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Expression of Catalytically Active Recombinant *Helicobacter pylori* Urease at Wild-Type Levels in *Escherichia coli*

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The genes encoding *Helicobacter pylori* urease, a nickel metalloenzyme, have been cloned and expressed in *Escherichia coli*. Enzymatic activity, however, has been very weak compared with that in clinical isolates of *H. pylori*. Conditions under which near wild-type urease activity was achieved were developed. *E. coli* SE5000 containing recombinant *H. pylori* urease genes was grown in minimal medium containing no amino acids, $NiCl_2$ was added to 0.75 μM, and structural genes *ureA* and *ureB* (pHP902) were overexpressed in *trans* to the complete urease gene cluster (pHP808). Under these conditions, *E. coli* SE5000(pHP808/pHP902) expressed a urease activity up to 87 μmol of urea per min per mg of protein (87 U/mg of protein), a level approaching that of wild-type *H. pylori* UMAB41 (100 U/mg of protein), from which the genes were cloned. Poor catalytic activity of recombinant clones grown in Luria broth or M9 medium containing 0.5% Casamino Acids was due to chelation of nickel ions by medium components, particularly histidine and cysteine. In cultures containing these amino acids, $^{63}Ni^{2+}$ was prevented from being transported into cells and was not incorporated into urease protein. As a consequence, M9 minimal medium cultures containing histidine or cysteine produced only 0.05 and 0.9%, respectively, of active urease produced by control cultures containing no amino acids. We conclude that recombinant *H. pylori* urease is optimally expressed when Ni^{2+} transport is not inhibited and when sufficient synthesis of urease subunits UreA and UreB is provided.

Helicobacter pylori has adopted a strategy for survival in the human gastric mucosa that includes production of a bacterial urease. This enzyme hydrolyzes urea, releasing ammonia, which may allow survival of the organism at a low pH. The urease has been demonstrated as essential for colonization of the gastric mucosa in the gnotobiotic piglet model of infection (11). In addition, urease is a key protein used for detection of the organism by measuring serum antibody to the protein (10, 35), enzyme activity in gastric biopsies (26), or release of labeled CO_2 with the urea breath test (5, 13).

H. pylori urease is a 550-kDa (19, 27) enzyme, consisting of two distinct subunits with apparent molecular masses of 29.5 kDa (UreA) and 66 kDa (UreB). The ratio of subunits, determined by densitometric scanning of stained polyacrylamide gels of purified preparations, is approximately 1:1, suggesting a stoichiometry of $(29.5–66\ kDa)_6$ for the native enzyme. Like all other ureases (28), nickel ions are present in the protein structure (16), probably as a component of the active site.

H. pylori urease genes have been cloned in *Escherichia coli* (7, 9, 17, 22). We have found that only *ureA* and *ureB* are required for the synthesis and assembly of the 550-kDa apoenzyme (17). Two additional genes (*ureC* and *ureD*) are required for the expression of weak and unstable urease activity in *Campylobacter jejuni* (22). However, these four genes, *ureC*, *ureD*, *ureA*, and *ureB*, are still not sufficient for urease activity in *E. coli*. Cussac et al. (9) reported that four additional open reading frames (*ureE*, *ureF*, *ureG*, and *ureH*), downstream of structural genes *ureA* and *ureB*, were required for recombinant *H. pylori* urease activity in *E. coli*. The urease activity reported by this group, however, was still very weak (<1 μmol of urea per min per mg of protein after 3 days of growth). Evidence that expression was under the control of the nitrogen regulatory system and that the catalytically active enzyme was synthesized only under nitrogen-limiting conditions was provided.

In this report, we describe conditions under which catalytically active *H. pylori* urease is expressed in *E. coli* at near wild-type levels. We found that maximal recombinant urease activity was not regulated by nitrogen limitation but could be achieved by (i) overexpressing the urease structural subunits, (ii) adding exogenous $NiCl_2$, and (iii) culturing bacteria in minimal medium that contains no Ni^{2+}-chelating agents.

(A preliminary account of this work has appeared previously [29].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* SE5000 [F^- *araD193* Δ(*argF lac*) *U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR recA56*], a maxicell strain, was used as a recipient for recombinant plasmids. Bacteria were grown in M9 salts medium (containing, per liter, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of NH_4Cl, containing 0.4% glucose, 0.002% thiamine-HCl, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$) at 37°C with aeration (200 rpm). Medium was supplemented with 0.5% Casamino Acids and various concentrations of $NiCl_2$ as indicated.

H. pylori UMAB41 was originally cultured from an endoscopic gastric biopsy obtained from a patient with complaints of abdominal pain and a history of peptic ulcer disease (27). Bacteria were cultured on brucella agar containing 10% sheep blood under microaerobic conditions (19).

