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The Novel *Helicobacter pylori* CznABC Metal Efflux Pump Is Required for Cadmium, Zinc, and Nickel Resistance, Urease Modulation, and Gastric Colonization

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Maintaining metal homeostasis is crucial for the adaptation of *Helicobacter pylori* to the gastric environment. Iron, copper, and nickel homeostasis has recently been demonstrated to be required for the establishment of *H. pylori* infection in animal models. Here we demonstrate that the HP0969-0971 gene cluster encoding the Czc-type metal export pump homologs HP0969, HP0970, and the *H. pylori*-specific protein HP0971 forms part of a novel *H. pylori* metal resistance determinant, which is required for gastric colonization and for the modulation of urease activity. Insertional mutagenesis of the HP0971, HP0970, or HP0969 genes in *H. pylori* reference strain 26695 resulted in increased sensitivity to cadmium, zinc, and nickel (czn), suggesting that the encoded proteins constitute a metal-specific export pump. Accordingly, the genes were designated *cznC* (HP0971), *cznB* (HP0970), and *cznA* (HP0969). The CznC and CznA proteins play a predominant role in nickel homeostasis, since only the *cznC* and *cznA* mutants but not the *cznB* mutant displayed an 8- to 10-fold increase in urease activity. Nickel-specific affinity chromatography demonstrated that recombinant versions of CznC and CznB can bind to nickel and that the purified CznB protein interacted with cadmium and zinc, since both metals competitively inhibited nickel binding. Finally, single *cznA*, *cznB*, and *cznC* mutants did not colonize the stomach in a Mongolian gerbil-based animal model. This demonstrates that the metal export functions of *H. pylori cznABC* are essential for gastric colonization and underlines the extraordinary importance of metal ion homeostasis for the survival of *H. pylori* in the gastric environment.

The gram-negative bacterial pathogen *Helicobacter pylori* colonizes the mucus layer of the human stomach, and lifelong colonization is associated with various disorders of the upper gastrointestinal tract (31). Initial infection, as well as long-term persistence in the hostile gastric niche, necessitates the expression of adaptive mechanisms, which enable *H. pylori* to effectively survive environmental changes. Maintaining metal ion homeostasis is a prerequisite for the establishment of the *H. pylori* infection (reviewed in reference 36), since various proteins involved in iron and copper metabolism are essential for gastric colonization in animal models (3, 22, 37, 38, 40, 41). In addition, magnesium homeostasis was shown to be essential for *H. pylori* viability in vitro (24). Nickel is a cofactor of two important *H. pylori* enzymes. These are the urease enzyme required for gastric acid resistance (12, 17) and the membrane-bound hydrogen uptake hydrogenase enzyme (21). Therefore, intracellular nickel homeostasis is essential for gastric adaptation via the modulation of urease and hydrogenase activity (6, 16, 20, 21, 42). On the other hand, free metal ions are able to inhibit the activity of many enzymes such as urease (8, 23) and catalyze the generation of toxic oxygen radicals (15).

In order to maintain metal homeostasis, *H. pylori* strictly regulates the import, storage, and efflux of different metal ions (36). In bacteria metal efflux is mediated by cation diffusion facilitators, P-type ATPases, and resistance-nodulation-cell division (RND)-type exporters (18). In *Ralstonia* sp. and other bacteria, the proton-driven RND-type metal efflux pump Czc, which is responsible for the resistance to cadmium, zinc, and cobalt, is composed of the inner membrane, periplasmic, and outer membrane proteins CzcA, CzcB, and CzcC. The genome of *H. pylori* contains two sets of genes for Czc-type exporters (32). The *czcB* homologs HP0970 (later renamed/called *cznB*) and HP1328 are located directly upstream of the corresponding *czcA* homologs HP0969 and HP1329 (Fig. 1A). Together with the flanking *crdA* and *crdB* genes, the *czcAB* gene pair HP01328/HP1329 forms a copper resistance determinant (Fig. 1A), which is strongly upregulated by copper via the CrdR/S two-component regulatory system (39, 40). However, the metal export functions of the second *czcAB* gene pair HP0969/HP0970 (Fig. 1B) have not been investigated thus far. Therefore, we inactivated the corresponding genes and determined their functions in metal resistance. Since a CzcC homolog is absent in the *H. pylori* genome, the HP0971 gene, located directly upstream of the CzcB homolog HP0970 (Fig. 1A and B), was included in the study. Computational analysis revealed

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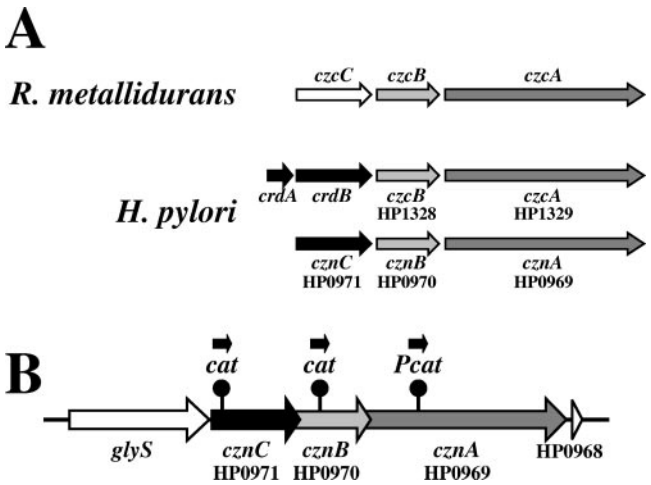

