Interactions among the seven *Helicobacter pylori* proteins encoded by the urease gene cluster

PETRA VOLAND,1 DAVID L. WEEKS,2 ELIZABETH A. MARCUS,2 CHRISTIAN PRINZ,1 GEORGE SACHS,2 AND DAVID SCOTT2

1Department of Medicine II, Technical University, 81675 Munich, Germany; and 2Department of Physiology, University of California Los Angeles and Veterans Affairs Greater Los Angeles Health System, Los Angeles, California 90073

Submitted 1 May 2002; accepted in final form 21 August 2002

Voland, Petra, David L. Weeks, Elizabeth A. Marcus, Christian Prinz, George Sachs, and David Scott. Interactions among the seven *Helicobacter pylori* proteins encoded by the urease gene cluster. *Am J Physiol Gastrointest Liver Physiol* 284: G96–G106, 2003. First published September 4, 2002; 10.1152/ajpgi.00160.2002.—Survival of *Helicobacter pylori* in acid depends on intrabacterial urease. This urease is a Ni\(^{2+}\)-containing oligomeric heterodimer. Regulation of its activity and assembly is important for gastric habitation by this neutralophile. The gene complex encodes catalytic subunits (ureA/B), an acid-gated urea channel (ureF), and accessory assembly proteins (ureE–H). With the use of yeast two-hybrid analysis for determining protein-protein interactions, UreF as bait identified four interacting sequences encoding UreH, whereas UreG as bait detected five UreE sequences. These results were confirmed by communoprecipitation and β-galactosidase assays. Native PAGE immunoblotting of *H. pylori* inner membranes showed interaction of UreA/B with UreI, whereas UreI deletion mutants lacked this protein interaction. Deletion of ureE–H did not affect this interaction with UreI. Hence, the accessory proteins UreE/G and UreF/H form dimeric complexes and UreA/B form a membrane complex with UreI, perhaps enabling assembly of the urease apoenzyme at the membrane surface and immediate urea access to intrabacterial urease to allow rapid periplasmic neutralization.

Urease is a Ni\(^{2+}\)-containing cytoplasmic enzyme that converts urea into NH\(_3\) plus CO\(_2\). Its inactivity at neutral pH prevents lethal alkalinization of the cytoplasm, whereas an increase of activity is used to combat acidic environments (6). For this acidic medium-dependent increase in activity, *H. pylori* has developed a unique mechanism to control access of the substrate to intrabacterial urease. Urea channels (UreI) present in the inner membrane are opened at pH levels less than −6.5, increasing delivery of medium urea to bacterial urease (38). Hence, *H. pylori* in acid produces large quantities of NH\(_3\) that diffuses into the periplasm and buffers this space due to the formation of NH\(_3\)\(_2\) (1, 29, 33). This mechanism allows efficient utilization of urease activity for the survival of the organism at acidic pH in vitro and in the stomach of animal models (6, 10, 11, 15, 18, 35, 36). Whereas the ureases of most bacterial species are composed of three subunits (UreA–C), *Helicobacter* species produce a urease with only two subunits, UreA and -B. Fusion of the ureA and the ureB genes of non-*Helicobacter* species has resulted in a single gene, ureA, in *Helicobacter* species, whereas ureB is homologous to ureC of other ureolytic bacteria (21).

In addition to the structural genes, the urease gene cluster in *H. pylori* contains accessory genes that are required for synthesis of catalytically active enzymes, namely ureE–H, the latter being homologous to ureD in other bacteria (21, 34). Extensive biochemical studies have deduced the function of the accessory proteins in *Klebsiella aerogenes*. These proteins are required for the assembly of the nickel metallocenter within the active center of the enzyme, and deletion of ureD, -F, or -G results in complete loss of urease activity, whereas deletion of ureE leads to a much reduced activity of the urease enzyme. UreD association with the urease apoenzyme does allow Ni\(^{2+}\) insertion into the apoenzyme at high medium Ni\(^{2+}\) concentrations, but UreE is the natural donor in the full assembly complex at normal Ni\(^{2+}\) concentrations (5, 23–26).

