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Characterization in *Helicobacter pylori* of a Nickel Transporter Essential for Colonization That Was Acquired during Evolution by Gastric *Helicobacter* Species

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Abstract

Metal acquisition is crucial for all cells and for the virulence of many bacterial pathogens. In particular, nickel is a virulence determinant for the human gastric pathogen *Helicobacter pylori* as it is the cofactor of two enzymes essential for *in vivo* colonization, urease and a [NiFe] hydrogenase. To import nickel despite its scarcity in the human body, *H. pylori* requires efficient uptake mechanisms that are only partially defined. Indeed, alternative ways of nickel entry were predicted to exist in addition to the well-described NixA permease. Using a genetic screen, we identified an ABC transporter, that we designated NiuBDE, as a novel *H. pylori* nickel transport system. Unmarked mutants carrying deletions of *nixA*, *niuD* and/or *niuB*, were constructed and used to measure (i) tolerance to toxic nickel exposure, (ii) intracellular nickel content by ICP-OES, (iii) transport of radioactive nickel and (iv) expression of a reporter gene controlled by nickel concentration. We demonstrated that NiuBDE and NixA function separately and are the sole nickel transporters in *H. pylori*. NiuBDE, but not NixA, also transports cobalt and bismuth, a metal currently used in *H. pylori* eradication therapy. Both NiuBDE and NixA participate in nickel-dependent urease activation at pH 5 and survival under acidic conditions mimicking those encountered in the stomach. However, only NiuBDE is able to carry out this activity at neutral pH and is essential for colonization of the mouse stomach. Phylogenomic analyses indicated that both *nixA* and *niuBDE* genes have been acquired via horizontal gene transfer by the last common ancestor of the gastric *Helicobacter* species. Our work highlights the importance of this evolutionary event for the emergence of *Helicobacter* gastric species that are adapted to the hostile environment of the stomach where the capacity of *Helicobacter* to import nickel and thereby activate urease needs to be optimized.
Author Summary

*Helicobacter pylori* is a bacterium that persistently colonizes the stomach of half of the human population worldwide. Infection by *H. pylori* is associated with gastritis, peptic ulcer disease and adenocarcinoma. To resist gastric acidity and proliferate in the stomach, *H. pylori* possesses an urease, this enzyme contains a nickel-metallocenter at its active site. Thus, the capacity of *H. pylori* to infect its host relies on important nickel supply despite its scarcity in the human body. To import nickel, *H. pylori* requires efficient mechanisms that were partially defined. In the present study, we identified a novel *H. pylori* nickel transport system that we designated NiuBDE. NiuBDE was directly demonstrated to import nickel into the bacterium. NiuBDE and the previously described NixA permease are the two sole nickel transporters in *H. pylori*. Urease activation by nickel depends on the NiuBDE and NixA systems and NiuBDE is essential for colonization of a mouse model by *H. pylori*. We found that NiuBDE and NixA have been acquired during evolution by *H. pylori* and other gastric-*Helicobacter* species. Thus, acquisition of nickel transporters by gastric *Helicobacter* was a key event for the emergence of one of the most successful bacterial pathogens, *H. pylori*.

Introduction

Transition metals are essential elements for living organisms because they are involved in many enzymatic reactions and physiological processes. For bacteria, metals have been shown to be essential for survival under various conditions including those faced during infection [1]. Tremendous efforts have been devoted to deciphering the mechanisms of iron uptake, trafficking and intracellular homeostasis control. Much less information is available on the acquisition and homeostasis of nickel, which is nevertheless an essential element for many bacteria [2]. Indeed it is the cofactor of at least nine enzymes involved in diverse cellular processes such as energy and nitrogen metabolism/detoxification and virulence [3], including [NiFe] hydrogenase, urease, Ni-SOD and CO dehydrogenase [4,5].

Nickel is a virulence determinant for *Helicobacter pylori*, a pathogen that colonizes the stomach of about half of the human population worldwide and causes a variety of gastric pathologies ranging from gastritis to adenocarcinoma [6,7] [8]. In order to successfully colonize the stomach, which is a highly acidic environment, *H. pylori* depends on urease [9]. Urease requires nickel to catalyze hydrolysis of urea into carbon dioxide and ammonia [10]. These buffering compounds are essential to maintain the pH close to neutrality in the bacterial cytoplasm [11,12]. Urease accounts for up to 6% of the soluble cellular proteins [13]. In *H. pylori*, nickel is also required for the activity of a second enzyme, a [NiFe] hydrogenase shown to be important for colonization, presumably because it provides an alternative respiratory pathway, allowing *H. pylori* to use molecular hydrogen as an energy source [14]. Since nickel is required for urease and hydrogenase activities, *H. pylori* has to acquire sufficient nickel ions, while preventing the toxic effects of non-physiological intracellular metal concentrations that would interfere with incorporation of other metals in essential proteins. Thus, *H. pylori* strictly controls intracellular nickel concentration with multiple mechanisms of sensing, transport and protection. Several proteins involved in these processes have been described. These include NikR, a nickel responsive pleiotropic transcriptional regulator [15–17], CznABC a metal efflux pump [18] and also three Histidine-rich proteins Hpn, Hpn-2 and HspA that are found only in the *Helicobacter* genus [19–22]. Hpn and HspA are involved in sequestration of nickel ions.
and the corresponding mutants are highly sensitive to nickel exposure [21,22]. In addition, the Hpn/Hpn-2 and HspA nickel-binding proteins contribute to the production of active urease and hydrogenase, respectively [22–24].

