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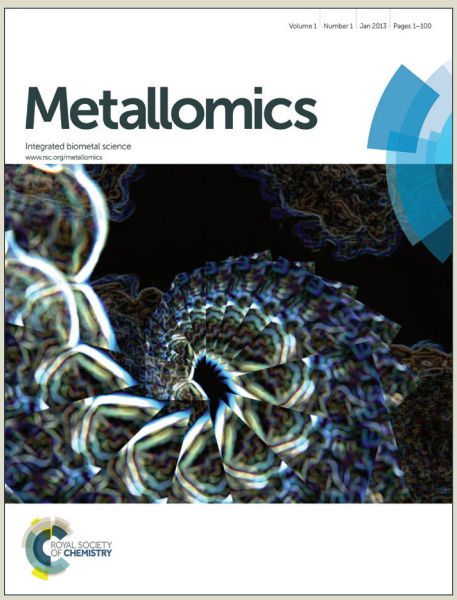

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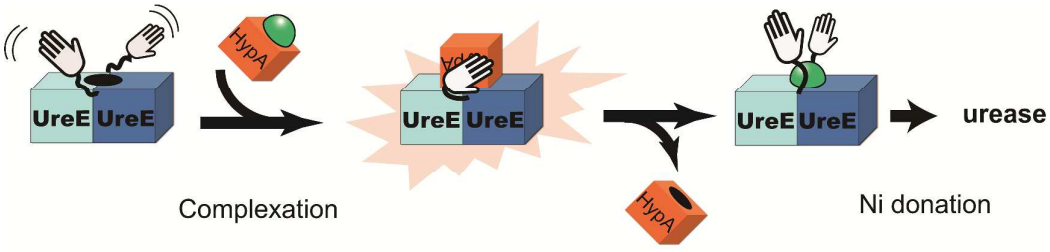

Novelty of the work:

Direct *in vitro* and *in vivo* evidence as well as molecular details of nickel translocation mediated by HypA-UreE interaction.

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ARTICLE

Nickel Translocation between Metallochaperones HypA and UreE in *Helicobacter pylori*

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Incorporation of nickel ions to the active sites of urease and hydrogenase is prerequisite for the proper functions of the metalloenzymes. Such a process requires the participation of several accessory proteins. Interestingly, some of them are shared by the two enzymes in their maturation processes. In this work, we characterized the molecular details of the interaction of metallochaperones UreE and HypA in *Helicobacter pylori*. We show by chemical cross-linking and static light scattering that UreE dimer binds to HypA to form a hetero-complex i.e. HypA-$(UreE)_2$. The dissociation constant (K_d) of the protein complex was determined by ITC to be 1 μM in the absence of nickel ions; whereas binding of Ni^{2+} but not Zn^{2+} to UreE resulted in *ca.* one fold decrease in the affinity. The putative interfaces on HypA unveiled by NMR chemical shift perturbation were found mainly at the nickel binding domain and in the cleft between α1 and β1/β6. We also identified that the C-domain of UreE, in particular the C-terminal residues of 158-170 are indispensable for the interaction of UreE and HypA. Such an interaction was also observed intracellularly by GFP-fragment reassembly assay. Moreover, we demonstrated using a fluorescent probe that nickel is transferred from HypA to UreE *via* the specific protein-protein interaction. Deletion of the C-terminus (residues 158-170) of UreE abolished nickel transfer and led to a significant decrease in urease activity. This study provides direct *in vitro* and *in vivo* evidence as well as molecular details of nickel translocation mediated by protein-protein interaction.