FIG. 1. Genetic organization of the *H. pylori czcAB* gene homologs. Genes represented by arrows are numbered according to the annotated genome sequences of *H. pylori* strain 26695 (32). The sequences and annotations were obtained from the TIGR microbial database (http://www.tigr.org). (A) Comparative view of *czcAB* homologs in *H. pylori* and *R. metallidurans*. Homology of *H. pylori* HP0969/HP0970 (*cznAB*) and HP1329/HP1328 genes to *R. metallidurans czcA*/*czcB* sequences is indicated by black and gray, respectively. The *H. pylori*-specific genes HP0971 (*cznC*), CrdB (HP1327), and CrdA (HP1326), located upstream of the corresponding *czcB* and *czcA* homologs, are shown in white. (B) Schematic overview and mutational analysis of the *H. pylori cznABC* genes. Genes are indicated by gray and white arrows, respectively. The insertion of *cat* and P*cat* resistance cassettes are marked by black circles. The directions of the resistance cassettes are displayed by black arrows.

that the HP0971 protein structurally belongs to the TolC-type efflux proteins (29), which catalyze the export of a variety of substrates in gram-negative bacteria (18). Export functions of *H. pylori* HP0971 were recently experimentally supported by the finding that a *H. pylori* double mutant lacking the TolC-like proteins HP0971 and HP0605 was more susceptible to metronidazole (33). The results of our investigations indicate that HP0971, together with the downstream encoded CzcB and CzcA homologs, constitutes a novel *H. pylori* metal ion efflux pump, which is required for metal resistance, urease modulation, and gastric colonization. According to the specificity for cadmium, zinc, and nickel (czn), we designated the HP0971, HP0970, and HP0969 genes *cznC*, *cznB*, and *cznA*, respectively (Fig. 1).

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Bacterial strains are listed in Table 1. *H. pylori* was routinely cultivated on blood agar with 10% horse serum in a microaerobic atmosphere as described earlier (3). Growth inhibition experiments with metal ions were performed in brucella broth supplemented with 5% fetal calf serum (BBF). Metal-enriched conditions were established by supplementation of BBF with chloride salts of nickel, zinc, cadmium, iron, cobalt, and copper and bismuth citrate (catalog numbers 339350, 211273, C-2544, F-2130, C-6641, 222011, and B-1654; Sigma). For growth inhibition experiments *H. pylori* wild-type and mutant strains were precultured in BBF medium to an optical density at 600 nm (OD_{600}) of 1.0 and diluted 1:100 in test medium supplemented with metal ions at increasing concentrations as described previously (38–40). After growth for 48 h, the influence of metal ions on bacterial growth was determined by measuring the OD_{600} photometrically. The growth inhibition experiments were performed in triplicate and were repeated independently at least three times. Control cultures were supplemented with sodium chloride at the highest metal concentrations to exclude the influence of osmotic stress or chloride ions on metal resistance. *Escherichia coli* was grown in Luria-Bertani (LB) medium. When appropriate, growth media were supplemented with 50 mg of ampicillin (Ap) or 20 mg of chloramphenicol (Cm)/liter.

DNA techniques and mutagenesis. Restriction and modifying enzymes (Roche Diagnostics, Germany) were used according to the manufacturer's instructions. Cloning was performed in *E. coli* according to standard protocols (2). Plasmids were isolated with a kit from QIAGEN. Sequences of the chloramphenicol acetyltransferase gene cat_{GC} with (*Pcat*) or without (*cat*) its own promoter were amplified by PCR with the primers CATS1 or CATS2 in combination with the primer CATAS1 as described earlier (3, 4). *Pcat* or *cat* genes were fused to upstream and downstream DNA regions of mutagenized genes (Fig. 1) by using a modified version of the megaprimer PCR protocol (28) as described earlier (30, 39, 40). Briefly, the upstream and downstream sequences of the *cznC* (HP0971) and *cznA* (HP0969) genes were amplified by PCR from DNA of *H. pylori* strain 26695 using primers carrying 5′ extensions complementary to the 5′ and 3′ ends of the *Pcat* and *cat* cassettes, respectively (Table 2). The resulting PCR products were purified with a kit from QIAGEN and subsequently mixed with PCR-amplified *Pcat* or *cat* cassettes to work as megaprimers in a second PCR containing only the flanking primers. The resulting PCR products carrying *cat* or *Pcat* inserted in the *cznC* (HP0971) or *cznA* (HP0969) gene were cloned into plasmid pZErO-2 (Invitrogen). In order to mutagenize the *cznB* (HP0970) gene (Fig. 1), the corresponding sequence was amplified from DNA of *H. pylori* strain 26695 and cloned in pGEM-T Easy (Promega). The *cznB* coding region in the resulting plasmid was interrupted by insertion of the promoterless *cat* gene in the unique EcoRV site. The resulting plasmids pCZNA-PCAT, pCZNB-CAT, and pCZNC-CAT (Table 1) were used for the mutagenesis of *H. pylori*. Correct construction of the plasmids was confirmed by sequencing or by restriction analysis with appropriate enzymes. Marker exchange mutagenesis of *H. pylori* was performed by electroporation or transformation according to standard procedures (7, 11). *H. pylori* mutants carrying the *cat* gene inserted into the chromosome were selected by growth on Dent blood agar containing Cm at concentrations of 20 mg/liter. Correct insertion of *cat* and *Pcat* in the *cznABC* genes (Fig. 1B) was verified by PCR analysis with the appropriate primers listed in Table 2 (data not shown).

