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An Intact Urease Assembly Pathway Is Required To Compete with NikR for Nickel Ions in *Helicobacter pylori*▽†

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We examined the effects of urease and hydrogenase assembly gene deletions on NikR activation in *H. pylori* strains 26695 and G27. The loss of any component of urease assembly increased NikR activity under Ni^{2+}-limiting conditions, as measured by reduced transcript levels and ^{63}Ni accumulation. Additionally, SlyD functioned in urease assembly in strain 26695.

A diverse complement of proteins is dedicated to the acquisition, trafficking, and regulation of intracellular transition metal ions. The mechanisms by which these activities are integrated to allocate the appropriate proportion of metal to different metal-binding proteins are not yet understood. Additionally, studies of the equilibrium metal-binding properties of transcriptional regulatory proteins important for metal homeostasis have revealed that they avidly bind their cognate metal ions (10^{-21} M $\leq K_d \leq 10^{-9}$ M [8, 9, 22, 33, 42]). These observations suggest that competition may exist between metalloenzyme assembly and metalloregulation. Detailed investigations of this hypothesis are encumbered by the presence of numerous essential metalloenzymes for metals such as zinc and iron. Microbial nickel physiology provides an ideal system for studying intracellular metal competition due to the small number of enzymes that require nickel ions (30) and their nonessentiality under laboratory growth conditions.

We have studied the effect of disrupting Ni^{2+}-dependent enzyme assembly pathways on nickel-dependent gene regulation in the gram-negative gastric pathogen *Helicobacter pylori* (3). The two Ni^{2+}-dependent enzymes of *H. pylori*, urease and hydrogenase, are required for efficient colonization of animal models of infection (15, 16, 31). Both enzymes require conserved, GTP-dependent pathways for metal cofactor assembly that include an absolute requirement for nickel insertion chaperones under metal-limiting conditions (30). Hausinger and coworkers identified UreE as the Ni^{2+}-binding protein required for urease assembly in *Klebsiella aerogenes* (10, 38). Similarly, Bock and coworkers identified two chaperones, HypA and HypB, required for the nickel insertion step of *E. coli* hydrogenases (21, 26). Recently, the *E. coli* SlyD protein was shown to associate with HypB and participate in hydrogenase assembly (25, 46). Interestingly, in *H. pylori*, the hydrogenase chaperones HypA and HypB function in both hydrogenase and urease assembly (32).

NikR regulates the expression of several *H. pylori* genes in response to increased nickel ion levels. Genetic and biochemical studies have shown direct NikR-dependent regulation of genes required for nickel import (*nixA* [5, 17, 44], *fecA3* [18], *frpB4* [14, 18], and *exbBD tonB* [18]), Ni^{2+}-dependent enzyme activity (*ureAB* [1, 5, 17]), and nickel storage (*hpn* [11]). Because NikR represses all currently known nickel import genes (*nixA*, *fecA3*, *frpB4*, and *exbBD tonB*) (14, 17, 18, 44), Ni^{2+}-dependent enzyme biosynthesis pathways must acquire Ni^{2+} before NikR, and the subsequent repression of nickel uptake genes. Such competition, if present, would be manifested as a change in NikR activity independently of a change in total nickel levels. In the absence of competition, NikR activity would correlate with a fixed total nickel concentration, independent of Ni^{2+}-dependent enzyme expression or biosynthesis. Competition between metalloenzymes and metalloregulatory proteins has not been tested. Demonstration of the nature of such competition would facilitate subsequent studies to understand the molecular basis of metal ion partitioning within cells.

We examined the effects of Ni^{2+}-dependent enzyme assembly pathway gene deletions on NikR activity using several assays. In each case, cells were grown under identical conditions and manipulated in the same way for the same length of time. Cells were grown for 20 h (26695) or 24 h (G27) to an optical density at 600 nm of 1.0 in brucella broth (BD Difco) with 5% fetal bovine serum (Sigma) and then exposed to either 100 μM dimethylglyoxime (DMG), a Ni^{2+}-selective chelator, or 100 μM $NiCl_2$ for 40 min. Cells were then assayed for transcript levels (20) or urease activity (6, 37) as previously described. ^{63}Ni content was measured as previously described (23) after 40 min accumulation in the absence of DMG treatment. Genes necessary for urease or hydrogenase assembly were individually deleted in strains 26695 or G27 using standard approaches (7, 13). Stop codons were introduced in all three frames to avoid polar effects due to gene disruption (see Tables S1 and S2 in the supplemental material for strains and oligonucleotide sequences).