Address for reprint requests and other correspondence: P. Voland, Dept. of Medicine II, Technical Univ., Ismaningerstrasse 22, Bau 520, Rm 20.01.12, D-81675 Munich, Germany (E-mail: petra.voland@lrz.tum.de).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpgi.org
Yeast two-hybrid analysis is a powerful method for detection of interactions of soluble proteins (4, 9, 37). While this work was in progress, two applications of yeast two-hybrid analysis to urease gene clusters appeared. One used an automated method and concluded yeast two-hybrid analysis to urease gene clusters appeared. While this work was in progress, two applications of yeast two-hybrid analysis to urease gene clusters appeared. One used an automated method and concluded yeast two-hybrid analysis to urease gene clusters appeared. While this work was in progress, two applications of yeast two-hybrid analysis to urease gene clusters appeared. One used an automated method and concluded that the product of one of the accessory genes, UreH, interacted with UreA and -F of *H. pylori* and that several proteins interacted with soluble fragments of UreI (27). A manual yeast two-hybrid method was used to detect interactions of the urease gene products in *Proteus mirabilis* (16).

However, neither the possibility of in vivo interactions between the catalytic and the accessory proteins nor their requirement for generation of active urease have been determined in *H. pylori*. We used a yeast two-hybrid screen to define protein interactions in the urease gene cluster, which were later confirmed by immunoprecipitation. This two-hybrid system in *Saccharomyces cerevisiae* is able to detect cytosolic protein-protein interactions. The method depends on reconstitution of a transcription factor in yeast that consists of two separate domains: a DNA-binding domain (BD) and a transcription activation domain (AD). Nuclear translocation of the complex is required to allow transcriptional activation and detection of interaction. Therefore, the method is generally not suitable for detecting interactions with membrane proteins, because these usually do not translocate into the nucleus.

For the expression of active urease *ureI* is not required but is essential for survival in animal models and in acid in vitro in the presence of urea (15, 33). Expression of this gene is essential for colonization of the normal gerbil stomach but not for infection of the acid-inhibited stomach (15). UreI is a polytopic integral inner membrane protein (28, 29, 31, 38, 39) functioning as a proton-gated urea channel. As a six-membrane segment protein with relatively short intersegment loops, *ureI* is present in *H. pylori* and other gastric *Helicobacter* species and is not present in *H. pylori* and other gastric *Helicobacter* species and is not present in *Helicobacter pylori* (17, 34, 38). Soluble fragments of these loops may not be able to detect cytosolic urease gene cluster with either the DNA-BD or the AD of the transcription factor GAL4 were generated by cloning the cDNA fragments into multiple cloning site (MCS) of pGBRT7, pGADT7 (Matchmaker yeast two-hybrid system, Clontech), and pPOBD80 that will be referred to as pGBk, pGAD, and pOD. The cDNA fragments were generated by PCR using plasmid pH808 (kindly provided by H. Mobley) containing the complete *H. pylori* urease gene cluster as the template with primers introducing appropriate restriction sites (Table 1). PCR reactions were carried out for 29 cycles using 1 unit ExTag DNA polymerase (Perkin-Elmer) as follows: 30 s at 94°C, 30 s at annealing temperatures ranging from 56 to 58°C, and 1 min at 72°C. An additional

**Table 1. PCR primers used to amplify Helicobacter pylori urease genes in yeast two-hybrid expression vectors**

<table>
<thead>
<tr>
<th>Gene/Vector</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ureB</em></td>
<td>pOBD80 5' AACGGCATGGAATCTAGAAATTAGATTAC 3'</td>
</tr>
<tr>
<td></td>
<td>5' TAACTGGAATGAACTGTTTTAG 3'</td>
</tr>
<tr>
<td><em>ureE</em></td>
<td>pOBD80 5' GGCTGGTCTACATGATTAGCCGTATTTAATAG 3'</td>
</tr>
<tr>
<td></td>
<td>5' TGTGGTCATCTTCCATGACCATATTAAAAT 3'</td>
</tr>
<tr>
<td><em>ureF</em></td>
<td>pOBD80 5' CGAAGAATGGAATGAAATTAGATTAC 3'</td>
</tr>
<tr>
<td></td>
<td>5' GAGACTGTCGACTACAGATTTAAAATTGACATG 3'</td>
</tr>
<tr>
<td><em>ureG</em></td>
<td>pOBD80 5' AGGCTTGCCATGCTTAGAATTACAGTATTAC 3'</td>
</tr>
<tr>
<td></td>
<td>5' GTGCTGTGACATTCCCATATTAAAATGACATG 3'</td>
</tr>
<tr>
<td><em>ureH</em></td>
<td>pOBD80 5' CGGAACCATGGAATGAAATTAGATTAC 3'</td>
</tr>
<tr>
<td></td>
<td>5' CCGGCTGTGACATTCCCATATTAAAATGACATG 3'</td>
</tr>
</tbody>
</table>

Restriction sites are underlined.
H. pylori genonic library. A genomic library of H. pylori strain ATCC no. 43504 was generated by Clontech. Briefly, genomic DNA was purified from bacteria and partially digested with restriction enzyme Sau3AI. Fragments with sizes between 0.4 and 4 kb were purified, their extremities were filled in using Klenow S1, and the products were ligated to EcoRI/blunt adaptors. These fragments were finally cloned into the EcoRI site of the MCS of pGAD10 vector, thus generating a fusion library with the AD of the GAL4 yeast transcription factor.