The concentration of nickel ions in the human body is low (1–11 nM) [25]. Consequently, H. pylori requires highly specific importers of Ni(II) ions. These mechanisms are only partially identified. In Gram-negative bacteria, energized transport of metabolites such as iron-siderophore complexes through the outer membrane (OM) relies on the TonB machinery and on TonB-dependent-transporters (TBDTs). We previously described FrpB4, the first TonB-dependent nickel transport system across a bacterial OM [26,27]. The activity of FrpB4 is acid-induced and its expression is repressed by nickel via NikR. This allows H. pylori to optimize nickel uptake and nickel-dependent activation of urease under conditions where ammonia production needs to be maximal. By analogy with siderophores, it is possible that a nickelophore, i.e. a small organic chelator of nickel, is required for transport by this TBDT. Studies highlighted that a nickelophore is required for nickel binding to the periplasmic NikA protein from Escherichia coli [28,29] or for nickel uptake by the Staphylococcus aureus Cnt transporter [30].

Transport of nickel ions across the cytoplasmic membrane is generally accomplished either by multiple-components, ATP-binding-cassette-containing transporters or by single component secondary transporters of the NiCoT family (nickel/cobalt transporter [31]) that use the physiochemical gradient of the cytoplasmic membrane as an energy source. The ABC-type NikABCDE transporter, first described in E. coli [32], is composed of NikA, a periplasmic binding protein, NikBC, two integral membrane components, and NikDE, two ATPases that energize substrate translocation through ATP hydrolysis. Another nickel ABC transporter has been identified in Campylobacter jejuni, that is composed of NikZYYXWV [33,34]. In S. aureus, nickel is taken up by two ABC transporters NikABCDE and CntABCDF in addition to the NixA permease [35–37].

In H. pylori, the only nickel transport system across the cytoplasmic membrane that has been reported so far is NixA, a permease of the NiCoT family [38,39], whose expression is repressed by nickel [17,40]. Inactivation of NixA renders H. pylori more resistant to nickel overload [41]. However, NixA deficient mutants were reported to retain urease activity [42] and are still able to colonize the mouse model at levels comparable to that of a wild type strain [43]. Several other proteins have therefore been proposed to participate in nickel uptake [44,45], but the role of none of them was confirmed.

The Helicobacter genus is composed of two subgroups, the enterohelial species (EH) that infect the liver or gastrointestinal tract of mammals and some birds and a small group of gastric Helicobacter species (including H. pylori) [46]. Only few information is available on the presence of nickel transporters in the non-pylori gastric Helicobacter and EH Helicobacter species. In Helicobacter mustelae that colonizes ferrets, NikH, a TBDT different from FrpB4 and a CeuE/FecDE ABC transport system were proposed to be involved in nickel uptake as the corresponding mutants are affected in urease activity and intracellular nickel content [47]. In the mouse-colonizing Helicobacter hepaticus, NikR regulates the expression of genes encoding homologues of the E. coli nickel ABC transporter (NikABCDE) and of the H. mustelae NikH [48].

In the present work, we applied a genetic screen to search for additional nickel transporters in H. pylori. We found that an ABC transporter, that we designated NiuBDE, transports nickel separately from NixA. In addition, we provide evidence that NiuBDE, but not NixA, also transports cobalt and bismuth, a metal currently used in H. pylori eradication therapy in humans. Phylogenomic analyses revealed that both transport systems have been acquired by the last common ancestor of the gastric Helicobacter species via horizontal gene transfer. Finally, we demonstrated that, contrary to NixA, the NiuBDE transporter is indispensable for colonization of the mouse model by H. pylori. We conclude that acquisition of nickel
transporters has been an important evolutionary event to allow *Helicobacter* to adapt to colonization of the hostile gastric environment.

**Results**

Selection and characterization of *H. pylori* mutants resistant to high Ni(II) concentrations

Nickel toxicity depends on import into the cell. Therefore, in order to search for new Ni(II)-uptake mechanism(s), we applied a genetic screen to select for *H. pylori* mutants able to survive exposure to high Ni(II) concentrations. A genomic library constructed by N.R Salama [49], in which *H. pylori* genes were randomly inactivated *in vitro* by the insertion of a transposable element conferring chloramphenicol resistance, was used to transform *H. pylori* strain G27 [50] and the isogenic Δhpn mutant that is more sensitive to nickel [22]. We first established the nickel concentrations at which the plating efficiencies of strains G27 and G27 Δhpn were strongly affected. This corresponded to 1 mM for wild type G27 strain and only 500 μM for the G27 Δhpn mutant. After transformation with the library, several mutants resistant to chloramphenicol and able to grow on plates in the presence of lethal concentrations of nickel were obtained in four independent selection experiments. Chromosomal DNA of these mutants was extracted and used to localize the transposon insertion sites by nested PCR [49]. Two independent insertions conferring nickel resistance in both the G27 and G27 Δhpn backgrounds were mapped inside the *hpG27_842* open reading frame.