Introduction

Nickel is essential for the survival and pathogenicity of the gastric pathogen *Helicobacter pylori,* which infects nearly half of the world population and is responsible for chronic gastritis, peptic ulcer and even stomach cancer.[1] Therefore *H. pylori* has developed an elaborate system to regulate nickel acquisition, storage, delivery (into target enzymes) and efflux through biosynthesis of a series of metalloproteins/chaperones.[2-4] For example, the bacterium produces two unique histidine-rich Ni^{2+}-binding proteins (Hpn and Hpnl) serving the function of nickel storage.[5-11] In addition, chaperonin GroES (HspA) from *H. pylori* possesses an additional histidine/cysteine-rich C-terminus, different from other species, with the capacity of nickel binding, involved in nickel homeostasis [12-15] apart from its typical function of assisting protein folding *via* its interaction with GroEL.[16-19] Among all the metalloproteins generated by the pathogen, two nickel-containing enzymes i.e. urease and [NiFe]-hydrogenase are vital for the survival and successful colonization of the bacterium with the former catalyzing the hydrolysis of urea to ammonia to neutralize the acidic local pH of the mucosa, and the later providing energy through oxidizing molecular hydrogen.[1, 20-23] The proper functioning of both enzymes relies heavily on the assembly of Ni^{2+}-containing centers, which requires cooperation of a battery of metallochaperones and accessory proteins.[2-4, 24]

Two metallochaperones HypA and HypB have been demonstrated previously to be critical for the maturation of [NiFe]-hydrogenase.[25-30] *Hp*HypA possesses a Ni^{2+}-binding site located at the N-terminus, and interacts with *Hp*HypB, facilitating Ni^{2+} transfer.[28, 31-34] In addition, another metallochaperone SlyD, which consists of an additional His/Cys-rich Ni^{2+} binding C-terminus,[35] may also participate in the enzyme maturation through interaction with HypB, possibly transfers nickel ions to HypB in *H. pylori*, resulting an elevated GTPase activity of HypB in *H. pylori* [36, 37] in spite of no observable effect on GTPase activity upon nickel binding in *E. coli*.[38,39] Interestingly, deletion of both *hypA* and *hypB* genes led to a reduced urease activity in the bacterial strains,[30] indicating that both HypA and HypB also participate in nickel insertion into urease.

Urease synthesis requires four accessory proteins i.e. UreE, UreF, UreG and UreH,[40-42] among which UreE appears to be an important metallochaperone, involves in nickel incorporation into urease *via* its interaction with UreG in *K. aerogenes*.[43, 44] The crystal structures of UreE from *H. pylori* reveal that apo-*Hp*UreE assumes as homodimeric architecture, consisting of N-terminal and C-terminal domains. However, the metal-bound *Hp*UreE arranges as a tetramer consisting of a dimer of dimers.[45, 46] His102 and possibly His152 involved in the metal binding, which causes the ordering of the C-terminus.[45, 46] The interaction of UreE with UreG both *in vivo* and *in vitro* was demonstrated in *H. pylori*.[40, 47-49] Moreover, the binding of *Hp*UreE to *Hp*HypA was found to be indispensable for urease

activation under low nickel conditions.[50] However the molecular detail of the interaction is lacking and it is also not fully understood how nickel is transferred between these two proteins.

In this report, we examined the molecular mechanism of nickel transfer mediated by the interaction of HypA and UreE by combined chemical, biochemical, biophysical and molecular biology techniques. We determined the binding affinity and further identified the binding interfaces on both proteins. We observed Ni^{2+} transfer from HypA to UreE by employing a home-made fluorescent probe and the C-terminus of UreE appears to be crucial for nickel transfer both *in vitro* and *in vivo*.

Results and discussion

Formation of HypA-$(UreE)_2$ Complex

Both HypA and UreE were overexpressed and purified (Fig. S1). The interaction of HypA and UreE was investigated by chemical cross-linking as shown in Fig. 1A, upon incubation of HypA and UreE with the BS^3 cross-linker, a band corresponding to a molecular weight of *ca.* 35 kDa was observed (Fig. 1A, lane 10), which was also seen previously and attributed to the formation of heterodimeric HypA-UreE complex (MW ~34 kDa) consisting of monomers of UreE and HypA.[50] Surprisingly, an additional band at a molecular weight of *ca.* 55 kDa was also observed and further MALDI-TOF MS/MS study confirmed the existence of both HypA and UreE proteins in the band (Fig. S2). Considering the observed MWs of the UreE dimer (~ 40 kDa) and the HypA monomer (~ 14 kDa), we assign this band to the protein complex of HypA-$(UreE)_2$, consisting of one UreE dimer and one HypA monomer.