Plasmids. Plasmid pHP808, which encodes the entire urease gene cluster, was constructed by subcloning an 11.0-kb *Sau*3A restriction fragment from a cosmid clone, pHP9D11, into the *Bam*HI site of pACYC184 as described previously (17). Subclone pHP902 was constructed by poly-

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merase chain reaction amplification of a 2.8-kb fragment carrying only the structural genes *ureA* and *ureB*. Oligonucleotide primers used for amplification included an *Eco*RI site upstream of *ureA* and a *Kpn*I site downstream of *ureB*. This fragment was cloned into *Eco*RI-*Kpn*I-digested pBluescript KSII(−) (17). New plasmid constructs are described in Results.

Urease assay. Rates of urea hydrolysis by soluble protein, derived from French press lysates, were measured by the spectrophotometric assay of Hamilton-Miller and Gargan (14), calibrated for the measurement of ammonia as described previously (30).

Maxicell labeling. Plasmid-encoded polypeptides were labeled with [^{35}S]methionine (specific activity, 800 to 1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) by using UV-treated *E. coli* SE5000(pHP802) and the method of Gherardini et al. (12). Labeled polypeptides were solubilized in sodium dodecyl sulfate (SDS)-gel sample buffer (3), electrophoresed on a 10 to 25% gradient SDS-polyacrylamide gel, and visualized by autoradiography.

Nickel incorporation and uptake. For measurement of nickel ion incorporation, *E. coli* SE5000(pHP808) was grown in M9 salts medium with and without 0.5% Casamino Acids, containing $^{63}NiCl_2$ (specific activity, 14.5 Ci/g; Amersham). After overnight incubation, a sample (50 μl) of the 100-ml culture was counted by liquid scintillation to determine total counts per minute. Bacterial cells were harvested by centrifugation (10,000 × *g*, 10 min, 4°C) and washed twice with 20 ml of 20 mM sodium phosphate (pH 6.8) and then were resuspended in 3 ml of 20 mM sodium phosphate (pH 6.8) buffer. A sample (20 μl) was counted by liquid scintillation, and the percentage of label that was incorporated into bacterial cells was calculated. The masses of the bacterial pellets were not significantly different and were not used in the calculations.

For measurement of nickel uptake, overnight cultures of *E. coli* SE5000(pHP808) were grown in M9 salts medium with and without 0.5% Casamino Acids but containing no $NiCl_2$. Cells were harvested by centrifugation, washed with M9 salts (containing, per liter, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of NH_4Cl), and suspended in that solution to an optical density at 600 nm of 1.3. To each 8-ml sample was added 0.75 μM $NiCl_2$ (75 nM $^{63}NiCl_2$ and 675 nM unlabeled $NiCl_2$). Then, samples (500 μl each) were taken at 0.75, 1.5, 2.5, 5, 10, and 20 min by vacuum filtration through 0.45-μm-pore-size filters (Millipore) and washed three times with 3 ml of phosphate-buffered saline (PBS). $^{63}Ni^{2+}$ retained on the filters was quantitated by liquid scintillation counting.

Nickel toxicity studies. Twofold dilutions of $NiCl_2$ at concentrations ranging from 0 to 16 μM and 20 amino acids at concentrations ranging from 0 to 12.5 mM were made in M9 medium, each alone and in combination in the wells of microtiter plates. Medium (150 μl) was inoculated with a 1:1,000 dilution of an overnight culture of *E. coli* SE5000(pHP808) and incubated for 18 h at 37°C. MICs of $NiCl_2$ and amino acids, defined as the lowest concentrations that completely inhibited growth, were assigned by visual inspection.

Immunoprecipitation. Overnight cultures were grown in M9 medium containing 1 mM histidine, cysteine, arginine, glutamine, or proline or with no amino acid and 1 μM $NiCl_2$ (250 nM $^{63}NiCl_2$ and 750 nM unlabeled $NiCl_2$). Cells, harvested by centrifugation (10,000 × *g*, 10 min, 4°C), were washed twice with 10 ml of 20 mM sodium phosphate (pH 6.8) and then were resuspended in 3 ml of 20 mM sodium phosphate (pH 6.8) and disrupted by a single passage through a precooled French pressure cell at 20,000 lb/in^2. Unbroken cells were removed by centrifugation (10,000 × *g*, 10 min, 4°C). Soluble protein was analyzed for urease activity and protein concentration. Synthesis of urease structural subunits (UreA and UreB) was assessed by Western blot (immunoblot) using antisera specific for UreA or UreB.