Protein analysis. *H. pylori* cultures grown to an OD_{600} of 1.0 to 1.2 in broth were harvested by centrifugation for 10 min at 4,000 × *g* at 4°C. Determination of protein concentrations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were performed as described earlier (3, 39). The *H. pylori* urease B subunit was detected with the specific antiserum SE744 (kindly provided by K. Melchers, ALTANA). Bound rabbit antibodies

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
H. pylori		
26695	Wild-type, reference strain	32
26695-*cznC*	26695, HP0971::*cat*; Cm^r	This study
26695-*cznB*	26695, HP0970::*Pcat*; Cm^r	This study
26695-*cznA*	26695, HP0969::*Pcat*; Cm^r	This study
P149	Wild-type, gerbil-adapted by four animal passages; Str^r	9
P149-*cznA*	P149, HP0969::*Pcat*; Cm^r Str^r	This study
P149-*cznB*	P149, HP0970::*cat*; Cm^r Str^r	This study
P149-*cznC*	P149, HP0971::*cat*; Cm^r Str^r	This study
E. coli BL21	F$^-$ *dcm ompT hsdS*(r_B^- m_B^-) gal	Stratagene
Plasmids		
pZErO-2	Cloning vector, MCS in *lacZ′*, *neo*; Km^r	Invitrogen
pGEM-T Easy	Cloning vector, MCS in *lacZ′*; Ap^r	Promega
pCZNC-CAT	pZErO-2, *cznC*::*cat*; Cm^r Km^r	This study
pCZNB-CAT	pGEM-T Easy, *cznB*::*cat*; Cm^r Ap^r	This study
pCZNA-PCAT	pZErO-2, *cznA*::*Pcat*; Cm^r Km^r	This study
pASK-IBA3	Expression vector, MCS, *Strep*-Tag II; Ap^r	IBA
pASK-IBA3-CznC	pASK-IBA3 with the *H. pylori cznC* gene; Ap^r	This study
pASK-IBA3-CznB	pASK-IBA3 with the *H. pylori cznB* gene; Ap^r	This study

[a] *cat*, promoterless cat_{GC} gene; *Pcat*, cat_{GC} gene with promoter; MCS, multiple cloning site; Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance; Str^r, streptomycin resistance; Km^r, kanamycin resistance.

TABLE 2. Oligonucleotides used in this study

Gene[a]	Application	Primer	Sequence (5′→3′)	Primer	Sequence (5′→3′)[b]
cznC	Mutagenesis, upstream	971-L1	TTATAAGGAGCGTGTTAAAC	CAT971-R1	1-CAGCTCCATTGAATTAATTT
	Mutagenesis, downstream	CAT971-L1	3-TCCCTATATTGAGAATGAAA	971-R1	CTTTAGCCGTTAGATCCAAA
	Protein expression	971ASK3-L1	ATGGTAGGTCTCAAATGA-GCATG TTTAGTGCGGGCATGC	971ASK3-R1	ATGGTAGGTCTCAGCGCT-ATGC AATTCTCCTAATCTGGTCAAA
cznB	Mutagenesis	NCCB-L1	GTGCGATTAGAACCTGAAGC	NCCB-R1	AAATCTTGCTGCGCTCTTCT
	Protein expression	NCCBASK3-L1	ATGGTAGGTCTC-AAATGAAGCGG GCGTTATTGTGGCTTAT	NCCBASK3-R1	ATGGTAGGTCTCAGCGCTTTCCT CCCCTAAATTGTTGATCATG
cznA	Mutagenesis, upstream	CZCA1-L1	GAATGATGCTCGCTTCCATT	PCATCZCA1-R1	2-CTAAATTAGTGGAAACGGTG
	Mutagenesis, downstream	CATCZCA1-L1	3-TTGCGAAAGTCATCAGCCAG	CZCA1-R1	TTTAAGACAAGAGAGCTGAC
cat	*cat* gene without promoter	CATS2	TCCGAGATTTTCAGGAG	CATAS1	TTACGCCCCGCCCTGCCA
Pcat	*cat* gene with promoter	CATS1	TCCGGTTTTTGTTAATCCGCC	CATAS1	TTACGCCCCGCCCTGCCA

[a] Gene numbers refer to the *H. pylori* 26695 genome sequence (32).

[b] The 5′ extensions used for fusion of PCR products to the cat_{GC} gene are indicated by numbers as follows: 1, (5′-CTCCTGAAAATCTCGGA), complementary to the 5′ region of the *cat* gene without promoter; 2, (5′-GGCGGATTAACAAAAACCGGA), complementary to the 5′ region of the *cat* gene with promoter; 3, (5′-TGGCAGGGCGGGGCGTAA), complementary to the 3′ end of the cat_{GC} gene.

were detected with a protein A-alkaline phosphatase conjugate, followed by incubation with nitroblue tetrazolium as the substrate. N-terminal sequencing of proteins was performed by Edman degradation according to standard procedures as described previously (3).