The *hpG27_842* gene has been annotated as *fecD* because of its sequence homology with the *E. coli* fecD gene whose product is involved in ferric citrate transport. *E. coli* citrate-mediated iron transport system is expressed by five genes grouped into a single operon, designated *fecABCDE* [51]. *fecA* and *fecB* encode a TBDT outer membrane protein and a periplasmic protein, respectively. The *fecC* gene and its paralogue *fecD* encode cytoplasmic membrane proteins, *fecE* codes for an ATP-binding protein associated with the cytoplasmic membrane. It has been shown that after FecA-mediated TonB-dependent transport of iron(III) dicitrate across the outer membrane, FecBCDE transport iron across the cytoplasmic membrane. In *H. pylori* strains, the annotated operon is restricted to two genes, *fecD* followed by *fecE*. In *H. pylori* G27 strain, the *fecDE* promoter region is a possible NikR target as it was pulled out in a NikR ChIP-Seq experiment [52]. The selection of *fecD* insertions during our screen prompted us to examine whether this protein could be involved in nickel transport across the inner membrane. Since our experiments confirmed this activity, this gene was renamed, for sake of clarity, *niuD* for nickel uptake protein D and the downstream gene, annotated *fecE*, was renamed *niuE*.

NiuD and NixA inactivation increases *H. pylori* tolerance to high nickel concentrations

Using a previously described method [53], we constructed unmarked deletions mutants of *niuD* (hpB8_663) in the B128-S and B128-S Δhpn strains, (a genetic background that we previously characterized [22], S1 and S2 Tables). Tolerance of these mutants to nickel exposure was evaluated at neutral pH by following growth inhibition in liquid medium. Surprisingly, we observed that the *niuD* deletion did not significantly increase nickel tolerance in the B128-S (Fig 1) or B128-S Δhpn background (S1 Fig). We then examined the effect of these mutations in combination with a deletion of the gene encoding NixA, the previously identified nickel permease. The ΔnixA mutation increased nickel tolerance in both *hpn*+ and Δhpn backgrounds but only at the lowest nickel concentration tested (Figs 1 and S1). We found that growth of the ΔnixA ΔniuD mutants was insensitive to nickel exposure up to 2 mM. Thus, the ΔnixA ΔniuD
mutants were significantly more tolerant to exposure to high nickel concentrations than the single ΔnixA mutants in both hpn+ and Δhpn backgrounds. To test complementation, the niuDE genes were cloned under the control of the ureI promoter in the pIRC(PureI) vector \[54\] and introduced at a neutral position on the chromosome of our different mutants (designated c-niuDE). The ΔnixA ΔniuD/c-niuDE strain thus constructed recovered nickel tolerance levels comparable to that of the parental ΔnixA mutant (Fig 1).

Altogether, these results strongly suggest that NiuD is part of a novel nickel transport system that is distinct from NixA.

NiuD and NixA inactivation results in reduced intracellular nickel content

To evaluate the role of NiuD in nickel uptake, we measured the total intracellular nickel content of our collection of mutants grown in the presence of various nickel concentrations by Inductively-coupled plasma optical emission spectrometry (ICP-OES) as previously described \[22\] (Fig 2A). No nickel could be detected in strains grown without nickel supplementation. In the wild type strain grown with 10 and 100 μM NiCl2, intracellular nickel content reached 800 and 1,300 μg/g prot, respectively. In the ΔnixA mutant, nickel content reached a constant value that was, under both conditions, 2-fold (at 10 μM NiCl2) and 3-fold (at 100 μM NiCl2) lower than...
that of the wild type strain. Similarly, the ΔniuD mutant presented a reduced nickel content equivalent to that of the ΔnixA mutant in the presence of 10 μM nickel. When grown with 100 μM nickel, intracellular nickel content of the ΔniuD mutant increased to a level that was still about 1.6-fold lower than that of the wild type strain under the same conditions. Importantly, nickel content of the ΔnixA ΔniuD double mutant was below the detection limit when the bacteria were exposed to 10 μM NiCl₂ and reduced by more than 25-fold with 100 μM nickel. Similar results were obtained in the Δhpn genetic background (S2 Fig). These data indicate that the ΔniuD and ΔnixA mutations have additive effects on intracellular nickel accumulation.