As expected, *nixA* and *frpB4* levels were repressed five- and sixfold (Fig. 1a to d), respectively, in a NikR-dependent manner (14, 17, 18, 44). *nixA* and *frpB4* levels in the *nikR* mutant strain treated with DMG or $NiCl_2$ were also increased relative to levels in the parent strain (Fig. 1b and d), suggesting that some level of NikR-dependent repression occurs under these

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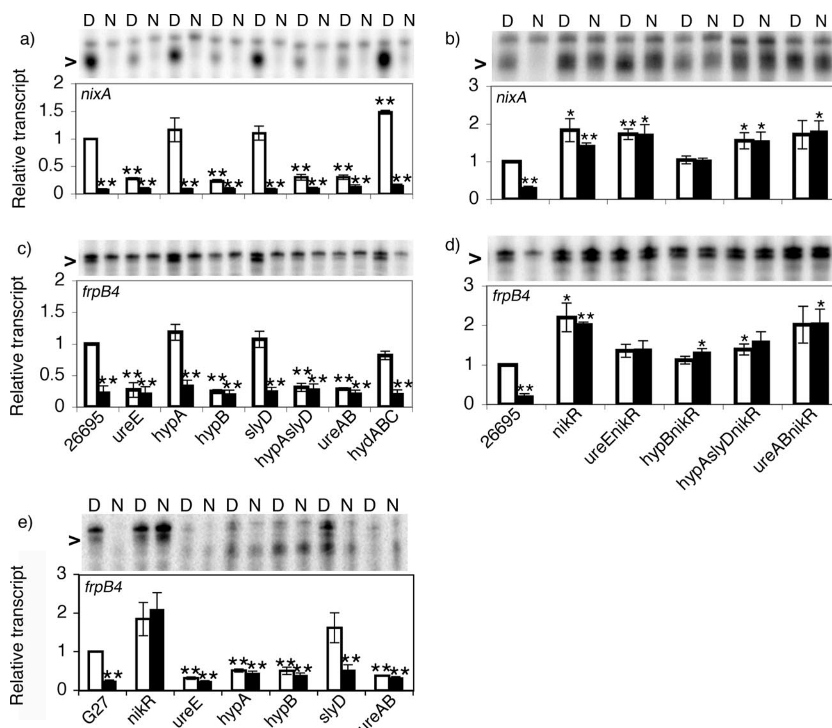

FIG. 1. Deletion of genes necessary for urease assembly results in NikR activation under Ni^{2+}-limiting conditions in strains 26695 and G27. Cells were exposed to DMG (D) or $NiCl_2$ (N) as described in the text. RNA was isolated from 26695 (a to d) or G27 (e) cells using Trizol (Invitrogen), and levels of *nixA* (a and b) or *frpB4* (c to e) transcript were measured using 10 μg input RNA. One representative gel (10% denaturing polyacrylamide) for each probe (input, 2×10^5 cpm) is shown, and arrowheads indicate bands corresponding to digested probes for *nixA* or *frpB4*. Parent and *nikR* strains were always assayed in parallel to control for variations in growth. Plotted data in each panel are the averages of three independent cultures normalized to the transcript level of the parent strain. Calculated standard errors are shown. Each experiment was repeated at least twice. The upper bands in all gels represent undigested probe. Lower bands in panel e likely represent cross-hybridization of the *frpB4* probe with one or more existing *frpB* paralogs present in *H. pylori* (4, 41). *, $P < 0.1$; **, $P < 0.05$.

conditions. Deletion of the *fur* gene did not alleviate nickel-dependent repression of *nixA* and *frpB4* under these conditions (data not shown). Deletion of *ureE*, *hypB*, or the urease structural genes (*ureAB*) resulted in significantly decreased *nixA* and *frpB4* levels after DMG treatment compared to the parent strain, whereas deletion of *hypA*, *slyD*, or the hydrogenase structural genes (*hydABC*) had no effect (Fig. 1a and c). The differential effect of the *hypA* and *hypB* deletions was unexpected, because both HypA and HypB participate in urease assembly (32). Therefore, we also measured NikR regulation in a *hypA slyD* double mutant, which behaved similarly to all other urease pathway mutants, suggesting that HypA and SlyD play redundant roles in urease assembly in strain 26695. Together, these data suggest that an intact urease assembly pathway is required to compete with NikR for intracellular nickel.

The decrease in transcript level was always NikR dependent (Fig. 1b and d). De novo synthesis of NikR was not required for the mutant phenotypes, because inhibition of translation by chloramphenicol or erythromycin had no effect (data not shown). Single or double mutant strains with deletions of genes involved in Ni^{2+} transport (*nixA* [28] and *exbBD tonB* [36]), Ni^{2+} storage (*hpn* and *hpn*-like [6, 19, 29, 37]), and Ni^{2+} efflux (*cznABC* [39]) also showed no changes in NikR-dependent regulation (data not shown). This contrasts with results from *E. coli*, where the Ni^{2+} efflux protein RcnA impedes NikR activation under nickel-limiting conditions (23).