To further delineate possible region of UreE involved in the binding, the two distinct domains of UreE i.e. *N*-UreE (1-76) and *C*-UreE (77-170) were prepared (Fig. S3) and their interactions with HypA were examined similarly. A band at a molecular weight of *ca.* 45 kDa was observed as HypA-(*C*-$UreE)_2$ complex (Fig. 1A, lane 11). Whereas no apparent band corresponding to HypA-*N*-UreE complex could be detected upon mixing *N*-UreE and HypA with the cross-linker, (Fig. 1A, lane 12). Taken together, we demonstrated that UreE dimer forms a complex with HypA through its C-domain.

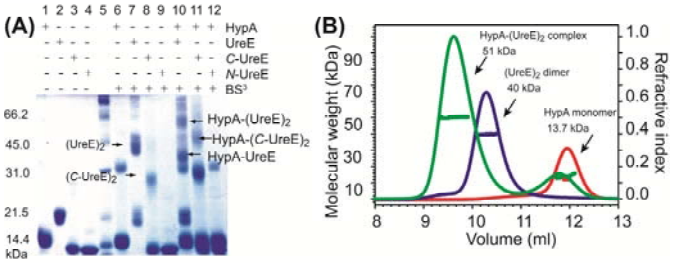

Fig. 1 Formation of HypA-UreE complex. (A) SDS-PAGE (12%) analysis of cross-linked products of HypA and UreE (or its variants). HypA was incubated with full-length UreE (lane 10) or its C-domain (*C*-UreE, lane 11) or N-domain (*N*-UreE, lane 12) in the presence of 0.1 mM BS^3. Complex bands were observed for full-length UreE and *C*-UreE, but not for *N*-UreE. (B) Determination of molecule weight of HypA-UreE complex by SEC/SLS. HypA-UreE complex was eluted at 51 kDa. Individual elution profiles of *Hp*UreE (blue) and *Hp*HypA (red) are shown for comparison.

The interaction of HypA and UreE under non-denaturing condition was monitored by Size Exclusion Chromatography and Static Light Scattering (SEC/SLS). Mixing equimolar amounts of HypA and UreE resulted in appearance of a new peak with a molecular weight of *ca.* 51 kDa accompanied by the disappearance of UreE dimer peak. Notably, the HypA peak could still be observed, indicating HypA was in excess in the mixture (Fig. 1B). This clearly suggests that each UreE dimer forms a complex with one monomer of HypA, consistent with our cross-linking results. The observation of heterodimeric complex HypA-UreE is likely due to incomplete cross-linked products.

The formation of HypA-UreE complex in living cells was also demonstrated by GFP-fragment reassembly assay (Fig. S4). Both HypA and UreE were fused to the N- and C-terminal fragments of EGFP respectively. The cells co-expressing the pET32a-NGFP-HypA and pBAD33-UreE-CGFP proteins displayed the emitted green fluorescence, clearly indicating the complexation of HypA and UreE in the complex cellular cytoplasm both in the absence and presence of nickel.

Regulation of the Binding Affinity of HypA-UreE by Nickel

We have shown previously that *Hp*HypA binds Ni^{2+} and Zn^{2+} at two distinct metal binding sites;[31] while *Hp*UreE binds Ni^{2+} *via* the conserved residue His102,[45-47] with a higher binding affinity than Ni^{2+}-HypA binding (Fig. S5). And two additional amino acids (Gly-Ser) in the N-terminus of HypA resulted from thrombin digestion showed negligible effects on Ni^{2+} binding (Fig. S5). Our SEC data showed that Ni^{2+} or Zn^{2+} has no obvious effect on the SEC profile of HypA-$(UreE)_2$ complex (Fig. S6). To further quantify the binding affinity of HypA-UreE as well as to examine the effects of metal ions on the binding affinity, ITC was applied in the present study. The dissociation constant of apo-HypA to apo-UreE was determined to be 1.1±0.4 μM and one dimer of apo-UreE binds to *ca.* 0.9 monomer of apo-HypA (Fig. 2A), which is consistent with our cross-linking results. Binding of Zn-HypA or Ni-Zn-HypA to apo-UreE gave rise to similar dissociation constants (1.2±0.2 and 1.2±0.3 μM respectively); indicating that neither zinc nor nickel binding to HypA altered its dissociation constants with apo-UreE. Given that apo-HypA is not very stable at a high concentration;[31] either Zn-HypA or Ni-Zn-HypA was used to investigate their binding affinities to the metal-bound UreE. The dissociation constant of Zn-HypA to Zn-UreE was determined to be 1.1±0.4 μM, similar to that of HypA binding to apo-UreE, indicative of negligible effect of Zn^{2+} on the stability of protein complex. Binding of Ni^{2+} to UreE led to slightly weaker interaction between HypA and UreE with the dissociation constants of Zn-HypA or Ni-Zn-HypA to Ni-UreE of 1.7±0.3 and 2.0±0.3 μM respectively (Fig. 2B).