Another way to evaluate the intracellular nickel concentration is to measure the expression of a reporter gene that is under the control of the nickel-responsive transcriptional regulator of H. pylori, NikR. The well-characterized PfecA3 promoter, which is repressed by NikR in response to nickel [17,55], was used to construct a pILL2157 derivative plasmid carrying a PfecA3::lacZ fusion. This construct was transformed into strain B128-S and its isogenic mutants ΔnixA, ΔniuD, ΔnixA ΔniuD and ΔnikR (Fig 2B). As expected, β-galactosidase activity of the ΔnikR mutant was strongly enhanced and insensitive to nickel addition, validating the reporter system. β-galactosidase activity measured in the wild type strain and the mutants was about 5,000 miller units in non-supplemented medium. Addition of 200 μM NiCl₂ to the wild type strain did not cause significant growth inhibition and resulted in a rapid decrease in β-galactosidase activity observable as soon as 4 hours after nickel addition. After 24 hours growth, the activity of the fusion was decreased about 5-fold in both the wild type strain and the ΔniuD mutant and about 2.5-fold in the ΔnixA mutant as compared to the control condition without added nickel (Fig 2B). Nickel addition to the ΔniuD ΔnixA mutant only resulted in a slight 1.3-fold reduction of β-galactosidase activity. These results are consistent with a diminished NikR-dependent repression of the reporter fusion as a consequence of minimal intracellular nickel accumulation in this double mutant.

Comparing the phenotypes of the mutants with the wild type strain, we found a good correlation between diminished nickel sensitivity, a decrease in total nickel content and derepression of the PfecA3 promoter. Our results show that inactivation of both the nixA and niuD genes resulted in a massive defect in accumulation of nickel into the cells, strongly indicating that NiuD and NixA are nickel uptake systems.

NiuD and NixA are involved in two separate nickel uptake pathways in H. pylori

To obtain definitive proof of the involvement of NiuD in nickel transport, we measured the uptake rates of radioactive ⁶³Ni(II) in the wild type and mutant strains in the presence of 10 μM total nickel at pH 5 (Fig 2C). As previously reported, radioactive ⁶³Ni(II) uptake is not measurable at pH 7 as it is, under the test conditions, presumably limited by the acid-activated FrpB4 OM transporter [26]. The ΔnixA mutant was dramatically affected with a nickel uptake rate reaching only 17% of that of the wild type, suggesting that NixA is a major contributor in nickel uptake under these conditions.

In the ΔniuD mutant, the uptake rates were 51.7% of that of the wild type. Most importantly, the ΔnixA ΔniuD mutant was completely deficient in nickel transport, with an uptake rate of 5%, similar to the background of non-specific nickel binding on filters in control experiments (Fig 2C). Complementation of the ΔnixA ΔniuD mutant with an ectopic chromosomal copy of the niuDE operon (c-niuDE) resulted in complete restoration of the Niu-dependent nickel transport, with an uptake rate close to that measured with the ΔnixA mutant. Together, these results demonstrate that NixA and NiuD are involved in two distinct nickel uptake systems and strongly suggest that no other nickel transport systems exist in the H. pylori cytoplasmic membrane.
NiuB1 and NiuB2 are the periplasmic binding proteins of the ABC nickel uptake system

Uptake ABC transporters function with high-affinity solute binding proteins that bind the substrate in the periplasm and allow its recognition by the inner membrane transporter proteins. In _H. pylori_, a gene annotated as _ceuE_ encodes a periplasmic protein that was a good candidate for this function. Although it was annotated as an iron-binding protein, recent publications showed that the _ceuE_ gene is regulated by NikR in response to nickel [52] and that the CeuE protein, whose structure has been solved, binds nickel [56]. Some _H. pylori_ strains such as B128 possess two consecutive and paralogous genes annotated _ceuE1_ (hpB8_1657) and _ceuE3_ (hpHPB8_1658) that share 85% identity, which we renamed _niuB1_ and _niuB2_. An unmarked deletion of the two genes, referred to as _ΔniuB_, was constructed. We found that this deletion alone did not influence nickel tolerance in the wild type B128-S strain (Fig 3A). However, as...
observed with the ΔnixD mutation, deletion of niiB increased nickel tolerance in the ΔnixA genetic background (Fig 3A). In addition, while expression of the \( \text{P}_{\text{fecA3::lacZ}} \) fusion was slightly affected in the \( \Delta \text{niiB} \) mutant as compared to the wild type strain, strong derepression was observed in the \( \Delta \text{nixA} \Delta \text{niiB} \) double mutant. This phenotype, indicative of a total absence of nickel uptake, was similar to that of the \( \Delta \text{nixA} \Delta \text{niiD} \) mutant (Figs 2B and 3B). Thus, the behavior of the \( \Delta \text{niiB} \) mutants fully parallels that of the \( \Delta \text{niiD} \) mutants and points to the participation of NiuB in nickel transport together with the NiuD and NiuE proteins.

