomultimeric complexes that are resistant to SDS (62). As-

sembly of these complexes in the outer membrane requires the presence of cognate lipoproteins that have been termed pilotins (27). However, outer membrane pore complexes in type IV secretion systems probably adopt different structures than the secretins (6). In the Cag system, the interaction between CagX and CagT requires the presence of CagM, which has no sequence similarity to other type IV secretion system components. Since our yeast two-hybrid data contained a strong interaction of CagM with itself, we speculate that CagM determines the oligomerization of the CagX-CagM-CagT complex at the outer membrane.

In conclusion, we provide a first low-resolution structural model comprising the complete set of Cag secretion apparatus proteins. This model may be used as a basis to examine the functions of Cag-specific components or of conserved type IV secretion components with Cag-specific domains. Further studies are necessary to understand these specialized and well-adapted functions in detail.

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