Yeast two-hybrid system. The yeast two-hybrid strategy applied for the library screen is the one described by Fromont-Racine et al. (13). Briefly, the MATα yeast strain Y187 was transformed using the lithium acetate method to -80°C. The MATα yeast strain CG-1945 was similarly transformed with either GAL4 bait plasmid, pODureG, or pODureF. For each screening experiment, bait and library expressing cell populations were mixed (2:1), concentrated onto sterile 0.22-μm pore size membrane filters (Millipore, Bedford, MA), and incubated on rich YPD agar for 4.5 h at 30°C before recovery into SDO medium lacking leucine and tryptophan and being spread on 70 SDO agar plates lacking histidine and amino acids. Cotransformation experiments were performed in yeast strain Y187 with potential interacting partners using the lithium acetate method in a small-scale application (14), including controls followed by β-galactosidase colony lift assay and quantitative β-galactosidase assay.

β-Galactosidase assays. β-Galactosidase colony lift assays were performed for qualitative evaluation of interactions. After 2–4 days of growth at 30°C, yeast transformants were transferred onto filter papers (VWR grade 410; Fisher, Tuscaloosa, WI), digested with the appropriate restriction enzyme (Promega), and ligated overnight to generate a fusion with the DNA-BD or the AD of the yeast transcription factor GAL4. Nucleotide sequence and reading frame of each construct was confirmed by automated DNA sequencing. The plasmids were amplified in E. coli and purified using Qiagen purification.

Plasmid isolation from yeast. Single yeast colonies were suspended in 200 μl lysis buffer (2% vol/vol Triton X-100, 1% vol/vol sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Acid-washed glass beads (0.3 g) (Sigma, St. Louis, MO) and phenol/chloroform/isoamyl alcohol (200 μl; 25:24:1) (GIBCO Life Technologies, Grand Island, NY) were added. Tubes were vortexed for 10 min followed by centrifugation at 20,000 g for 10 min. DNA was ethanol precipitated at -80°C for 30 min by adding 2.5 volumes of ethanol and 1/10 volume of 3 M NaAc, pH 5.2. After centrifugation, the DNA pellet was washed with 70% (vol/vol) ethanol, air dried, and resuspended in 15 μl of nuclelease-free H2O.

Plasmid rescue and analysis in E. coli. The plasmid DNA isolated from positive yeast was transformed into E. coli for restriction enzyme and sequencing analysis. Transformation was performed by electroporation. Plasmids were amplified in E. coli, purified using Qiagen purification columns according to the manufacturer’s protocol, and digested with EcoRI to recover the genomic sequence inserted into the AD plasmid pGAD10. DNA sequencing was performed to identify the insert. Transformed E. coli were grown on selective media and the AD inserted sequence was in frame with the DNA-AD of GAL4.

In vitro transcription/translation and coimmunoprecipitation. To confirm the interactions found with the two-hybrid system, the proteins were expressed by in vitro transcription/translation using the TNT T7 polymerase-coupled reticulocyte lysate system (Promega) in the presence of 35S-labeled cysteine using PCR products for ureF and -H subcloned into vectors of the Matchmaker hybrid system MM3 (Clontech) referred to as pGBKTT7-ureF and pGADT7-ureH. These vectors contain a T7 promoter providing the expressed proteins with the in fusion epitope tags, c-Myc (pGBK7T7) and HA (pGADT7), used subsequently for in vitro coimmunoprecipitation with specific antibodies. The system provides vectors encoding known interacting proteins, SV40 large T antigen (pGADT7-T/H/A) and murine p53 (pGBK7T7–53/c-Myc), as positive control as well as a vector encoding human lamin C (pGBK7T7-Lam/c-Myc) known not to interact with most other proteins as negative control. One microgram of plasmid DNA was used in the reaction according to the manufacturer’s protocol. Five to twenty microliters of each in vitro transcription/translation reaction mixture were mixed with 66 μl (single incubations) or 133 μl (double incubations), respectively, of “Colrain” buffer ([in mM] 100 KCl, 25 HEPES, pH 7.5, 10 MgCl2, 1 DTT, 0.1 EDTA, 1 PMSF, and 10% glycerol, 0.1% Nonidet P-40, 1 mg/ml leupeptin, and 1 mg/ml aprotinin), incubated for 30 min at room temperature, diluted with 233 or 466 μl of “washing” buffer ([in mM] 50 Tris-HCl, pH 7.5, 250 NaCl, 5 EDTA, 1 DTT, 1 PMSF, 0.1 EDTA, and 0.5% Nonidet P-40, 1 mg/ml leupeptin, and 1 mg/ml aprotinin), and incubated for 30 min at room temperature. Samples for incubations with two antibodies were divided into two tubes, and the antibodies were added, namely monoclonal anti-c-Myc and polyclonal anti-HA (Clontech). After 30 min incubation at room temperature, 20 μl protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added to conjugate the immunocomplexes overnight at 4°C. The resulting agarose pellets were washed five times with 500 μl of washing buffer, resuspended in 20 μl 2× SDS electrophoresis sample buffer, and boiled shortly. The supernatants were analyzed by 12.5% tricine SDS-PAGE and subsequent autoradiography.