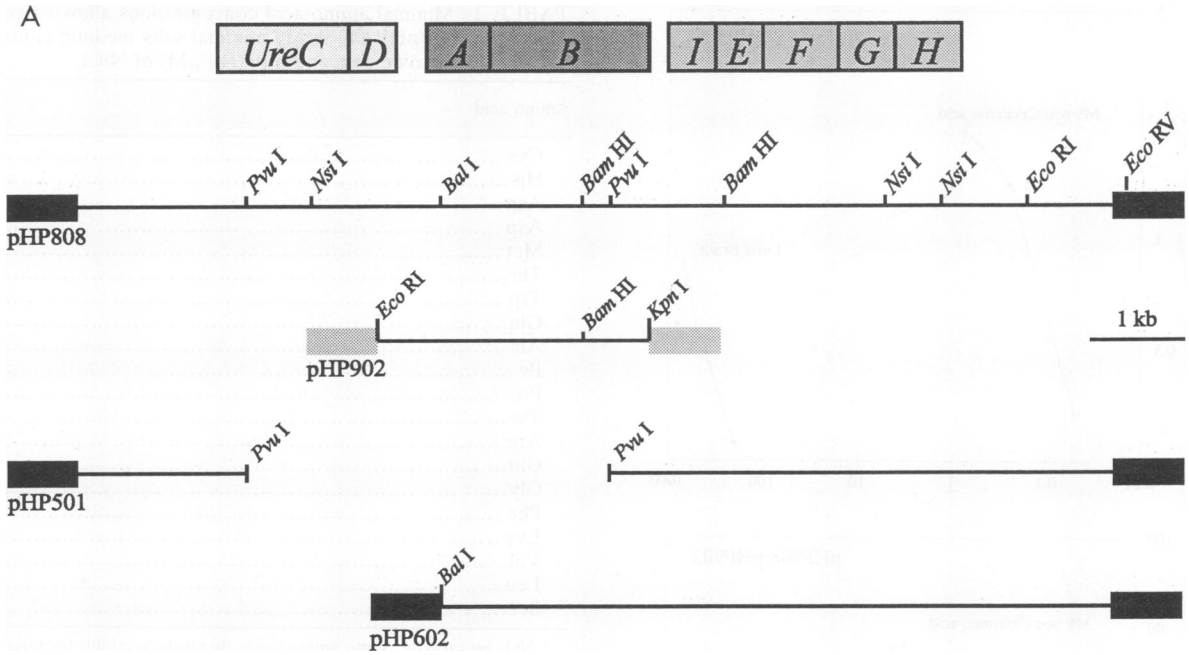
For immunoprecipitation, cell lysates (100 μl) were incubated with either anti-UreB (1:20) or anti-UreA (1:10) in a total volume of 750 μl of 2% Triton X-100–50 mM Tris–0.15 mM NaCl–0.1 mM EDTA (pH 8.0) (37) at 4°C for 18 h. Following this incubation, 75 μl of a 10% (wet wt/vol) suspension of nonviable *Staphylococcus aureus* Cowan cells in 0.04 M sodium phosphate buffer (pH 7.2)–0.15 M NaCl containing 0.005% sodium azide (Sigma) was added to the precipitation mixture and incubated on ice for 1 h. After centrifugation in a microcentrifuge for 5 min, the pellet was washed twice in PBS (8 g of sodium chloride, 0.2 g of potassium chloride, 1.15 g of disodium hydrogen phosphate, and 0.2 g of potassium dihydrogen phosphate per liter, pH 7.4) containing 0.1% SDS and 1% Triton X-100 and was washed once in 10 mM Tris (pH 8.0). The immunoprecipitate was extracted from the pellet with 200 μl of 1% SDS in 10 mM Tris–1 mM EDTA (pH 8.0) by heating at 100°C for 10 min and centrifuging in a microcentrifuge for 10 min (3). Supernatant (150 μl) was added to 7 ml of Ready Protein$^+$ scintillation cocktail (Beckman) and counted by liquid scintillation.

Western blot. Protein was electrophoresed on 15% polyacrylamide (*N*,*N*′-methylene-bis-acrylamide–acrylamide, 0.8:30) gels and blotted onto nitrocellulose. Western blots were developed as described by Towbin et al. (41) with the modifications of Batteiger et al. (4). All incubations were carried out at room temperature. Rabbit antisera raised against each of the purified *H. pylori* UreA and UreB urease subunit polypeptides were diluted in 1% sodium caseinate in PBS (pH 7.4). Nitrocellulose filters were incubated in antisera for 1 h. After being washed in 0.05% Tween 20 in PBS, filters were incubated in 1:1,000 conjugated goat anti-rabbit immunoglobulin G–alkaline phosphatase for 1 h. Filters were washed as described above, and the color reaction was developed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma). Densitometric tracings of Western blots were done with ImageQuant software (Molecular Dynamics).

RESULTS

Plasmid constructs. Plasmids used in this study are shown in Fig. 1A. Plasmid pHP808 carries the entire *H. pylori* urease gene cluster on an 11.0-kb *Sau*3A fragment cloned into the *Bam*HI site of pACYC184 (17). Plasmid pHP902 carries only *ureA* and *ureB* cloned into pBluescript KS(−) (17). Plasmid pHP602 was constructed by deleting a 3.8-kb *Bal*I fragment, encoding *ureC* and *ureD*, from pHP808. Plasmid pHP501 was constructed by deleting a 3.9-kb *Pvu*I fragment, encoding *ureC*, *ureD*, *ureA*, and *ureB*, from pHP808. The 5′ end of *ureC* and the 3′ end of *ureB* remain in the latter construct.