As mentioned before, some \( H. \text{pylori} \) strains harbor two NiuB paralogues. To analyze the individual contribution of the two paralogues, we cloned the \text{niuB1} and \text{niuB2} genes separately and transformed them into the \( \Delta \text{nixA} \Delta \text{niiB} \) mutant. Both \text{niuB1} or \text{niuB2} genes expressed either from the chromosome (designated \( c- \)) or from a plasmid under the control of an IPTG inducible promoter (designated \( p- \)) restored nickel sensitivity to levels comparable to those of

Fig 3. NiuB1 and NiuB2 control the nickel sensitivity and uptake in \( H. \text{pylori} \). Panel A: Effect of nickel on growth of \( H. \text{pylori} \) B128-S wild type strain and isogenic mutants. \( \Delta \text{niiB} \) corresponds to a \( \Delta \text{niiB1-\Delta niiB2} \) double mutant. Results are presented as in Fig 1. Panel B: β-galactosidase activity of the \( \text{P}_{\text{fecA3::lacZ}} \) reporter fusion in different backgrounds presented as in Fig 2. Panel C: Measurements of radioactive nickel uptake rates in wild type and mutant strains. On the right scale, the uptake rates were normalized with respect to the value measured for the wild type strain. The mutant strains were either complemented by \( c-\text{niuB1} \) or \( c-\text{niuB2} \) (\( c- \) for chromosomally inserted) inserted at a neutral site on the chromosome under the control of the \( \text{P}_{\text{ureI}} \) promoter or by \( p-\text{niuB1} \) and \( p-\text{niuB2} \) (\( p- \) for inserted on a plasmid) expressed from derivatives of plasmid pILL2157 under the control of an IPTG-inducible \( \text{P}_{\text{ureI}} \) promoter in the presence of IPTG. In these different experiments, the data correspond to the mean value of three independent experiments and error bars represent the standard deviation. *** indicates that the mean value is significantly different (\( P \leq 0.001 \)) from that of the wild type strain.

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the ΔnixA mutant (see below). Radioactive nickel uptake rates measured in the ΔniuB was 38% that of the wild type strain and less than 5% for the ΔnixA ΔniuB mutant (Fig 3C). This again paralleled the results obtained for the ΔniuD and ΔnixA ΔniuD mutants, further confirming that NiuB and NiuD are indeed part of the same transport system (Fig 3C). Intriguingly, when expressed from the chromosome (c- niuB1 and c- niuB2 in Fig 3C), NiuB1 but not NiuB2 was able to restore 65Ni(II) uptake in the ΔnixA ΔniuB mutant to the level of the genetically equivalent ΔnixA mutant (compare Fig 3C to Fig 2C). In the ΔnixA ΔniuB double mutant, IPTG-induced overexpression of NiuB1 from plasmid pILL2157 (p- niuB1) resulted in a strong increase of nickel uptake rate reaching 95% of wild type level. In contrast, no restoration of nickel uptake was observed with NiuB2 even when overexpressed under the same conditions (p- niuB2). Thus, NiuB1 appears to be the major periplasmic component for nickel uptake by the NiuBDE ABC transport system.

Role of NiuD, NiuB1-2 and NixA in nickel-dependent urease activation

We then evaluated the role of the two nickel uptake systems in the activation of the nickel-dependent urease in strains incubated in BBβ medium, which contains 0.2 μM nickel [40]. Urease activity was tested in intact live cells exposed to pH 5 or pH 7 by measuring ammonia produced from urea hydrolysis in wild-type and mutant strains as previously described [57] (Fig 4). We first found that urease activity did not decrease in the ΔnixA mutant at neutral pH and rather slightly increased under pH 5 acidic conditions (140%) compared to the parental wild type strain. At pH 5, urease-dependent ammonia production of the niuD and niuB mutants were similar and only slightly affected (70% of the wild type strain). In contrast, at pH 7, these two mutants presented a strong decrease in urease activity, reaching only 15% of the wild type value and suggesting that the remaining NixA transporter is not supporting nickel uptake under this condition. This suggests that NiuD activity is independent of the pH, while NixA is only efficient under acidic pH conditions. Finally, at both pH values ammonia production of the ΔnixA ΔniuD and ΔnixA ΔniuB double mutants was dramatically decreased to a negligible level under both conditions (<10% of the wild type values). This indicates that in these mutants, there is a strong defect in nickel delivery to urease as a consequence of deficient nickel transport.

When the niuDE operon was expressed ectopically in the ΔnixA ΔniuD double mutant (c- niuDE), urease activity was restored to levels similar to that of the ΔnixA single mutant. Plasmidic expression of either NiuB1 or NiuB2 (p- niuB1, p- niuB2) in the ΔnixA ΔniuB mutant restored urease activity under the two pH conditions. However, only expression of NiuB1 could complement the double mutant to wild type levels, while NiuB2 only partially restored urease activity. This again argues in favor of NiuB1 being more efficient in nickel transport than its NiuB2 parologue.

Overall, these results indicate that H. pylori possesses two nickel uptake machineries, NiuBDE and NixA, that function separately from each other to deliver nickel to the major virulence factor urease. Contrary to NiuBDE that functions at both pH 5 and 7, NixA is much more active under acidic conditions than at neutral pH.