Construction of deletion mutants for ureE–H in H. pylori. H. pylori genomic knockouts were generated by homologous recombination of gene-disrupting cassettes into the urease gene cluster. pBluescript (Stratagene) containing the gene that codes for an antibody specific to the epitope for α-β-galactosidase activity detector kit according to the manufacturer’s instructions (Clontech). Activity is expressed in relative light units normalized to 1 OD600 of the yeast culture, and values reported are the means of three independent cotransformants tested for each protein pair.
for kanamycin resistance (kanR) in the MCS was used as a scaffold to construct knockout cassettes. Of interest were 400–600 bp flanking the 5’ and 3’ ends of the gene, which were amplified from genomic H. pylori DNA by PCR. The 400-bp segments were designed to contain a site for restriction by XbaI on the 5’ end and the ATG start codon and a site for restriction by SalI on the 3’ end. Care was taken to minimize disruption of the operon other than the gene replacement by joining these segments to the 800-bp kanR-cassette in a way that only 5 bp separated the end of kanR and the ATG of the subsequent gene in the operon. Hence, the gene of interest was completely removed and replaced by the kanR-cassette that is transcribed and translated using the same H. pylori promoters and ribosome binding sites. Knockout plasmids were introduced into H. pylori by natural transformation. Recombinants were selected on brain-heart infusion agar containing 10 μg/μl kanamycin.

Urease activity assays. Urease activity of the H. pylori wild-type and the knockout mutants was determined qualitatively using the rapid urease test by suspending bacteria in sucrose, and centrifuged at 100,000 g for 1 h. The supernate was removed, layered onto 5-ml 20% sucrose, and centrifuged at 3,000 g through a French press at 20,000 psi. Lysate was centrifuged for 1 h. Sucrose was then resuspended in native gel sample buffer (750 mM 6-aminon-caproic acid, 50 mM bis-Tris, and 1% dodecylmaltoside) pH 7.0. Solubilized membranes were then separated by nondenaturing native gel electrophoresis using 4–12% gradient gels. Molecular weight size standards were not used, because membrane proteins separated on native gels do not strictly run according to their molecular mass, and the ternary structure with dodecylmaltoside as the solubilizing agent gives incorrect molecular weights. The anode buffer contained 50 mM bis-Tris-HCl at pH 7.4, and the cathode buffer contained 50 mM tricine, 15 mM bis-Tris, and 0.02% Coomassie blue at pH 7.0. In some experiments, the Coomassie blue was omitted. After electrophoresis, the gel was destained for 1 h in 40% methanol/10% acetic acid, followed by two 10-min washes in water. Finally, to facilitate transfer to the membranes for immunoblotting, the gel was soaked in 0.1% SDS (Sigma) in 1 M Tris-HCl for 1 h. The gel was then transferred to nitrocellulose (Bio-Rad) for Western blot analysis. The blot for each strain was probed with three antibodies against UreA (38), UreB, and UreC (kindly provided by Dr. Harry Mobley, Baltimore, MD) using the enhanced chemiluminescence method (Amersham, Munich, Germany). In the case of the ureI deletion mutant, although protein was present on the native gel as detected by Coomassie or Ponceau staining, no reactivity with either urease antibody was detected.

SDS gel analysis of H. pylori membranes. The band in the location corresponding to that detected in the immunoblot was identified by Coomassie or Ponceau staining, cut out, run on a 10–20% tricine gradient gel using SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF; Bio-Rad) for protein sequencing to confirm their identity.