[Ni^{2+}]-dependent urease activity in recombinant clones. *E. coli* SE5000, a maxicell strain, transformed with plasmid pHP808, displayed weak urease activity (<0.05 U/mg of protein), which could be only qualitatively detected when cultures were grown on minimal salts agar containing no Casamino Acids supplement (17). When M9 medium con-



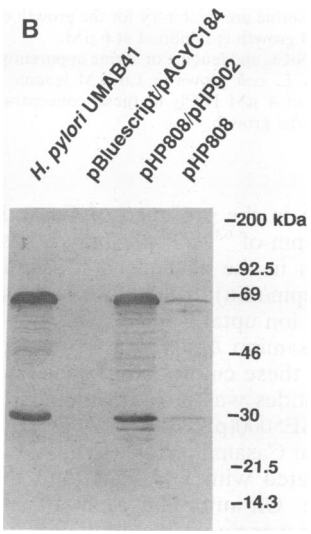


FIG. 1. *H. pylori* urease clones used in this study. (A) Plasmid pHP808 is a pACYC184 derivative containing the entire urease gene cluster on an 11-kb insert (17). Plasmid pHP902 is a pBluescriptII KS(−) derivative containing only *ureA* and *ureB* on a 2.8-kb insert. Plasmids pHP602 and pHP501 are deletion mutants of pHP808 and are described in Results. Urease genes, shown at the top, are those described by Cussac et al. (9) and have been aligned as described previously (17). Partial vector sequences are shown as thick lines. (B) Western blot of soluble protein (100 μg) from *H. pylori* UMAB41, *E. coli* SE5000(pHP808), and *E. coli* SE5000(pHP808/pHP902), electrophoresed on an SDS–10 to 17.5% polyacrylamide gel. The primary antibodies were anti-UreA and anti-UreB. Blots were developed as described in Materials and Methods.

taining no Casamino Acids was supplemented with various concentrations of $NiCl_2$, measurable urease activity was detected (Fig. 2) in the soluble protein of this strain. Urease activity was maximal (1.4 U/mg of protein) at a medium concentration of 0.75 μM $NiCl_2$.

To further increase urease activity, *E. coli* SE5000 (pHP808) was cotransformed with plasmid pHP902, which encodes only structural genes *ureA* and *ureB*. When *E. coli* SE5000(pHP808/pHP902) was grown in 1 μM $NiCl_2$, urease activity was increased more than 50-fold to 76 U/mg of protein compared with *E. coli* containing subclone pHP808 alone (Fig. 2). This value approached urease activity measured for wild-type *H. pylori* UMAB41, which was 100 U/mg of protein (19).

Increase in urease activity was paralleled by an increase in urease protein recognized on a Western blot with antiserum to UreA and UreB (Fig. 1B). Compared with wild-type *H. pylori* UMAB41, *E. coli*(pHP808) produced only 15% of the urease of the native organism as estimated by scanning densitometry of the blot using the intensity of UreB (66-kDa band) as an index. On the other hand, *E. coli*(pHP808/pHP902) produced 82% of the urease synthesized by wild-type *H. pylori*.

There was no detectable urease activity when clones were grown either in M9 medium containing Casamino Acids or in Luria broth. This lack of urease activity in clones grown in M9 medium containing Casamino Acids or in Luria broth could be reversed by addition of higher concentrations of $NiCl_2$. When the $NiCl_2$ concentration was increased to 300 μM in M9 medium containing Casamino Acids, subclone pHP808 demonstrated urease activity of 1.2 U/mg of protein. *E. coli*(pHP808) and *E. coli*(pHP808/pHP902) had maximal activities of 0.9 U/mg of protein at 700 μM $NiCl_2$ and 32.8 U/mg of protein at 800 μM $NiCl_2$, respectively, in Luria broth. Neither reached the activities observed with M9 medium containing no Casamino Acids. This raised the possibility that availability of nickel to the cytosol accounted for differences in urease activity.