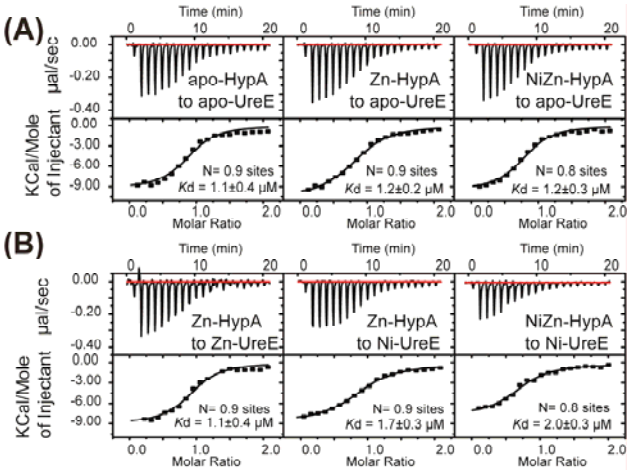

Fig. 2 Binding affinities of HypA to UreE by isothermal titration calorimetry. HypA (0.2 mM) either in apo-, or metal-bound forms was injected stepwise into cells containing apo-UreE (20 μM as dimers) (A) or Zn^{2+}- /Ni^{2+}-bound UreE (B) and the heat of binding was recorded for every injection. Ni^{2+} but not Zn^{2+} binding to UreE resulted in a slightly weaker HypA-UreE interaction.

Similarly, the binding affinity of HypA to the metal-binding domain of UreE (*C*-UreE) was also investigated. In comparison with the full length UreE, apo-*C*-UreE binds to Zn-HypA with a dissociation constant of 1.6 ± 0.3 μM. Preloading of nickel to *C*-UreE also led to weaker interaction between Ni-*C*-UreE and Zn-HypA (3.7± 1.0 μM) or Ni-Zn-HypA (4.4± 0.9 μM) (Fig. S7) as observed for the full length protein. Taken together, these results imply UreE may regulate its binding to HypA through sensing of Ni^{2+}.

The affinity of *Hp*HypA-*Hp*UreE was found to be at a level of micromoles despite that a much higher affinity was reported by biolayer interferometery and surface plasmon resonance previously.[48] Given that the complex requires dissociation upon delivery of nickel ions, it is conceivable for the weak or moderate association between chaperones. The associations between metallochaperones HypA-HypB,[33] SlyD-HypB[37] and UreE-UreG[47] were also reported weakly with dissociation constants at levels of micromolars. In addition, such weak interactions were also noted between copper chaperones such as Atx1 and Ccc2 ATPase,[51] CopZ and CopA.[52]

Identification of UreE Binding Interfaces on HypA by NMR Spectroscopy

Taking the advantage of previous NMR assignment on HypA,[31] we explored the residues of HypA involved in the interaction with UreE by NMR spectroscopy.[53] A series of 2D [^{1}H-^{15}N] HSQC spectra of ^{15}N-HypA (0.3 mM) were recorded upon each titration of unlabeled UreE protein (up to a molar ratio of UreE to HypA of 0.8:1). It is noted that certain cross-peaks underwent significantly broadening (data not shown), indicating that the bound- and free- forms of the protein are in an intermediate exchange on the NMR time scale.[33, 54] To quantify the extent of line broadening, the peak intensities of 101 well-resolved peaks (out of total 125 assigned peaks) were plotted against UreE concentrations and the resulted slopes for each residue were plotted and are shown in Fig. 3A. Residues experiencing large perturbation (with slopes > 3.1) include Tyr6, His19, Lys27, Leu114 and Ala118; while others that displayed moderate perturbation (3.0<slopes<3.1) consist of Glu5, Val8, Ala14, Cys16, Lys22, Arg30, Val32, Ile35, Ser48, Thr52, Phe53, Glu56, Ile64, Met116 and Glu119. These residues are almost exclusively located at the Ni^{2+} binding domain in the cleft between α1 and β1/β6 (Fig. 3B).