RESULTS

Interactions of UreF/H and UreG/E detected with the yeast two-hybrid system. A set of GAL4 DNA-BD fusion proteins containing the proteins of the urease gene cluster was investigated in the yeast two-hybrid system (see Table 1). The construct containing the entire sequence for UreF in fusion with the DNA-BD (pOD-ureF) was used as “bait” to screen a genomic DNA library of H. pylori in fusion with the DNA-AD of the GAL4 transcription factor. Screening of ~3 × 10^6 diploid cotransformants revealed 53 blue clones by colony lift assay, implying that these clones were positive for transcriptional activation of the HIS3 and LacZ reporter genes. Clones contained four different sequences that were compared with the database. All four sequences were identified as a portion of or as the entire sequence of ureH of H. pylori in frame with the DNA-AD of GAL4. The result of the alignment is shown in Fig. 1A with the four inserts shown in different patterns. As depicted in the figure, the different inserts had varying parts of the flanking gene, ureG, at the 5’ end. However, these sequences were not in frame, and therefore, translation of this region would not yield a peptide containing parts of UreG. The interaction of UreF with UreH was quantified using the chemiluminescent β-galactosidase assay. The four independent clones of ureH in pGAD10 were cotransformed again with the bait pOD-ureF into yeast in parallel with the appropriate controls. Controls were cotransformations of each individual pGAD10-ureH “prey” clone and the BD vector without insert and of the bait pOD-ureF with the empty pGAD10. Interacting proteins showed 10–250 times higher values for β-galactosidase activity than for controls (Fig. 1B), confirming the interaction of UreF/H. The magnitude of the chemiluminescent signal was severalfold larger compared with the values obtained for the interacting α- and β-subunits of the gastric H^+ -K^+ -ATPase determined previously in our laboratory, also using the yeast two-hybrid technique (20).

The second construct (pOD-ureG) used as bait for a library screening contained the entire sequence for UreG in fusion with the DNA-BD. In this experiment, 1.5 × 10^6 diploid cotransformants were screened and 36 blue clones could be detected by colony lift assay containing five different inserted sequences. After recovery and amplification of the clones in E. coli, DNA sequencing revealed that all five clones contained the entire sequence for ureE in frame with the DNA-AD of GAL4. The result of the alignment with the gene for ureE is shown in Fig. 2A. Quantification of this
interaction with the chemiluminescent β-galactosidase assay performed on the five independent ureE prey clones cotransformed with the bait pOD-ureG showed 20–55 times higher values for β-galactosidase activity than controls. The 4 clones of ureH in pGAD10 were cotransformed with the bait pOD-ureF into yeast Y187 in parallel with appropriate controls (cotransformations of each individual pGAD10-ureH prey clone plus BD vector without insert and bait pOD-ureF plus empty pGAD10). ATG, start codon; IS, insert relating to insert number in A; RLU, relative light units; number of tests = 3 ± SE.

Immunoprecipitation of ureF/H and ureE/G complexes in vitro. UreF/HA (39 kDa) was detected in immunocomplexes with UreH/c-Myc (37 kDa) using the anti-c-Myc or the polyclonal anti-HA (Fig. 3A, lanes 2 and 3). No coimmunoprecipitation of UreH/c-Myc with T-AG/HA (90 kDa) (lane 5) and UreF/HA with the control protein C-lamin/c-Myc (32 kDa) (lanes 6 and 7) could be detected. Lanes 1 and 4 show the immunoprecipitation of UreF/HA and UreH/c-Myc using the antibody corresponding to their own tag as additional negative controls. Results of the coimmunoprecipitation confirm those of the yeast two-hybrid assay indicating interaction of UreF with UreH.

With the use of 10–20% gradient tricine gel, we also detected the interaction of UreE and -G. As illustrated in Fig. 3B, UreE tagged with c-Myc (31 kDa) could be precipitated with the specific antibody against c-Myc (lane 1). When using the anti-HA antibody, UreG/HA was precipitated as a single band at the correct size of ~33 kDa (lane 4). In the simultaneous presence of UreG/HA and UreE/c-Myc, no interaction could be observed by precipitating with the anti-c-Myc antibody (lane 2). When precipitating with the anti-HA antibody, a protein band appeared (lane 3) due to complex formation between UreE and -G.

Characterization of the H. pylori knockout mutants. Knockout mutants of H. pylori were generated by an in frame insertion of a kanamycin cassette to delete the gene of interest. PCR was performed to confirm the
successful deletion of the single genes (data not shown).