As expected, deletion of *ureE*, *ureAB*, or *hypB* decreased urease activity to ≤1% of the parent strain level (Table 1). Deletion of *hypA* resulted in ~30% activity, while the *hypA slyD* double mutant had <1% activity. These data provide the first functional evidence of SlyD participation in *H. pylori* Ni^{2+}-dependent enzyme assembly and suggest partially overlapping

TABLE 1. Urease activity in different *H. pylori* strains

Genotype	Urease activity (μmol NH_3 min^{-1} mg $protein^{-1}$)[a]			
	26695		G27	
	DMG	$NiCl_2$	DMG	$NiCl_2$
Wild type	13.55 (0.52)	20.71 (1.88)	25.76 (1.98)	36.65 (2.25)
nikR	17.64 (1.43)*	26.80 (0.89)*	29.56 (0.95)*	30.23 (0.64)**
ureE	0.11 (0.03)**	0.24 (0.07)**	0.13 (0.02)**	0.16 (0.00)**
hypA	4.76 (0.31)**	10.52 (0.53)*	0.05 (0.02)**	0.01 (0.00)**
hypB	0.09 (0.00[b])**	0.23 (0.02)**	0.12 (0.00)**	0.17 (0.02)**
slyD	13.96 (0.56)	25.80 (0.58)	32.92 (2.53)*	27.00 (0.69)*
hypA slyD	0.07 (0.01)**	0.23 (0.04)**	0.01 (0.00)**	0.01 (0.00)**
ureAB	0.04 (0.04)**	0.01 (0.00)**	0.01 (0.00)**	0.01 (0.00)**
hydABC	15.21 (5.05)	25.26 (3.15)	ND	ND

[a] Values are the averages for three independent cultures (standard error are in parentheses) for each strain and condition. Cells were lysed by sonication and either used directly (low activity) or diluted 20-fold (high activity) in 50 mM HEPES (pH 7.0). Samples were incubated for 10 (high activity) or 30 min (low activity) at 37°C. ND, not determined. * and **, $P < 0.1$ and $P < 0.01$ for mutant versus parent strain.

[b] Error is <0.01. The lowest measurable value by this method (A_{625} = 0.001) corresponds to ~0.5 to 1 nM NH_3.

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TABLE 2. Short-term ^{63}Ni accumulation in *H. pylori* strains

Genotype	Total ^{63}Ni/cell[a]			
	26695		G27	
	Low Ni[b]	High Ni[c]	Low Ni[b]	High Ni[c]
Wild type	134.3 (1.9)	8.52 (0.34)	536.5 (40.3)	6.45 (0.23)
nikR	156.0 (7.5)*	10.19 (0.79)	867.5 (28.0)**	6.99 (0.23)*
ureE	92.2 (7.1)*	6.86 (0.19)*	196.8 (2.1)**	5.26 (0.11)*
hypA	128.1 (11.7)	7.82 (0.45)	191.2 (1.9)**	8.71 (0.28)**
hypB	124.6 (8.8)**	5.46 (0.63)**	213.6 (5.4)**	5.18 (0.22)*
slyD	134.0 (2.0)	7.34 (0.36)	564.0 (38.0)	6.75 (0.17)
hypA slyD	96.1 (1.8)**	6.33 (0.41)**	156.4 (5.5)**	9.75 (0.08)**
ureAB	120.7 (1.7)**	7.68 (0.29)	155.7 (5.1)**	6.60 (0.35)
hydABC	137.9 (5.1)	8.09 (0.44)	ND	ND

[a] Values are the averages for three independent cultures (standard errors are in parentheses) for each strain and condition. Cells were incubated with $^{63}NiCl_2$ as described in the text, harvested by centrifugation (16,000 × *g*, 1 min), washed with 900 μl of 50 mM HEPES (pH 7.0)–50 mM EDTA, and resuspended in 200 μl 10 mM acetic acid before being mixed with 1 ml scintilliation fluid. ^{63}Ni levels were determined by scintillation counting using a preprogrammed 10-min acquisition window (0 to 1.31 MeV). Counts per minute were converted to atoms of ^{63}Ni/CFU using a standard curve determined for cells grown under identical conditions. ND, not determined. * and **, $P < 0.1$ and $P < 0.05$ for mutant versus parent strain.

[b] 10 nM $^{63}NiCl_2$.

[c] 50 nM $^{63}NiCl_2$ plus 100 μM $NiCl_2$. Values are in hundred thousands.

functions of HypA and SlyD in urease assembly in this strain. The loss of urease activity itself was not linked to NikR function, because treatment of 26695 cells with fluorofamide, a competitive inhibitor of holo-urease (2, 34), had no effect on *nixA* and *frpB4* levels in the 26695 parent or *ureAB* strains but decreased urease activity >100-fold in the parent strain (data not shown).