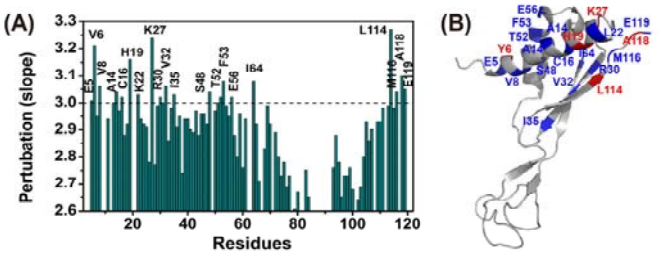

Fig. 3 Identification of protein-protein interfaces on *Hp*HypA by NMR spectroscopy. (A) Perturbation of resonance intensities of ^{15}N-labeled *Hp*HypA by *Hp*UreE. The residues with the slopes larger than 3.0 are labeled. (B) Cartoon (Left) and surface (Right) models of *Hp*HypA structure. Those residues perturbed by UreE are highlighted in red (slope >3.1) and blue (3.1> slope >3.0).

Recognition of HypA by the C-terminus of UreE

Based on crystal structures of *Hp*UreE, the C-terminal region is highly disordered in the apo-form of the protein, but acquired significant ordering in the presence of metal ions driven by coordination of His152 to metal ions. Thus the C-terminus has been implicated in molecular recognition and metal ion delivery.[46, 47]

In order to unveil the residues of UreE involved in the HypA-UreE interaction and to avoid disruption of Ni^{2+} coordination,[46] several UreE mutants were constructed including UreEΔ158-170 (residues 1-157), *C*-UreE (residues 77-170) and *C*-UreEΔ158-170 (residues 77-157). The interactions of the UreE mutants with HypA were examined by chemical cross-linking similarly as described above. Surprisingly, no bands corresponding to molecular weights of either HypA-(UreE)$_2$ or HypA-(*C*-UreE)$_2$ were observable upon the deletion of residues 158-170 of UreE (Fig. 4), indicating that the C-terminus (residues 158-170) is indispensable for the formation of HypA-(UreE)$_2$ complex, which was confirmed by both SEC and ITC respectively (Fig. S8)

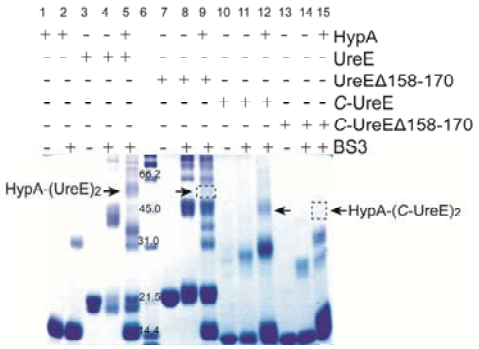

Fig. 4 Identification of protein-protein binding interfaces on UreE. Purified HypA was incubated with UreE/*C*-UreE (lane 5, 12) or their C-terminus truncated mutants (lane 9, 15) in the presence of 0.1 mM BS^3. The bands corresponding to the protein complexes are indicated. Note that neither HypA-(UreEΔ158-170)$_2$ nor HypA-(*C*-UreEΔ158-170)$_2$ was observed.