All knockout mutants except the ureE knockout lost their urease activity completely. The ureE knockout mutant still exhibited slight urease activity in the rapid urease test determined to be 1% of wild type by quantitative urease assay (wild type 2.66 ± 0.07 μmol·min⁻¹·mg protein⁻¹; ureE knockout 0.05 ± 0.01 μmol·min⁻¹·mg protein⁻¹). Therefore, we used a polyclonal peptide antibody against a synthetic peptide (amino acids 26–41) of UreE to determine whether the ureE gene had been successfully deleted in the knockout mutant (Fig. 4). Identical 5 μg amounts of protein were loaded onto each lane. This showed that no UreE protein was produced, hence that deletion of the ureE gene did not result in totally inactive urease.

**Combined native/reducing gel electrophoresis of H. pylori membranes.** Purified wild-type H. pylori membranes were separated under nonreducing conditions by native polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose membrane. Western blot analysis using antibodies against UreA, -B, and -I detected a single band of the same molecular weight with all three antibodies (Fig. 5A). This represents a large molecular weight complex.

The ureI nonpolar deletion mutants were generated, and identical membrane preparations were obtained. Western blot analysis using antibodies against UreA, -B, and -I was also performed. Although protein was detected on the native gel as shown by Ponceau S staining of the membrane at the region corresponding to the wild-type protein stain and immunoreactivity (Fig. 5B, left lane), no reaction with the urease antibodies was detected in the UreI knockout membrane preparation (Fig. 5B, center lanes). These results show that interaction between UreA and -B with the inner membrane of the organism requires expression of UreI. No change in the pattern was observed with any of the knockouts of ureE–H (data not shown), showing that UreI interacted with the apoenzyme.
Bands on the immunoblot with anti-UreA and anti-UreB after precipitation of *H. pylori* wild-type membranes with anti-UreI (Fig. 5C) were additionally identified by NH₂-terminal sequencing. Coomassie blue staining showed two protein bands that were cut out, run on a 10–20% tricine gradient gel using SDS-PAGE, and transferred to PVDF (Bio-Rad) for protein sequencing that identified these bands as UreA and -B.

**DISCUSSION**

Urease production by *H. pylori* is essential for survival under acidic conditions. Urease is an enzyme that consists of an oligomeric heterodimeric complex of the subunits UreA and -B. Nickel has to be inserted into the apoenzyme for urease activity. This is achieved by the action of the four accessory proteins. These proteins in the urease gene cluster of *H. pylori* have been identified as UreE–H (21, 34).

In the present study, knockout mutants were used to determine the functional importance of each subunit of the urease gene cluster. Accessory proteins of the urease gene cluster were expressed by in vitro transcription/translation in the presence of 35S-labeled cysteine, providing the expressed proteins with in fusion epitope tags, c-Myc and HA. These tags were used subsequently for in vitro coimmunoprecipitation with specific antibodies. A: we mixed UreF with UreH, anti-c-Myc (lane 3), or anti-HA (lane 2); added antibodies; and analyzed interactions by SDS-PAGE and autoradiography. With the use of c-Myc as a tag for UreH (or HA as a tag for UreF), two bands at the expected molecular mass were detected (lanes 2 and 3), indicating the interaction of these two proteins with each other. UreF and -H alone, precipitated by their specific antibodies, were used as negative controls (lanes 1 and 4). Large T-cell AG and human C-lamin were used as negative controls for the interacting proteins (lanes 5–7) and did not show interaction with UreH and -F, respectively. B: With the use of identical techniques, the interaction of UreE with -G was determined. UreE was mixed with UreG, anti-c-Myc (lane 2), or anti-HA (lane 3); antibodies were added; and interactions were analyzed by SDS-PAGE and autoradiography. UreE and -G alone, precipitated by their specific antibodies, were used as negative controls (lanes 1 and 4). Molecular mass of marker proteins are shown on the left.

---

**Fig. 3.** Coimmunoprecipitation of the interacting proteins of the urease gene cluster. Accessory proteins of the urease gene cluster were expressed by in vitro transcription/translation in the presence of 35S-labeled cysteine, providing the expressed proteins with in fusion epitope tags, c-Myc and HA. These tags were used subsequently for in vitro coimmunoprecipitation with specific antibodies. A: we mixed UreF with UreH, anti-c-Myc (lane 3), or anti-HA (lane 2); added antibodies; and analyzed interactions by SDS-PAGE and autoradiography. With the use of c-Myc as a tag for UreH (or HA as a tag for UreF), two bands at the expected molecular mass were detected (lanes 2 and 3), indicating the interaction of these two proteins with each other. UreF and -H alone, precipitated by their specific antibodies, were used as negative controls (lanes 1 and 4). Large T-cell AG and human C-lamin were used as negative controls for the interacting proteins (lanes 5–7) and did not show interaction with UreH and -F, respectively. B: With the use of identical techniques, the interaction of UreE with -G was determined. UreE was mixed with UreG, anti-c-Myc (lane 2), or anti-HA (lane 3); antibodies were added; and interactions were analyzed by SDS-PAGE and autoradiography. UreE and -G alone, precipitated by their specific antibodies, were used as negative controls (lanes 1 and 4). Molecular mass of marker proteins are shown on the left.