Ni^{2+} incorporation and uptake. To analyze Ni^{2+} ion incorporation by *E. coli* SE5000(pHP808), bacteria were grown for 18 h in M9 medium with and without 0.5% Casamino Acids, containing 0.75 μM $NiCl_2$ (75 nM $^{63}NiCl_2$ and 675 nM unlabeled $NiCl_2$). Only 2.4% of total $^{63}Ni^{2+}$ was incorporated by bacterial cells grown in M9 medium containing 0.5% Casamino Acids. However, bacterial cells grown in M9

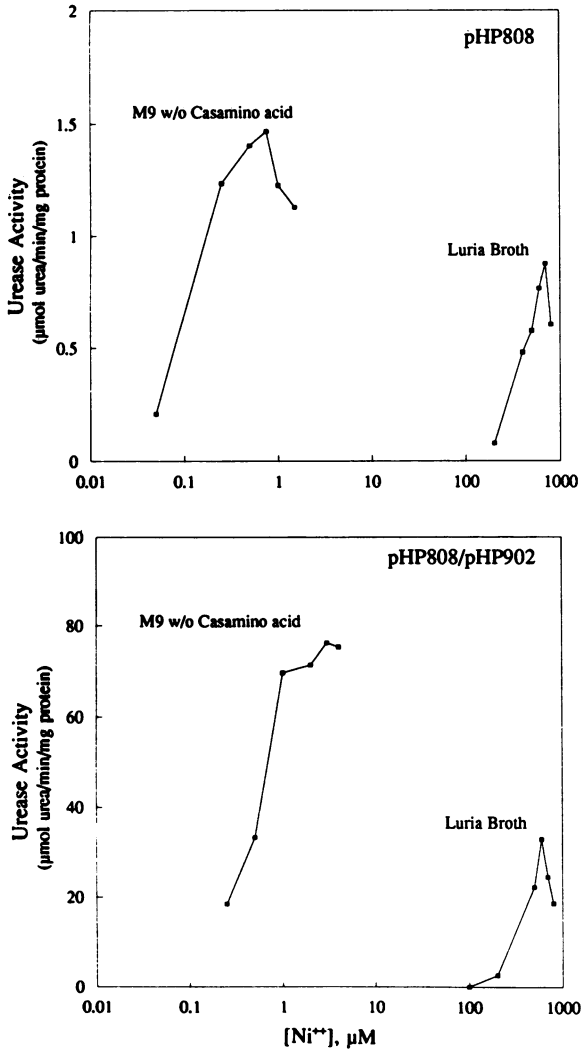


FIG. 2. $[Ni^{2+}]$-dependent urease activity of recombinant *H. pylori* urease expressed in *E. coli* SE5000. *E. coli* SE5000 containing cloned *H. pylori* urease genes (pHP808 alone [top] or cotransformed with pHP902 [bottom]) was grown in either M9 medium without (w/o) Casamino Acids for 18 h or Luria broth for 22 h, both containing different concentrations of $NiCl_2$. Urease activity in soluble protein derived from French press-ruptured cells from each of the cultures was determined by the phenol red spectrophotometric urease assay. Note the different urease activity scales for the two panels.

medium with no Casamino Acids incorporated 50.9% of total $^{63}Ni^{2+}$ into the cells. These data implied either that the absence of Casamino Acids was activating genes necessary for uptake of Ni^{2+} ions or, simply, that Ni^{2+} ions were being chelated by Casamino Acids in the medium, thus preventing uptake.

To distinguish between these two possibilities, *E. coli* SE5000(pHP808) was cultured in M9 medium with and without Casamino Acids for 18 h. Cells were harvested and washed with and suspended in M9 medium containing no Casamino Acids. The rate of nickel ion uptake was calculated for the first 1.5 min and was found not to differ between cells grown in the presence of Casamino Acids and those grown in their absence (a standardized suspension of cells that was grown in the presence of Casamino Acids transported 26,907 cpm of $^{63}Ni^{2+}$ per min; a suspension of cells that was grown in the absence of Casamino Acids transported 26,776 cpm/min). These data suggested that expression of a nickel ion uptake system was not regulated by the presence of Casamino Acids.

TABLE 1. Minimal amino acid concentrations allowing growth of *E. coli* SE5000(pHP808) in M9 minimal salts medium containing a toxic concentration (16 μM) of $NiCl_2$

Amino acid	Concn (mM)
Cys	<0.05
His	<0.05
Asn	0.8
Asp	0.8
Met	0.8
Thr	0.8
Trp	0.8
Gln	1.6
Ala	3.1
Ile	3.1
Pro	3.1
Tyr	3.1
Arg	6.3
Glu	6.3
Gly	6.3
Phe	6.3
Lys	12.5
Val	NG[a]
Leu	—[b]
Ser	—[b]

[a] NG, no growth. Some amino acids themselves inhibit bacterial growth. Asparagine, cysteine, histidine, isoleucine, leucine, phenylalanine, tryptophan, tyrosine, and serine are inhibitory for the growth of *E. coli* at >25 mM. For valine, bacterial growth is inhibited at 6 μM.

[b] The mixture of $NiCl_2$ and leucine or serine apparently increases nickel ion toxicity to bacteria. *E. coli* grows in 1.6 mM leucine and 0.2 mM serine. However, addition of 4 μM $NiCl_2$ to these concentrations of leucine and serine inhibits bacterial growth.