Nickel Transfer from HypA to UreE Mediated by Specific HypA-UreE Interaction

In spite of importance of nickel in the maturation of urease, it is not fully understood how it is delivered among the accessory proteins. Previous attempt on the deciphering nickel translocation between HypA and UreE both *in vivo* and *in vitro* was not successful. Here we investigated nickel transfer between HypA and UreE using our previously synthesized heterobifunctional fluorescence probe, consisting of a fluorophore, nitrilotriacetic acid moiety and arylazide as an anchor, which could label and light up the Ni^{2+}-bound proteins even under denatured condition, as described in supporting materials. Aliquots of apo-UreE samples were incubated with increasing amounts of Ni-HypA for 2 h to allow potential nickel transfer, and then treated with the fluorescent probe for 30 min to label Ni-binding proteins. Upon UV irradiation for 10 min to enable formation of covalent linkage between the probe and proteins *via* photo-activation, the samples were subjected to SDS-PAGE and fluorescence gel analysis. As shown in Fig. 5, Ni-HypA but not apo-UreE exhibits strong fluorescence (Fig. 5A, lanes 1 and 4). While incubation of apo-UreE with Ni-HypA resulted in the shift of lit-up bands from HypA to UreE, and the intensities of lit-up bands increased with the increases in the amounts of Ni-HypA (Fig. 5A, lane 5-8), implying that Ni^{2+} has been transferred from HypA to UreE. In contrast, the incubation of apo-HypA with Ni-UreE gave

rise to a fluorescence band at a molecular weight corresponding to UreE, indicating that Ni^{2+} could not be transferred from UreE to HypA (Fig. 5A, lane 10).

To examine if the C-terminus of UreE is essential for nickel transfer, we performed a similar experiment using apo-UreEΔ158-170, the incubation of apo-UreEΔ158-170 and Ni-HypA did not lead to lit-up bands of UreEΔ158-170 (Fig. 5B, lane 3-6), indicative of no occurrence of nickel transfer due to the fact that there is no interaction between HypA and UreE upon deletion of C-terminus of UreE. Taken together, we demonstrated that nickel ions can be transferred from HypA to UreE *via* the specific protein-protein interaction and the C-terminus of UreE is essential for nickel delivery.

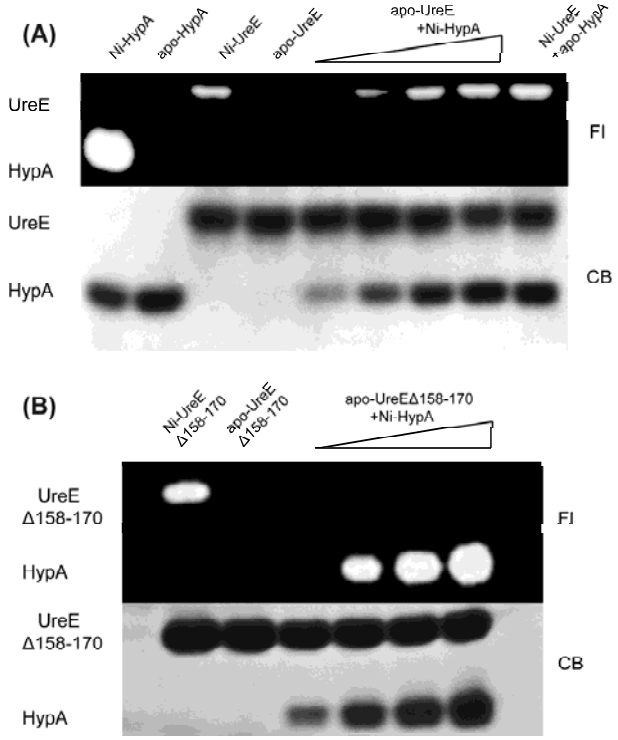

Fig. 5 Nickel transfer between HypA and UreE monitored by a fluorescence probe. Ni-HypA was incubated with apo-UreE (A) or apo-UreEΔ158-170 (B) then treated with a fluorescent probe, and was subsequently analyzed by 15% SDS-PAGE. Florescence images were captured after UV irradiation and the gels were then stained by Coomassie blue. Only Ni^{2+} bound proteins exhibit fluorescence. FI: Fluorescence Image; CB: Coomassie Blue.