Production of recombinant proteins and analysis of the nickel-binding capacity. Recombinant versions of the *H. pylori* CznC and CznB proteins were produced in *E. coli* using the *Strep*-Tag protein expression system (IBA, Germany) according to the manufacturer's instructions (http://www.iba-go.de). The *cznC* and *cznB* coding sequences from *H. pylori* strain 26695 were PCR amplified using appropriate primer pairs (listed in Table 2) and cloned via the BsaI restriction sites added as 5′ extensions (underlined) into plasmid pASK-IBA3 (IBA, Germany). The plasmids were transferred to *E. coli* BL21, and expression was induced with 0.2 mg of tetracycline/liter. The bacteria were harvested by centrifugation, and the recombinant proteins were purified to homogeneity on a *Strep*-Tactin column according to the manufacturers' instructions. For the analysis of nickel binding capacities of CznC and CznB, *E. coli* containing the recombinant proteins was lysed by sonication as recommended by the manufacturer. The lysates were incubated with 2.5 ml of nickel-nitrilotriacetic acid (NTA) agarose (QIAGEN) at 37°C. Proteins that did not bind to agarose were removed by washing with 4 ml of washing buffer (50 mM disodium hydrogen phosphate, 300 mM sodium chloride [pH 8.0]) containing 20 mM imidazole. Nickel-binding proteins were eluted with 250 mM imidazole in elution buffer (50 mM disodium hydrogen phosphate, 300 mM sodium chloride [pH 8.0]). Proteins in collected fractions were separated by SDS-PAGE and visualized by immunoblotting with *Strep*-Tactin-alkaline phosphatase (AP) conjugate.

Urease activity. *H. pylori* cultures grown to an OD_{600} of 1.0 to 1.2 in broth were harvested by centrifugation for 10 min at 4,000 × *g* at 4°C and were lysed with 0.1% SDS. Protein concentrations were determined with the Bradford protein assay (2). Urease activity in fresh lysates was determined by measuring ammonia production from urea hydrolysis with the Berthelot reaction as described previously (34, 35). The amount of ammonia present in samples was calculated from a standard NH_4Cl concentration curve. Urease activity was expressed as micromoles of urea hydrolyzed per minute per milligram of total protein.

Gastric colonization studies using the gerbil animal model. Experiments with the gerbil animal model were performed as described previously (9, 38). The Mongolian gerbils used here originate from the breeding of the Max von Pettenkofer-Institute (Munich, Germany). The animal model is registered at the Regierung Oberbayern (211-2531-43/02). Up to three animals were housed in each cage at a constant room temperature of 22°C with a 12-h light-dark cycle. For the infection experiment the *H. pylori* wild-type strain P149 (9) and its isogenic *cznC*, *cznB*, and *cznA* mutants (Table 1) were grown on serum agar (GC-agar, 8% horse serum and a complex vitamin mixture) supplemented with 250 mg of streptomycin (Str^{250})/liter. The animals were infected orogastrically by feeding with 0.3 ml of bacterial suspension in brucella broth (OD_{550} of 3.3) through a feeding needle, corresponding to a final infection dose of ca. 10^9 bacteria/gerbil. The infection was performed three times on subsequent days. For each infecting strain, two or three animals were kept for 3 weeks. After sacrifice of the gerbils in a CO_2 chamber, the stomach was removed from the animal, dissected, and cleared from the gastric contents. The washed stomach specimen was homogenized in 2 ml of brucella broth by using a glass homogenizer. Then, 100 μl of a 1:10 and a 1:100 dilution were plated on serum agar (Str^{250}) in duplicates. After 4 to 5 days, the colonies were counted and the bacterial loads were determined.

RESULTS

Role of the *cznA*, *cznB*, and *cznC* genes in metal ion homeostasis. To study the possible functions of the HP0971, HP0970, and HP0969 proteins in *H. pylori* metal metabolism, we inactivated the corresponding genes in the chromosome of the reference strain 26695 by marker exchange mutagenesis (Fig. 1B). To minimize possible polar effects on the downstream genes, the HP0971 and HP0970 genes were inactivated by insertion of a *cat* resistance cassette with a ribosome-binding site but without promoter or terminator sequences (Fig. 1B). The HP0969 gene was inactivated by insertion of a *cat* gene with promoter (but without terminator) to secure expression of the downstream genes (Fig. 1B). Subsequently, we investigated the functions of all three genes in metal metabolism by using growth inhibition experiments. Therefore, the inhibitory concentrations of cadmium, zinc, nickel, iron, cobalt, copper, and bismuth were determined by measuring the growth of the mutants and the *H. pylori* wild-type strain 26695 in BBF medium supplemented with the metal ions at increasing concentrations. In nonsupplemented broth, the growth of the mutants was comparable to the wild-type strain, indicating that the mutations do not generally limit bacterial fitness (Fig. 2). The inhibitory concentrations for iron, cobalt, copper, and bismuth were identical in the mutants and in the wild-type strain (data not shown). However, significant differences in growth inhibition indicated that all three mutants were more sensitive to cadmium, zinc, and nickel compared to the wild-type strain (Fig. 2). Sodium chloride, at the highest metal concentration of 1.2 mM, had no influence on growth (data not shown). This indicates that the metal sensitivity of the mutants was not caused by osmotic stress. The fact that all three mutants displayed increased sensitivity to the same metal ions at comparable levels supports the hypothesis that the HP0971, HP0970, and HP0969 proteins act together and form part of a novel *H. pylori* metal-specific export pump for cadmium, zinc,