The effect of these culture conditions on the synthesis of urease polypeptides was also examined with *E. coli* maxicells. *E. coli* SE5000(pHP808) was grown in M9 medium with and without Casamino Acids to an optical density at 600 nm of 0.5, treated with UV light, and then labeled with [^{35}S]methionine. On autoradiographs of an SDS-polyacrylamide gel, there was no visible difference in the number and intensity of the labeled polypeptides encoded by the urease gene cluster, including bands with apparent molecular sizes of 66 (UreB), 47 (UreC), and 29.5 (UreA) kDa. This finding suggested that urease genes were not expressed differentially in the presence and absence of Casamino Acids.

Protection from $NiCl_2$ toxicity by amino acids. To detect the specific amino acid in the Casamino Acids mixture responsible for chelating Ni^{2+}, *E. coli* SE5000(pHP808) was grown in M9 medium with different $NiCl_2$ concentrations (0 to 64 μM) and different concentrations of each of the 20 common amino acids (0 to 12.5 mM). Bacterial cells did not grow at $NiCl_2$ concentrations higher than 16 μM in M9 medium containing no Casamino Acids or other amino acid supplement. However, 17 of 20 amino acids displayed a range of concentrations that allowed growth of the bacteria in 16 μM $NiCl_2$. The lowest concentration of each amino acid that allowed growth is shown in Table 1. By far, histidine and cysteine provided the best protection. Bacteria were protected from $NiCl_2$ toxicity by <0.05 mM cysteine or histidine. Asparagine, aspartic acid, methionine, threonine, and

tryptophan at <1 mM protected bacterial growth from nickel ion toxicity.

Nickel ion incorporation into urease correlates with urease activity. To analyze the relationship between urease activity, amino acid protection from $NiCl_2$ toxicity, and Ni^{2+} incorporation into urease, *E. coli* SE5000(pHP808/pHP902) was cultured in M9 medium containing 1 μM $NiCl_2$ (250 nM $^{63}NiCl_2$ and 750 nM unlabeled $NiCl_2$) along with 1 mM each of five amino acids: histidine, cysteine, arginine, glutamate, and proline. Cells cultured with histidine and cysteine displayed urease activities of only 0.05 and 0.9%, respectively, of the control culture (no amino acid added) at 87 U/mg of protein (Fig. 3A). Arginine, glutamate, and proline decreased the urease activity only to 88.7, 62.6, and 81.5% of control activity, respectively.

Differences in urease activities were not due to different levels of expression of the structural subunits of urease. Soluble protein (100 μg), derived from French pressure lysates of each culture, was electrophoresed on an SDS-polyacrylamide gel and transferred to nitrocellulose. Urease protein was detected by immunoblotting with anti-UreB and anti-UreA antisera. Urease subunits were produced with the same intensity regardless of which amino acid was used to supplement the growth medium (Fig. 3B).

Urease activity correlated with the amount of $^{63}Ni^{2+}$ that was incorporated into the enzyme structure. After overnight incubation, $^{63}Ni^{2+}$-containing bacterial cells were washed thoroughly and lysed in a French press. Urease was immunoprecipitated from soluble protein with anti-UreB and anti-UreA antisera, and $^{63}Ni^{2+}$ counts per minute in the precipitate were determined (Fig. 3A). $^{63}Ni^{2+}$ incorporated into urease was detected at baseline levels for *E. coli* SE5000(pHP808/pHP902) grown in M9 medium with histidine or cysteine. The higher counts of $^{63}Ni^{2+}$ paralleled higher urease activities for bacteria grown in M9 medium containing arginine, glutamine, or proline. For bacterial cell cultures with histidine, cysteine, arginine, glutamate, proline, and no amino acid, 6.4, 3.4, 36.2, 39.6, 37.5, and 37.9%, respectively, of total $^{63}Ni^{2+}$ was incorporated into the cells. These data show that histidine and cysteine prevent nickel ions from entering the bacterial cell and, consequently, prevent the ion from incorporating into urease.

Urease activity of other constructs. By using conditions found to be optimal for expression of active urease (M9 medium without Casamino Acids, supplemented with 0.75 to 1.00 μM $NiCl_2$), constructs shown in Fig. 1A were tested for urease activity. *E. coli*(pHP602) hydrolyzed urea at three times the rate of *E. coli*(pHP808); *E. coli*(pHP602/pHP902) displayed 90% of the activity of *E. coli*(pHP808/pHP902). *E. coli*(pHP501) was urease negative but could be complemented in *trans* with pHP902; *E. coli*(pHP501/pHP902) had an activity that was 69% of that of *E. coli*(pHP808/pHP902).