**Fig. 4.** Western blot analysis of UreE expression in *H. pylori* wild-type (WT) and the UreE knockout (KO) mutant. Whole cell lysate (5 μg) of *H. pylori* wild-type and the UreE knockout mutant were separated on a 10% tricine SDS gel and transferred to nitrocellulose. A polyclonal peptide antibody against a synthetic peptide EWFETRKKFIARFKTRQ (amino acids 26–41) of UreE was used to detect UreE protein.
the urease protein complex. Knockout mutants ureF, -G, and -H lost urease activity entirely, whereas the ureE knockout mutant had a residual activity of ~1% of wild type. The results obtained here in H. pylori generally parallel findings obtained in K. aerogenes (5, 17, 22, 23, 25, 26). Mutation of different genes in the urease gene cluster of K. aerogenes introduced into a bacterial system (24) resulted in a urease-negative phenotype (deletion of ureD -F, or -G), whereas mutation of ureE in K. aerogenes led to a significantly reduced urease activity, comparable with our results obtained with the knockout mutants in H. pylori. UreE exists as a homodimer and contains internal essential nickel binding sites and has, therefore, been ascribed the function of serving as the primary nickel donor in the assembly complex (5). In H. pylori, the terminal histidine-rich binding sites are absent, but the internal histidine sites are present, probably functioning as nickel donors.

Automated high-throughput yeast two-hybrid analysis of a H. pylori library recently determined interaction of proteins in H. pylori (27). With the use of this technique, an interaction map of 261 H. pylori proteins was developed. Among numerous other protein interactions, specific interactions were described between UreA/B, UreF/H, and UreE/G as well as interaction of the latter two pairs with UreA. However, these interactions were not confirmed or quantified by biochemical or immunological assays. A cytoplasmic loop of UreI also interacted with a protein suggested to respond to the proton motive force (27).

The manual yeast two-hybrid system used here identified strong interactions between UreF/H and between UreE/G. These data confirm the results obtained in the automated high-throughput assay but also show quantitatively that these interactions are very strong. These results parallel the findings in P. mirabilis obtained by immunoprecipitation and manual yeast two-hybrid screening. In this species, yeast two-hybrid analysis detected interaction between UreD (UreH in H. pylori) and UreF. However, in contrast to the automated assay and consistent with our data obtained here in H. pylori, no interaction of UreA was found with the accessory proteins in P. mirabilis (16).

A role as “chaperone” protein has been described for K. aerogenes UreD, corresponding to UreH in H. pylori, (25). In contrast, the automated yeast two-hybrid assay (27) did not detect the interaction of UreB with -E and -H in H. pylori but rather interactions with UreA. Although we also detected a tentative interaction of UreE and -H with -B with our methods (not shown), the background was too high to reach firm conclusions, but we detected no interaction at all with UreA, in general agreement with the yeast two-hybrid data in P. mirabilis (16).

Additional biochemical evidence for the protein-protein interactions was found by coimmunoprecipitation of UreF with -H as well as UreE with -G, consistent with the yeast two-hybrid analysis. It is known from extensive biochemical analysis in K. aerogenes that stable complexes consisting of different proteins of the urease gene cluster are formed during urease assembly and are required for incorporation of nickel ions into the apoenzyme (26). In K. aerogenes, UreD binds to the apoenzyme (25), and complexes exist of UreD-F with the apoenzyme (23). UreG is the most highly conserved of the accessory proteins and contains a P-loop GTP binding site that might be involved in urease assembly. A mutation in this region prevented formation of active urease and of a soluble UreD/F/G complex normally present in wild-type cells (22).

Other studies in Klebsiella led to the hypothesis that UreD-apoenzyme complexes as well as UreD/F- and UreD/F/G-apoenzyme complexes exist and that the latter, with the addition of UreE, is the assembly system for active urease (5, 26). However, possible sequential interaction of the pairs of the accessory gene proteins and the nature of the structural subunit to which these pairs bind has not been determined by biochemical analysis.