A consequence of increased NikR activity was decreased Ni^{2+} accumulation, consistent with reduced *frpB4* and *nixA* transcript levels. ^{63}Ni accumulation was measured as previously described (23) in cells exposed to either low (10 nM $^{63}NiCl_2$; specific activity, 9.87 mCi mg^{-1}; Perkin-Elmer, Boston, MA) or high (50 nM $^{63}NiCl_2$ plus 100 μM $NiCl_2$) Ni^{2+} concentrations. The *ureE*, *hypB*, *hypA slyD*, and *ureAB* strains accumulated less ^{63}Ni relative to the parent strain under low- and high-Ni^{2+} conditions, whereas the *hypA*, *slyD*, and *hydABC* strains took up levels of ^{63}Ni similar to those in the parent strain (Table 2). Interestingly, the chaperone mutants showed a greater difference relative to the parent strain than the *ureAB* strain. This suggests that the Ni^{2+}-binding capacity of the cell does not reside entirely with UreAB. Deletion of *nikR* in each mutant background restored ^{63}Ni accumulation to levels similar to those of a *nikR* mutant strain (~120 to 150% of the level in the parental strain). These data provide further evidence that NikR activity is increased in the urease pathway mutants and indicate that the decrease in *nixA* and *frpB4* levels results in decreased NixA and FrpB4 protein levels during this assay, consistent with a previous report of the rate of NixA turnover under similar conditions (44).

H. pylori is well known for interstrain variability in gene content and physiology (24, 35). Gene deletions were also constructed in *H. pylori* strain G27, a clinical isolate used in laboratory studies (12), to determine if urease assembly in this genetic background similarly affects NikR activity. Deletion of *ureE*, *hypB*, or *ureAB* had the same effect on G27 *frpB4* transcript levels, urease activity, and ^{63}Ni accumulation under Ni^{2+}-limiting conditions as in strain 26695 (Fig. 1e; Tables 1 and 2). However, in G27, deletion of *hypA* alone decreased *frpB4* levels, urease activity, and short-term ^{63}Ni accumulation, while deletion of *slyD* had no effect in any assay. These data suggest that HypA is essential for urease assembly and nickel competition in strain G27 but not in strain 26695. HypA is also essential for urease assembly in strain 43504 (32), although activity was assayed under different conditions. A comparison of the predicted HypA, HypB, and SlyD sequences from the 26695 and G27 strains (4, 41) indicates that HypA is absolutely conserved, while HypB and SlyD both contain a few key amino acid differences that occur in domains of each protein necessary for a HypB-SlyD interaction that is required for hydrogenase assembly in *E. coli* (25). It is also possible that differences between strains could result from changes in protein levels of these chaperones or other proteins not examined in this study.

Our results indicate that the intact urease assembly pathway of *H. pylori* is required to compete with NikR for nickel ions. This competition occurs despite the substantial apparent difference in Ni^{2+} affinities between the chaperones and NikR ($K_d = 10^{-6}$ M [6, 27] versus 10^{-9} [45] to 10^{-12} M [1, 5]). Hausinger and coworkers have shown that *K. aerogenes* UreE inserts Ni^{2+} into apo-urease in the presence of strong Ni^{2+} chelators (iminodiacetic or nitrilotriacetic acid; $K_d = 10^{-9}$ and 10^{-12} M, respectively [38]), indicating that UreE, in conjunction with the urease assembly complex, can shield nickel ions from chelation (38). This observation provides a plausible model for the competition that we have observed, wherein a set of Ni^{2+} transfer reactions is refractory to competition from Ni^{2+} scavengers, such as NikR. Additionally, localized urease assembly near the inner membrane (43) could provide spatial separation from NikR, and different local concentrations of nickel ions would be sensed by each pathway within the cell.

The specific roles of the chaperones in urease assembly are not fully established. A recent study with strain 26695 identified SlyD in a complex with other urease chaperones, including HypB (40), further suggesting that SlyD functions in urease nickel insertion. The role of different chaperone functions in preventing NikR activation can now be assessed by genetic approaches using specific point mutations known to abrogate metal-binding and other activities, such as GTP hydrolysis. Additionally, the effects of the amino acid substitutions in the SlyD and HypB proteins of G27 and 26695 on urease assembly and nickel competition can be examined using both genetic and biochemical approaches.

The dynamics of intracellular metal trafficking are poorly understood. We have taken advantage of the prominent nickel physiology of *H. pylori* to begin to examine mechanisms of metal partitioning. Different transition metals are found either sparingly within cells (copper), used for only a few specific functions (nickel and manganese), or widely required (iron and zinc), making it likely that the nature of the competition between regulators and metalloenzymes will differ. Nonetheless, common mechanistic features are likely to emerge for the trafficking of different metals in cells.

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