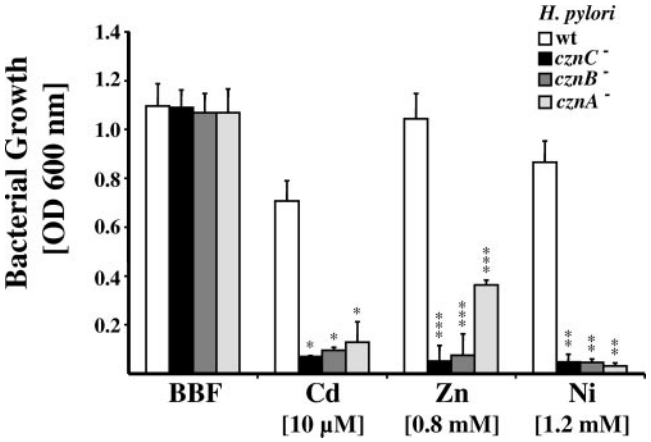

FIG. 2. Role of *cznA*, *cznB*, and *cznC* genes in *H. pylori* metal resistance. The growth of *H. pylori* strain 26695 (white) and of the isogenic mutants 26695-*cznC* (black), -*cznB* (dark gray), and -*cznA* (light gray) was determined by measuring the OD_{600}. The medium was supplemented with cadmium, zinc, and nickel at concentrations indicated on the *x* axis. The data represent mean values from three independent determinations. Standard deviations and significance levels determined by using the Student *t* test are indicated. ✻, $P < 0.05$; ✻✻, $P < 0.01$; ✻✻✻, $P < 0.001$.

and nickel. According to the existing nomenclature for metal export systems in other bacteria, we designated the corresponding genes *cznC*, *cznB*, and *cznA* for resistance to cadmium, zinc, and nickel (czn).

Influence of *cznA*, *cznB*, and *cznC* mutations on urease activity. Since free nickel ions in the cytoplasm activate the *H. pylori* urease enzyme (34), we investigated the possible influence of the Czn-mediated nickel export functions on urease modulation. Determination of the urease activity in the wild-type strain and in the *cznABC* mutants revealed that in the *cznC* and *cznA* mutants urease activity is 8- to 10-fold increased, whereas the *cznB* mutant displayed activity levels comparable to the wild-type strain 26695 (Fig. 3A). Detection of the urease B subunit by immunoblot revealed that the levels of urease protein are similar in the wild-type and in the *cznABC* mutants (data not shown), indicating that the increased urease activity in the *cznC* and *cznA* mutants is not caused by nickel-mediated induction of urease synthesis. Supplementation of the growth media with nickel at sublethal concentrations of 10 and 100 μM did further increase the urease activity in the *cznC* mutant (Fig. 3B), indicating that the elevated activity is most likely caused by accumulating nickel ions, which raise the nickel availability for incorporation into the urease apoprotein. The fact that nickel induced the urease activity of *cznB* and *cznA* mutants to levels similar to the wild-type strain excluded possible polar effects of the mutations. Supplementation of the growth medium with 0.8 mM zinc led to a significantly decreased urease activity, both in the wild-type strain and in the *cznABC* mutants (Fig. 3C). This demonstrates that the catalytic activity of *H. pylori* urease is inhibited by zinc ions.

Nickel binding by recombinant versions of CznC and CznB. In order to determine possible metal binding properties of the CznABC proteins, we investigated the nickel binding capacity of recombinant CznC, CznB, and CznA proteins on nickel-