Urease Activity Assay to Study the Role of HypA-UreE Interaction in Urease Maturation

To further explore physiological relevance of the interaction of UreE-HypA and the role of the C-terminus (residues 158-170) of UreE, plasmids containing genes *ureE/mutants* and *hypA* were co-transformed into *E. coli* strain KM1603 harboring plasmid pHP8080Δ*ureE*, which contains the full set of urease genes, *ureABFGHI* (except *ureE*), and the cells were grown in M9 minimal medium without or with supplement of nickel ions (1 μM) and urease activity was determined using a standard method.[50, 55] In the absence of either UreE, or both HypA and UreE, low levels of urease activity still could be detected, which may be due to the utilization of unidentified accessory proteins for urease activity *in vivo* as reported previously.[56] Co-expression of HypA-UreE resulted in an elevated urease activity in *E. coli* only under low Ni^{2+} condition (Fig. 6A), in consistence with a previous report;[50] while co-expression of *C*-UreE with HypA could also partially restore urease activity compared to the full length UreE, indicating that the C-domain of UreE is able to fulfill the function of the protein, to an extent. In contrast, cells co-expressing UreEΔ158-170 and HypA exhibited significant reduction in urease activity when grown in nickel deficient medium (Fig. 6A), implicating the importance of the C-terminus of UreE in nickel insertion into apo-urease. Upon supplementation of 1 μM Ni^{2+} to the medium, the urease activities of all strains were enhanced to a similar level and the discrepancy in urease activity between strains were completely abolished (Fig. 6B), in agreement with a previous study,[50] suggesting that the protein-protein interaction plays an important role in nickel delivery.

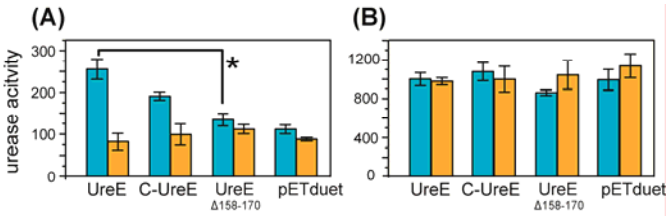

Fig. 6 Urease activities in *E. coli* (KMl603) harboring plasmids pHP8080Δ*ureE* and pET-derivatives plasmid in the absence (A) and presence of 1 μM Ni^{2+} (B). Urease activities are defined as nmol ammonia produced min^{-1}(mg total protein)$^{-1}$. Cells containing pET-derivatives plasmids pET-*ureE* (or variants)-*hypA* were used to co-express UreE (or variants) and HypA proteins (cyan bars), cells with pET-*ureE* (or variants) expressing only UreE (or variants) proteins were for comparison (yellow bars). Note: cells expressing only HypA, or neither HypA nor UreE were used as negative controls and denoted as pETduet in cyan or yellow bars respectively. Significant reduction ($P<0.05$) in urease activity was observed in pET-*ureEΔ158-170-hypA* expressing cells (marked in asterisk) and addition of only 1 μM Ni^{2+} in the medium abolished the differences of the urease activities between strains.

Conclusions

In summary, we have characterized the interaction between metallochaperones HypA and UreE from *Helicobacter pylori*. We show that each dimer of UreE binds to one monomer of HypA with a dissociation constant at micromolar levels. The C-domain of UreE, in particular the C-terminal residues of 158-170 are essential for the recognition of HypA, resulting in HypA conformational changes at the proximity of Ni^{2+} binding site. Such a binding also occurred in cellular cytoplasm. Significantly, the interaction of metallochaperones HypA and UreE facilitates nickel transferring from HypA to UreE both *in vitro* and *in vivo*, and subsequently to downstream partner proteins possibly UreG, which is currently not well understood and may warrant for further studies. The participation of HypA and UreE in the nickel incorporation into urease *via* the specific protein-protein interaction is intriguing. Such a "cross-talk" between the accessory proteins of urease and [NiFe]-hydrogenase might be related to the special niches that bacteria resides, leading to its unique evolution to adopt special environment for survival and pathogenesis. This phenomenon that bacteria employ certain strategy to cope with specific living condition for survival was also exemplified by the habitat-related occurrence of histidine-rich proteins, which were produced to detoxify heavy metal ions in certain prokaryotes bacteria.[57] Our study provides direct *in*

vitro and *in vivo* evidence as well as molecular details of nickel trafficking mediated by the protein-protein interaction.

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Notes and References

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