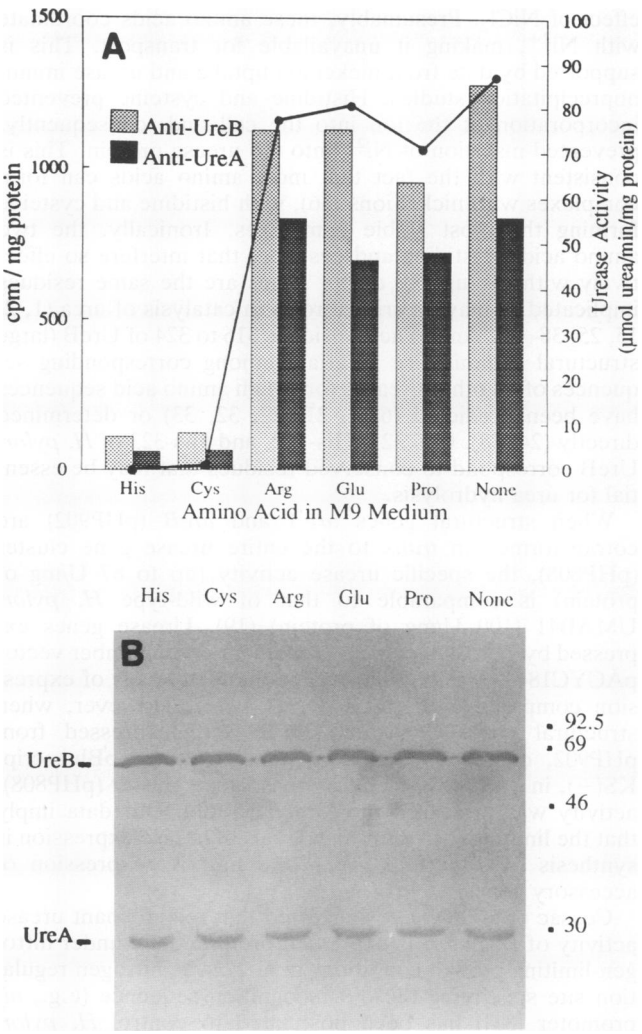


FIG. 3. Incorporation of $^{63}Ni^{2+}$ into urease after culture in the presence of various amino acids. *E. coli* SE5000(pHP808/pHP902) was grown for 18 h in M9 medium with one of five different amino acids: histidine, cysteine, arginine, glutamate, and proline. A control culture contained no amino acid. Each culture also contained 1 μM $NiCl_2$ (250 nM $^{63}NiCl_2$ and 750 nM unlabeled $NiCl_2$). (A) A line connecting urease activity values (●) is drawn for clarity only and does not imply a continuous function; $^{63}Ni^{2+}$ counts per minute immunoprecipitated by anti-UreA and anti-UreB antisera are shown as bars. (B) Western blotting. Soluble protein (100 μg) derived from each culture was electrophoresed on an SDS–15% polyacrylamide gel, transferred to nitrocellulose, and reacted with antisera directed against both the large (UreB) and the small (UreA) subunits of *H. pylori* urease. The migration of protein standards is shown on the right in kilodaltons (Rainbow protein molecular size markers; Amersham).

DISCUSSION

Urease genes, isolated and subcloned from *H. pylori*, were expressed in *E. coli* SE5000 at near wild-type activity. High levels of expression of a catalytically active urease required three conditions: (i) $NiCl_2$ must be supplied to growing cultures, (ii) *E. coli* containing cloned *H. pylori* urease genes must be grown in a minimal salts medium lacking compounds that chelate nickel ions, and (iii) structural subunits of the urease enzyme, UreA and UreB, must be synthesized at high levels.

Optimal expression of a catalytically active urease by *E. coli* SE5000(pHP808) and *E. coli* SE5000(pHP808/pHP902) (1.4 and 76 U/mg of protein, respectively) cultured in M9 medium without Casamino Acids was observed at $NiCl_2$ concentrations of 0.75 and 1 μM, respectively (Fig. 2). Cultures grown in the absence of $NiCl_2$ synthesized an apourease that had minimal enzymatic activity (<0.05 U/mg of protein).

When the 20 common amino acids were analyzed for the ability to protect bacteria from $NiCl_2$ toxicity, histidine and cysteine provided, by far, the best protection from the toxic

effect of $NiCl_2$. Presumably, these amino acids coordinate with Ni^{2+}, making it unavailable for transport. This is supported by data from nickel ion uptake and urease immunoprecipitation studies. Histidine and cysteine prevented incorporation of the ion into the cell and, consequently, prevented insertion of Ni^{2+} into the urease protein. This is consistent with the fact that most amino acids can form complexes with nickel ions (36), with histidine and cysteine forming the most stable complexes. Ironically, the two amino acids, histidine and cysteine, that interfere so effectively with the uptake of Ni^{2+} ions are the same residues implicated as playing critical roles in catalysis of urea (1, 2, 15, 25, 38–40). Amino acid residues 316 to 324 of UreB (large structural subunit) are invariant among corresponding sequences of all other ureases for which amino acid sequences have been predicted (6, 7, 21, 22, 32, 33) or determined directly (24, 38). Cys-321, His-322, and His-323 of *H. pylori* UreB correspond to conserved residues that may be essential for urea hydrolysis.