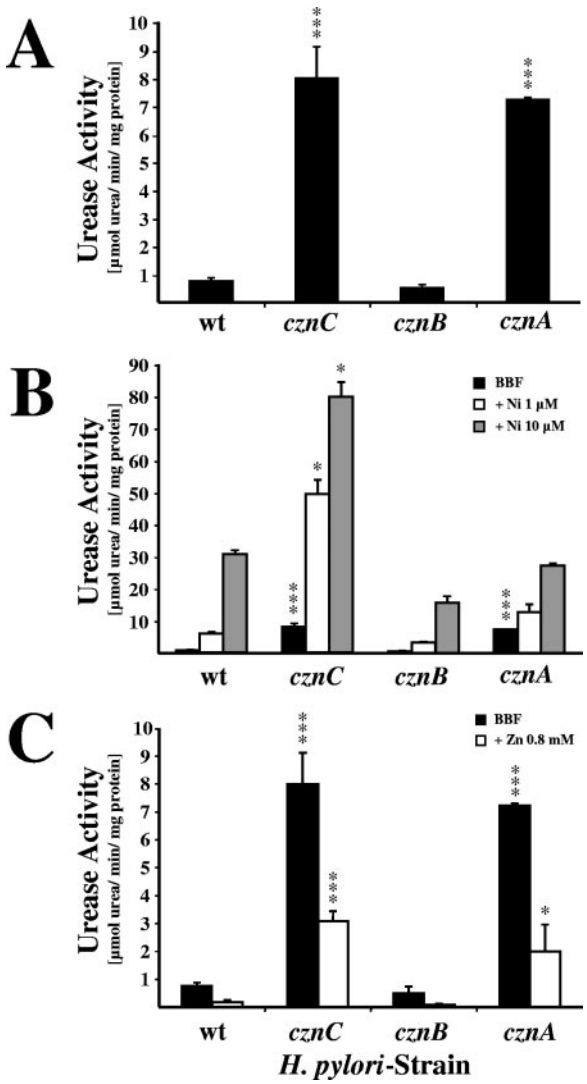

FIG. 3. Role of the *cznA*, *cznB*, and *cznC* genes in modulation of urease activity. The *H. pylori* wild-type strain 26695 and the isogenic 26695-*czcC*, -*czcB*, and -*czcA* mutants were grown in BBF medium, and the urease activity was determined as described in Materials and Methods. (A) Determination of urease activity in nonsupplemented BBF medium. The *H. pylori* wild-type strain 26695 and the isogenic 26695-*czcC*, -*czcB*, and -*czcA* mutants were grown in BBF medium. (B) Determination of urease activity in BBF medium supplemented with nickel. The *H. pylori* wild-type strain 26695 and the isogenic 26695-*czcC*, -*czcB*, and -*czcA* mutants were grown in BBF medium (■) and in BBF medium supplemented with 10 μM (□) or 100 μM (▨) nickel. (C) Determination of urease activity in BBF medium supplemented with zinc. The *H. pylori* wild-type strain 26695 and the isogenic mutants 26695-*czcC*, -*czcB*, and -*czcA* were grown in BBF medium (■) and in BBF medium supplemented with 0.8 mM zinc (□). The data represent mean values from three independent determinations. Standard deviations and significance levels determined by using the Student *t* test are indicated. ✻, $P < 0.05$; ✻✻✻, $P < 0.001$.

NTA agarose (QIAGEN). This technique was used earlier for enrichment and purification of the *H. pylori* Hsp60 protein (1). For the production of the recombinant proteins, containing a *Strep*-Tag for purification with *Strep*-Tactin, the coding regions of the *cznABC* genes were amplified by PCR with appropriate primers (Table 2) and cloned into the expression vector pASK-

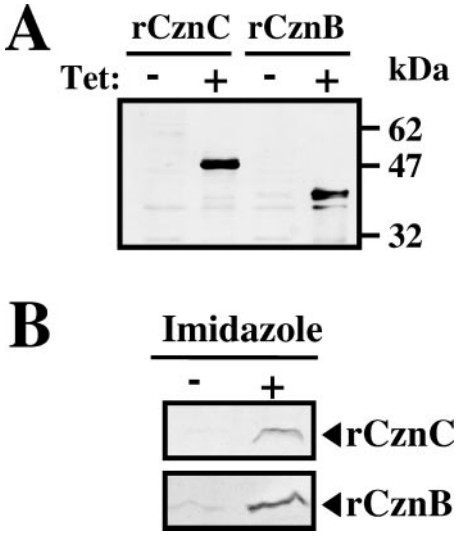

FIG. 4. Determination of the nickel-binding capacity of recombinant *H. pylori* CznC and CznB proteins by nickel affinity chromatography. (A) Production of recombinant *H. pylori* CznC and CznB proteins. *E. coli* carrying the coding sequences of *H. pylori cznC* and *cznB* genes cloned into the expression vectors pASK-IBA3-CznC and pASK-IBA3-CznB, respectively, were grown in LB medium with tetracycline (+), and the recombinant rCznC and CznB proteins were detected by immunoblotting with *Strep*-Tactin-AP. Cultures without tetracycline treatment served as controls (−). (B) Binding of *H. pylori* rCznC and rCznB proteins to nickel agarose. Total protein lysates from *E. coli* producing rCznC and rCznB from *E. coli* were incubated with nickel-NTA agarose, and the nickel binding capacity was determined by washing without (−) or with imidazole (+).

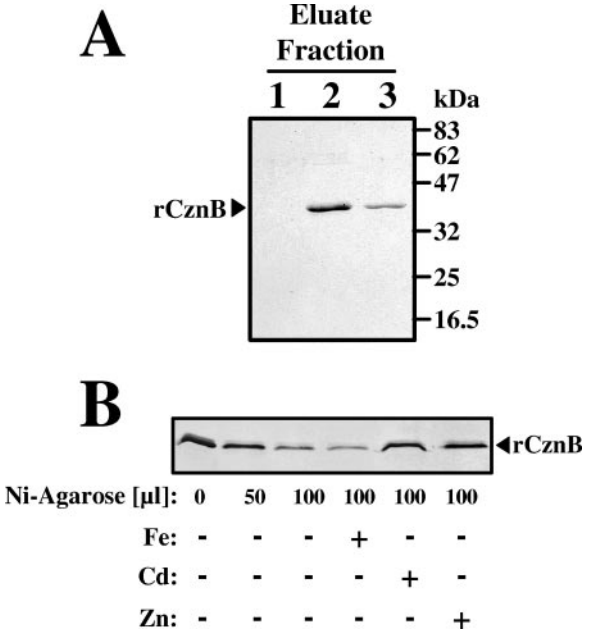

FIG. 5. Determination of the metal-binding capacity of purified rCznB. (A) Purification of the rCznB protein. The *H. pylori* rCznB protein was isolated from lysates of *E. coli* carrying the expression vector pASK-IBA3-CznB by affinity chromatography on *Strep*-Tactin columns. Proteins in samples taken from eluate fractions after stringency washing were separated by SDS-PAGE and visualized by staining with Coomassie blue. (B) Competition assay demonstrating binding of rCznB to cadmium and zinc. Purified rCznB protein was incubated with increasing amounts of nickel-NTA magnetic agarose beads in the presence or absence of metal ions as indicated. The unbound CznB protein was detected in supernatants of binding reactions after the removal of magnetic beads.