Both NiuBDE and NixA are required for survival to acid exposure

We further examined the respective importance of the two nickel uptake systems in the H. pylori survival capacity under extreme acidic conditions, mimicking those encountered in the gastric environment (Fig 5). Wild-type B128-S strain and mutants were tested for their ability to enhance the pH of a highly acidic buffer (PBS at pH 2, as in [58]) through urea breakdown and ammonia synthesis. In addition, survival of the cells was assessed with an Alamar blue-based viability test [59]. Without added urea, no strain survived a 40 minutes-long exposure at
pH 2 and the pH of the medium remained acidic. In the presence of 6 mM exogenous urea, wild type strain and the ΔnixA, ΔniuD and ΔniuB single mutants survived and their ammonia production resulted in pH increase to values close to neutrality, in line with their urease activity measurements (Fig 5). In contrast, the ΔnixA ΔniuD and ΔnixA ΔniuB double mutants were unable to increase the pH and, as a result, did not survive high acidity exposure. This phenotype was restored to wild type when an ectopic niuDE copy (c-niuDE) was introduced on the chromosome of the ΔnixA ΔniuD mutant. Interestingly, both niuB1 and niuB2 genes expressed from plasmids (p-niuB1 and p-niuB2) complemented the ΔnixA ΔniuB mutant to wild type phenotype. This demonstrates that under these conditions, both periplasmic NiuB1

Fig 4. Role of NixA and NiuBDE in urease activity of H. pylori at pH 5 and 7. Urease activity was measured on whole cells of the different mutants strains by measuring ammonia production. For each condition, ammonia production is expressed as a percentage of the wild type strain put at 100%. These data indicate that NiuBDE functions at both pH 5 and 7, while NixA is mainly active at pH 5. ΔniuB corresponds to a ΔniuB1-ΔniuB2 double mutant. The mutant strains were either complemented by c-niuDE (c- for chromosome) inserted at a neutral site on the chromosome under the control of the P ureI promoter or by p-niuB1 and p-niuB2 (p- for plasmid) expressed from derivatives of plasmid pILL2157 under the control of an IPTG-inducible P ureI promoter in the presence of IPTG. The data correspond to the mean value of three independent experiments and error bars represent the standard deviation. *** and ** indicate that the mean value is significantly different from that of the wild type strain (P ≤ 0.001 and P ≤ 0.01, respectively). For the complemented strains, ΔnixA ΔniuD + c-niuDE or ΔnixA ΔniuB p-niuB1/2, the P values correspond to comparison with the corresponding parental ΔnixA ΔniuD and ΔnixA ΔniuB mutants, respectively.

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and NiuB2 proteins are functional to mediate sufficient nickel entry within the cells. Thus, under conditions mimicking those of the stomach, NixA or NiuBDE is sufficient for survival to extreme acidity.

**Metal specificity of the NiuBDE transporter**

To determine whether the novel *H. pylori* NiuBDE transport system is involved in uptake of metals other than nickel, sensitivity tests were carried out with our collection of mutants. The effect of addition of zinc, iron, copper or manganese on growth of the ΔniuD, ΔnixA and ΔnixA ΔniuD mutants did not differ from that on the parental wild type strain. This suggests that the NiuBDE and NixA transport systems do not participate in uptake of these metals.

### Fig 5. NixA and NiuBDE are necessary for resistance to extreme acidity in the presence of urea. Wild type strains and mutants were exposed during 40 min at pH 2, without or with 6 mM urea. ΔniuB corresponds to a ΔniuB1-ΔniuB2 double mutant. Controls were performed at pH 7 without urea. Survival of the bacteria was assessed using an Alamar blue-based test, in which a pink staining reveals metabolically active cells, while a blue staining means that cells are metabolically inactive. The pink staining is proportional to the amount of metabolically active cells. The pH was evaluated for each strain and results were compared to control cells (exposed at pH 7) and to a pH-scale (right side of the figure). A reminder of the results obtained in other experiments for urease activity and ⁶³Ni(II) uptake is given at the bottom of the figure for comparison.

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We then performed tests with cobalt and bismuth (Fig 6). The sensitivity of the ΔnixA mutant to cobalt did not significantly differ from that of the parental strain (in agreement with [41]). In contrast, the ΔniuD, ΔniuB and ΔnixA ΔniuD mutants presented the same strong increase in tolerance to 0.01 mM cobalt exposure. Complementation of the ΔniuD and ΔnixA ΔniuD mutants with c-ΔniuDE rendered the strains more sensitive to cobalt than the wild type strain, indicating that more cobalt accumulated under these conditions. These results suggest that NiuBDE, but not NixA, is involved in cobalt uptake in H. pylori.

For bismuth exposure, no significant difference was observed with the ΔnixA mutant while tolerance of the ΔniuD, ΔniuB and ΔnixA ΔniuD mutants was enhanced, although this phenotype was less marked than with cobalt or nickel. However, the increased sensitivity of strains overexpressing niuDE strongly suggests that uptake of bismuth is mediated by the NiuBDE transporter and not by the NixA permease.