When structural genes *ureA* and *ureB* (pHP902) are cotransformed in *trans* to the entire urease gene cluster (pHP808), the specific urease activity (up to 87 U/mg of protein) is comparable to that of wild-type *H. pylori* UMAB41 (100 U/mg of protein) (19). Urease genes expressed by pHP808, cloned on medium-copy-number vector pACYC184, are not sufficient for the high levels of expression compared with those of *H. pylori*. However, when structural genes *ureA* and *ureB* were expressed from pHP902, cloned on high-copy-number vector pBluescript KS(−), in *trans* to the entire urease gene cluster (pHP808), activity was increased more than 50-fold. Our data imply that the limiting factor for high levels of urease expression is synthesis of structural genes and not overexpression of accessory genes.

Cussac et al. (9) have suggested that recombinant urease activity of *H. pylori* is expressed preferentially under nitrogen-limiting growth conditions in *E. coli*. A nitrogen regulation site specifying the σ^{54} recognition sequence (e.g., *nif* promoter [31]) has been postulated to control *H. pylori* expression in *E. coli*. A sequence 310 bp upstream of the *ureD* and *ureA* genes displays homology with the σ^{54} binding site (22). Under nitrogen-limiting growth conditions, active urease clones were identified (9, 17). Although we cannot rule out that nitrogen regulation plays a role in urease synthesis in wild-type *H. pylori*, we do not observe this effect when recombinant clones are expressed in *E. coli*. We agree that when phenotype alone (i.e., urease activity) is examined, there is the appearance of nitrogen regulation. For example, at 1 μM $NiCl_2$, when *E. coli* SE5000(pHP808) or *E. coli* SE5000(pHP808/pHP902) is grown under conditions in which nitrogen is not limited (M9 medium plus 0.5% Casamino Acids or Luria broth), no urease activity is observed. This effect, however, can be reversed by addition of more (300 to 700 μM) $NiCl_2$ to the medium prior to inoculation. Furthermore, by maxicell analysis, nitrogen limitation does not affect synthesis of [^{35}S]Met-labeled plasmid-encoded polypeptides encoded by the urease gene cluster compared with samples labeled under nitrogen-rich conditions. The loss of urease activity under nitrogen-sufficient conditions actually appears to be due to chelation of Ni^{2+} ions by amino acids. Under these conditions, $^{63}Ni^{2+}$ is unable to gain entry into the cells. If these cells are washed and suspended in M9 salts containing no amino acids, however, Ni^{2+} ion uptake proceeds normally.

Ureases from several gram-negative bacterial species, including *Providencia stuartii* (30), *Proteus mirabilis* (20, 21), *Proteus vulgaris* (32), *Morganella morganii* (18), *E. coli* (8), *Klebsiella aerogenes* (23, 33, 34), *Ureaplasma urealyticum* (6), and *H. pylori* (7, 9, 17, 22), have been cloned in *E. coli*. With the exception of *U. urealyticum*, all of these enzymes are expressed in *E. coli*, but activities of the recombinant ureases vary considerably. Some, like *P. mirabilis*, express a reasonable amount of catalytically active urease in Luria broth containing no supplemental $NiCl_2$, while others, like *M. morganii*, produce an enzyme that is only weakly active. Since the genetic backgrounds were similar (i.e., *E. coli* strains) for all of these clones, the urease genes from different species may have differing efficiencies of capturing and inserting Ni^{2+} ions into the urease protein. This could result from the relative binding efficiencies of the accessory proteins involved in nickel processing or could reflect the efficiencies at which the urease apoenzymes accept Ni^{2+} for coordination into the active site.

The urease produced by in vitro-cultured clinical isolates of *H. pylori* is extremely active. It follows that, if nickel ions are inserted into the apoenzyme while the protein is in the cytoplasm, some mechanism must exist for wild-type *H. pylori* to transport Ni^{2+} ions into the cell. This must occur in the presence of external Ni^{2+} ion-chelating compounds present in the rich media used for cultivation of the organism. Once nickel has entered the cell, the ions must then be transferred to the newly synthesized urease apoenzyme. On the other hand, if the urease is assembled in the periplasmic space, then there would be no requirement for an active nickel transport system.

The high level of urease activity expressed by *H. pylori* is due to a high level of enzyme production. The protein per se is not more active than ureases from other species, but simply, more of the enzyme is produced (19). Nevertheless, the single chromosomal copy of the *H. pylori* urease gene cluster is overexpressed in the wild-type strains. The mechanism for this is not known but may involve an optimal promoter, an optimal ribosomal binding site, or a highly stable mRNA transcript.

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