IBA3 (Table 1). Despite repeated attempts, we were unsuccessful in expressing the *cznA* gene, suggesting that the encoded membrane protein could be toxic for *E. coli*. However, expression vectors containing *cznC* and *cznB* coding regions could be transferred to *E. coli*, and the recombinant proteins designated rCznC and rCznB were correctly produced, as demonstrated by specific immunoblot detection with *Strep*-Tactin-AP conjugate after induction of gene expression (Fig. 4A). However, during the following purification procedure, the rCznC protein was repeatedly fragmented by proteolytic cleavage and could not be used for further experiments. Therefore, we analyzed the nickel-binding capacities of intact CznC and CznB proteins directly in *E. coli* cells expressing rCznC or rCznB by affinity chromatography with nickel-agarose. After removal of the nonbound proteins by washing, nickel binding was investigated by the elution of proteins with the nickel chelator imidazole. The detection of both proteins in the corresponding imidazole elution fractions by immunoblotting with *Strep*-Tactin-AP conjugate revealed that both rCznC and rCznB are able to interact with nickel (Fig. 4B).

Cadmium- and zinc-binding properties of recombinant CznB. In contrast to rCznC, the rCznB protein could be purified to homogeneity by affinity chromatography on a *Strep*-Tactin column (Fig. 5A). To assess the specific binding of rCznB to cadmium and zinc ions, we analyzed the metal-binding properties in a modified nickel-binding assay. To establish the approach, the rCznB protein was incubated with nickel-NTA magnetic agarose beads (QIAGEN) at increasing concentrations. After magnetic removal of the beads, the remaining protein in the supernatant was detected by immunoblot analysis with *Strep*-Tactin-AP conjugate. This analysis revealed that rCznB bound to nickel, as indicated by the disappearance of the protein in the supernatants after incubation with nickel-NTA agarose beads (Fig. 5B). To determine the binding of rCznB to other metals, we analyzed the binding of rCznB to nickel-NTA magnetic agarose beads in the presence or absence of cadmium or zinc. The results show that both cadmium and zinc prevented the binding of rCznB to the nickel-NTA agarose beads, whereas iron at identical concentrations was ineffective in this respect (Fig. 5B).

The *H. pylori cznABC* mutants do not colonize a gerbil model of infection. The role of metal homeostasis mediated by the *cznABC* metal exporter in gastric colonization was investigated in the gerbil-based animal model (9, 41). For this purpose, the *cznABC* genes were inactivated in the gerbil-adapted *H. pylori* strain P149 (Table 1). Gerbils were orally infected with the wild-type strain P149 ($n = 8$) and with the isogenic *cznC* ($n = 2$), *cznB* ($n = 3$), and *cznA* ($n = 3$) mutants, respectively. The eight animals challenged with the wild-type strain were colonized with an average bacterial load of 1.02×10^5 bacteria/g of stomach. In contrast, stomachs of the eight animals challenged with comparable inocula of the *cznC*, *cznB*, or *cznA* mutants were completely free of *H. pylori* 3 weeks after inoculation (Fig. 6).

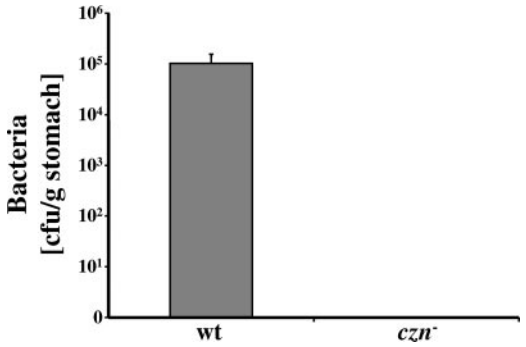

FIG. 6. Colonization properties of the *H. pylori cznABC* mutants in the gerbil model of infection. Eight animals were orally infected either with the *H. pylori* wild-type strain P149 (wt) or with *cznC* ($n = 2$), *cznB* ($n = 3$), and *cznA* ($n = 3$) mutant strains (combined in the bar labeled "*czn*$^-$"), respectively. After 3 weeks, *H. pylori* bacteria were reisolated from the stomach, and the bacterial load was determined by plating and counting the colonies (CFU). The results are presented as the mean, and the standard deviations are indicated.

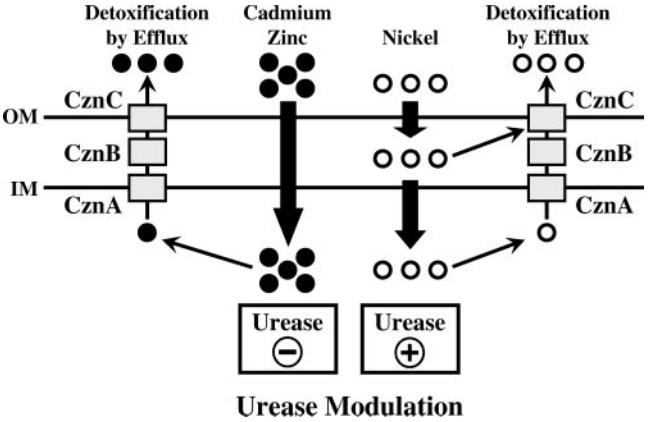

FIG. 7. Model of *H. pylori* Czn architecture and overview of the modulation of urease activity by export of individual metal ions. Metal fluxes are indicated by arrows. The Czn system is shown as tripartite complex similar to the CzcABC system in *R. metallidurans*. The effects of metal ion fluxes on urease activity are indicated. The efflux of cadmium and zinc results in the activation of urease, whereas the removal of nickel inhibits urease activity.