Similar experiments were carried out to determine which, if any, NiuB periplasmic protein is required for bismuth and cobalt uptake. We tested the ΔniuD ΔniuB and ΔnixA ΔniuD ΔniuB mutants complemented with a plasmid expressing either niuB1 or niuB2 under the control of an IPTG-inducible promoter (p-ΔniuB1, p-ΔniuB2). Without IPTG, only the ΔnixA ΔniuB and ΔnixA ΔniuB Δhpn mutants complemented with ΔniuB1 recovered wild type sensitivity to cobalt and bismuth, suggesting that Niub1 plays a major role in uptake of these metals, under these conditions. However, when ΔniuB1 or ΔniuB2 expression was induced by IPTG, cobalt and bismuth sensitivity was restored to wild type level of the ΔnixA Δhpn mutant, indicating that both Niub1 and Niub2 can function in cobalt and bismuth uptake.

We concluded that cobalt and bismuth are taken up by the NiuBDE transporter in H. pylori. The existence of an additional transporter for these metals can however not be excluded.

**NiuD and Niub1-2 but not NixA are essential for colonization of the mouse model**

The role of Niud and Niub1-Niub2 during gastric colonization was evaluated using the mouse model. H. pylori SS1 mutants carrying complete deletions of the niuD or niuB1 niuB2 (ΔniuB) genes and/or nixA genes were constructed. The ΔniuD mutant was recomplemented on the chromosome with a niuDE copy (c-ΔniuDE). The ΔniuB mutant was recomplemented with either ΔniuB1 or ΔniuB2 (c-ΔniuB1, c-ΔniuB2). Each strain was orogastrically inoculated in six NMRI mice. One month later, colonization was assessed by quantitative cultures of stomach homogenates (Fig 7). The geometric mean of the colonization loads of mice infected with the ΔnixA mutant was only slightly below that of the wild type strain, indeed half of the mouse were colonized at the level of the parental strain. In contrast, the ΔniuD and ΔniuB mutants were completely deficient in their capacity to colonize the mouse stomach, indicative of their major role in pathogenicity. Each of the complemented strain, SS1 ΔniuD c-ΔniuDE, SS1 ΔniuB c-ΔniuB1 and SS1 ΔniuB c-ΔniuB2 recovered close to wild type capacity to colonize the murine stomach. These results show that NixA is not essential for *in vivo* colonization. In contrast, the NiuBDE system is indispensable for colonization of the mouse stomach and *in vivo* can function with either Niub1 or Niub2.

**Distribution and phylogeny of the nickel transporters in the Helicobacter species**

The Helicobacter genus is composed of two subgroups, the enterohepatic (EH) species and a small group of gastric Helicobacter species including H. pylori that exclusively colonize the stomach of diverse mammals and depend on urease activity for their multiplication in this niche. The central role of NiuBDE suggested by our work prompted us to establish its
Fig 6. Sensitivity to cobalt and bismuth of wild type strain and isogenic mutants. Effect of NiCl₂, CoCl₂ and bismuth subcitrate potassium on growth of *H. pylori* B128-S wild type strain, isogenic mutants and complemented.
distribution in the *Helicobacter* genus, to search for other predicted inner membrane nickel transporters and to compare their distribution. We screened full proteomes of available *Helicobacter* genomes (NCBI) (11 EH species, 8 *non-pylori* gastric *Helicobacter* species and the 434 *H. pylori* strains analyzed in [22]). The MycoHIT program [60,61] was used to search for NixA, subunits of NiuBDE, NikABCDE, NikZYXWV, urease (UreB) and hydrogenase (HydA). The identified proteins were then mapped onto a previously-determined phylogenetic tree of *Helicobacter* species [22] (Fig 8). First, we observed that both NixA and NiuBDE are

![Graph showing bacterial loads and complemented strains](image)

Fig 7. *NiuD* and *NiuB* are required for mouse colonization by *H. pylori* strain SS1. Each point corresponds to the colonization load for one mouse one month after infection with the strain indicated below. Horizontal bars represent the geometric means of the colonization load for the wild type, each mutant and the chromosomally complemented mutants (designated c-). Δ*niiB* corresponds to a Δ*niiB1*–Δ*niiB2* double mutant. The results presented correspond to a representative experiment out of two. The detection limit is shown by a dashed horizontal line. *** indicates that the geometric value is significantly different (*P* ≤ 0.001) from that of the wild type strain.

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conserved in every *H. pylori* strain. As expected, urease is present in all gastric species and in only 5 EH species (S3 Fig). Hydrogenase is ubiquitous. The distribution of NixA and NiuBDE is not correlated with the presence of urease but rather follows the gastric/EH tropism of the *Helicobacter* species. Indeed, NixA is present in every gastric *Helicobacter* species and is not detected in EH species. The picture is more complex for NiuBDE. The 3 subunits were unambiguously detected in 5 gastric species but not in *H. felis, H. bizzozeronii, H. suis, H. heilmannii* (heilmannii-like group) and in only one EH species, *H. fennelliae*. Instead, 10 EH species out of 14 examined are equipped with a NikABCDE nickel transporter.