The current molecular biological studies, as well as biochemical data, suggest that heterodimers or oligomers of UreE/G and UreF/H are formed first and that these accessory protein complexes are probably

AJP-Gastrointest Liver Physiol • VOL 284 • JANUARY 2003 • www.ajpgi.org
the functionally significant structures. From the biochemical analysis of *K. aerogenes*, the pair UreF/D (H) binds to the apoenzyme first (UreD/F-apoenzyme complex) followed by UreG/E, and then the ternary complex catalyzes Ni²⁺ insertion from UreE. In *P. mirabilis* similar interactions were found with UreB, not UreA, using a manual yeast two-hybrid analysis method (16). A similar mechanism may be present in *H. pylori*, because we obtained preliminary evidence for interaction with UreB and not UreA.

Because UreI is a membrane protein, protein-protein interactions using yeast two-hybrid technology can only be used with cytoplasmic fragments of the protein and results rest on the assumption that the tertiary structure of these fragments reflects their structure when membrane tethered by the membrane-inserted segments. With the use of high throughput yeast two-hybrid analysis, the third cytoplasmic loop of UreI was thought to interact with ExhD, a member of a protein complex that transduces membrane potential to outer membrane proteins (27). No interactions of UreI with urease or any of the urease accessory proteins were detected using our yeast two-hybrid method. However, in the present study, using native gel Western blot analysis, an interaction between urease and the proton-gated urea channel UreI was found that was not revealed by the yeast two-hybrid method.

Intracellular urease activity is required for acid resistance by *H. pylori*. This activity is regulated by the proton-gated urea channel UreI by permitting urea entry only under acidic conditions to prevent lethal alkalization during times of relative neutrality (38). Measurement of membrane potential in acid after urea addition indirectly showed that the periplasm was selectively buffered to a pH of ~6.2, a pH that is consistent with normal functioning of a neutralophile, such as *H. pylori* (31). Periplasmic alkalization under the same conditions, for example at pH 5.5, was observed directly using pH-sensitive fluorescent probes, confirming the results calculated from the membrane potential experiments (1). This experiment was performed under conditions of slight acidity, under which urease is not essential for survival of the bacterium. These findings predict that urea hydrolysis may be in the vicinity of the membrane to allow directed diffusion of ammonia into the periplasm without a large increase in cytoplasmic pH. The UreAB/I complex may, therefore, act to selectively buffer the periplasm by coupling urea entry and hydrolysis at the inner membrane, with the resulting ammonia diffusing into the periplasm either through UreI or through the inner membrane, raising periplasmic pH. Interaction of the membrane protein, UreI, and urease was only detected by biochemical analysis. However, it was not possible to determine whether UreI interacted directly with UreA or with UreB, or with both subunits simultaneously. Deletion of any of the accessory proteins did not affect this interaction showing that the apoenzyme interacted with UreI.

Figure 6 summarizes the data presented here along with those found in other organisms. The accessory proteins form heterodimers composed of UreE/G and of UreF/H. This interaction is shown by the data generated in the present study, as well as observations made in other systems. The dimers, rather than the individual proteins, presumably bind to UreB via UreE/H recognition sites, an assumption that is based on observations and homologies in other bacteria. This has therefore been marked in the figure with an arrow pointing toward UreB. Binding of the accessory proteins allows Ni²⁺ insertion into the apoenzyme consisting of UreA/B heterodimers in an oligomeric complex. Our current observations further favor the assumption that the UreA/B dimeric apoenzyme is able to interact with UreI at the surface of the inner membrane of *H. pylori*. The accessory proteins may not be involved in this interaction, but an unknown protein may in fact mediate this association. The close association between urease and the urea transporter perhaps facilitates rapid activation of urease in response to acid and may also allow more rapid movement of NH₃ into the periplasm, decreasing possible elevation of cytoplasmic pH.

Because urease is the most abundant protein in *H. pylori* (~10% of the total protein) and UreI has fewer copies per cell, the complex between UreI and the UreA/B complex probably represents a minor fraction of the total urease, although the enzyme appears to be a dodecameric heterodimer. Even a 12-fold difference...
in expression of UreI and UreA/B would still result in a significant fraction of urease being membrane associated if it exists as a dimeric dodecamer in the organism. It is not necessary for this interacting urease to be active, because the complex was still detected when ureE–H were deleted. In H. pylori incubated at neutral pH, only approximately one-third of the urease is active (30), and perhaps this fraction is preferentially associated with UreI to facilitate NH₃-dependent buffering of the periplasm when the organism is exposed to acidic media.

This study was supported, in part, by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-46917, DK-46872, and DK-41301 and Deutsche Forschungsgemeinschaft Grants DFG-PR-411/7–1 and DFG-PR-411/9–1 (to C. Prinz).

REFERENCES