DISCUSSION

Depending on the diet, the gastric mucosa represents a highly variable habitat, in which changes in the environmental metal ion concentration occur within minutes. The average daily requirement for trace metals in the milligram range and the ionic content of drinking water leads to the assumption that *H. pylori* is exposed to metal ions in the micromolar range. Alterations in metal ion availability are thought to occur via the release of ions from food or by the cation-chelating activity of gastric mucus or host proteins (25). For its continuous persistence in the human stomach, *H. pylori* has evolved an extended repertoire of adaptive mechanisms, which allow the maintenance of cytoplasmic metal ion homeostasis even if the environmental conditions change drastically. Therefore, *H. pylori* contains genes for a multitude of metal ion transport systems, which differ in regulation and in ion specificity (36). The identification and characterization of the *H. pylori* metal export system Czn in the present study further corroborates the biological relevance of cytoplasmic ion homeostasis in gastric adaptation. The complete colonization defect of *cznABC* mutants in the gerbil stomach indicates that metal efflux by the Czn system is essential for gastric adaptation and shows for the first time that metal ion export plays a fundamental role in the successful establishment of *H. pylori* infection. The significant homology of *H. pylori* CznAB proteins to CzcAB proteins of other bacteria (Fig. 1A) provides strong evidence that the architecture of the *H. pylori* Czn system is similar to the Czc-type transenvelope transporters in *Ralstonia* sp. and in other bacteria (Fig. 7). In *Ralstonia* sp. CzcA is thought to be a transmembrane protein that functions as a cation-proton antiporter across the cytoplasmic membrane. While CzcB might span the periplasm, CzcC is probably attached to the outer membrane, where it might contact a hypothetical outer membrane protein, OmpY (19, 27). The Czn system of *H. pylori* shows most likely the same organization and localization of the three subunits (Fig. 7). The CznA protein consists of two hydrophobic domains and has homology to integral membrane proteins. Although most parts of the CznB and the CznC proteins are rather hydrophilic, the CznC protein contains a predicted domain that belongs to the OEP family (outer membrane efflux protein) (5, 29).

The phenotypes displayed by the *H. pylori cznC*, *cznB*, and *cznA* mutants in vitro demonstrated that the Czn system mediates metal ion efflux specific for cadmium, zinc, and nickel (Fig. 2). This was further supported by the binding of recombinant *H. pylori* CznC and CznB proteins to nickel (Fig. 4B) and the competitive inhibition of nickel binding to CznB by cadmium and zinc (Fig. 5B). Furthermore, determination of the zinc concentration of *H. pylori* lysates by using mass spectroscopy supports the zinc-exporting functions of *H. pylori* CznABC. The zinc concentration of 4.2 ± 0.4 in the wild-type strain was significantly elevated to 6.0 ± 0.4, 6.2 ± 0.4, and 5.2 ± 0.4 μg of zinc/mg of protein in the *cznA*, *cznB*, and *cznC* mutants, respectively. The nickel and cadmium concentration of the lysates was below the detection limit and could therefore not be analyzed. Nickel export by CznA and CznC was confirmed by the finding that the urease activity is enhanced in the *cznC* and *cznA* mutants, but not in the *cznB* mutant (Fig. 3A). This indicates that at low nickel concentrations, CznC and CznA are able to compensate for the nickel export deficiency caused by the *cznB* mutation. The fact that only the *cznC* mutant, but not the *cznA* mutant, displayed an elevated rise of the urease activity after nickel supplementation (Fig. 3B), suggests that the CznC protein is of particular importance for nickel export. Zinc supplementation reduced, but did not completely inhibit, urease activity both in the wild-type strain and in the *cznABC* mutants (Fig. 3C). This finding supports the assumption that the zinc export function of the Czn system is partially compensated for by other metal efflux systems. Since cadmium and zinc are also transported by the P-type ATPase CadA of *H. pylori* (8, 13), this export system might act in concert with Czn, as described for the Czc efflux pump and the P-type ATPases ZntA and CadA in *Ralstonia metallidurans* (10). It was reported that *H. pylori* double mutants, lacking CznC and HP0605, displayed increased metronidazole sensi-

tivity (33), suggesting a more global role of the Czn system in multidrug detoxification. However, the E-test analysis of the resistance of the *cznABC* mutants to different antibiotics revealed that the susceptibilities of our mutants to tetracycline, clarithromycin, amoxicillin, ciprofloxacin, and metronidazole did not differ from the wild-type strain (data not shown). This indicates that the Czn system alone is not required for maintaining *H. pylori* antibiotic resistance.

Finally, although the *H. pylori* CznABC system transports cadmium, zinc, and nickel, the biological consequences of the exported ions can differ considerably. Whereas nickel efflux results in a reduction in urease activity, the removal of cadmium and zinc prevents inhibition of the urease (Fig. 7). In reference to this, the export of cadmium and zinc from the cytoplasm is beneficial for *H. pylori* in the presence of acid. At neutral pH the export of nickel and the subsequent reduction of the urease activity could be advantageous for the bacterium, since high urea concentrations are toxic for *H. pylori* in the absence of gastric acid (14, 26). The fact that *H. pylori* is faced with both situations, depending on its localization in the stomach, might explain the essential function of Czn-mediated metal ion export in gastric colonization.

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