**Fig 8. Evolutionary history of Helicobacter nickel inner membrane transporters.** The core proteome-based neighbor-joining phylogenetic tree of *Helicobacter* species was obtained in a previous study [22]. Enterohepatic (EH) and gastric species are indicated and the latter are highlighted with a light green background. Names of each species are indicated. Important nodes (N1, N2 and N3) are indicated in red. For each strain, the table indicates the presence (green circles) or the absence (white circles) of the proteins indicated: transporters (NixA, NiuBDE, NikABCDE and NikZOppBCDE), Ni-dependent enzymes (urease, hydrogenase) or Ni-binding proteins (Hpn and Hpn-2). Grey circles indicate low identity orthologs that were detected in strains but that have no direct phylogenetical relationship to the proteins studied, according to the maximum likelihood (ML) trees. Arrows and comments describe the evolutionary events that account for the observations and phylogenetical analyses. At node N1, the last common ancestor of gastric species, in which Hpn emerged [22], also acquired NixA and NiuBDE transporters. NikABCDE was vertically inherited by the subsequent gastric species. NikZOppBCDE, a possible NikZYXWV-like system.

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Interestingly, in all _heilmannii_-like species tested, a NikZ protein (>60% identity) was detected, its corresponding _nikZ_ gene being part of a conserved operon of five genes encoding a predicted Opp-type ABC-transporter (S3 Fig). These gastric _Helicobacter_ species probably possess a NikZYXWV-like nickel transporter [33,34], as an alternative to the Niu transporter.

The observation that NixA is restricted to the gastric _Helicobacter_ species can either correspond to gene acquisition by these species or gene loss in the EH _Helicobacter_ species. To obtain a picture of the evolutionary history of both systems, we established the phylogeny of the NixA, NiuB and NiuD proteins. The maximum likelihood (ML) phylogenetic tree of NixA protein sequences shows that they emerge from within the Firmicutes, suggesting an acquisition of _nixA_ via horizontal gene transfer (HGT) from _Paenibacilli_ to the last common ancestor (LCA) of the gastric _Helicobacter_ species (S4 Fig). ML trees of NiuB and NiuD revealed a patchy taxonomic distribution, indicative of a complex evolutionary history (S5 and S6 Figs).

However, NiuB and NiuD from gastric _Helicobacter_ species form a clade distinct from other EH species and Epsilonproteobacteria, and closely related to Thermodesulfobacteria and Deltaproteobacteria, together with those from the _H. fennelliae_ (EH species) and _Campylobacter sputorum_. Importantly, the NiuB and NiuD proteins display very similar evolutionary histories (S7 Fig). Comparison of gene synteny with phylogenies reveals that _niuB_ and _niuD_ genes were originally in an operon and were likely transferred together from Thermodesulfobacteria to _C. sputorum_ and then to _H. fennelliae_ (Epsilonproteobacteria). Then, the _niuBDE_ operon would have been transferred further from an EH species related to _H. fennelliae_ to the LCA of gastric species. This operon would have been vertically inherited in other gastric species, except for _heilmannii_-like species, where the NiuBDE cluster was most likely lost and/or functionally replaced by a NikZYXWV-like system.

Among the gastric _Helicobacter_ species, _H. pylori_ is the only species that presents either one or two copies of NiuB depending on the strain. _niuB1_ is always located upstream of _niuB2_ and corresponds to the single _niuB_ from species having only one copy. The two copies are always in tandem suggesting a recent duplication event.

In conclusion, our phylogenomic analysis revealed that the two nickel transporters of _H. pylori_ were acquired by HGT in gastric _Helicobacter_ species. This acquisition was probably an important step in evolution to optimize the capacity of _Helicobacter_ strains to thrive in the stomach.

**Discussion**

Pathogenicity of bacteria depends on their capacity to acquire sufficient metals to survive within their hosts. In the case of _H. pylori_, nickel is a virulence determinant, since it is a cofactor of urease, which is essential for surviving the acidic environment of the stomach, and therefore for gastric colonization. Acquisition of nickel from the extracellular environment is a major challenge for _H. pylori_ since its concentration is very low in the human body [25]. Besides an outer membrane nickel transporter (FrpB4) [26], _H. pylori_ possesses a high affinity nickel permease of the NiCoT family in the inner membrane (NixA) that has been extensively described [38,62]. A _nixA_ deletion mutant retains urease activity and is still able to colonize the mouse model [43]. Therefore, it was proposed that another nickel transport system would be functional to fuel the bacterium with this metal.

A genome-wide genetic screen allowed us to identify a novel ABC transporter involved in nickel uptake in _H. pylori_. This system, that we named Niu (Nickel Uptake), is composed of three proteins: the periplasmic solute binding protein NiuB, together with NiuD and NiuE that are predicted to act as a permease and ATPase subunit, respectively (for a model of nickel transport in _H. pylori_, see Fig 9). The function of the Niu system as a nickel transporter was demonstrated in _H. pylori_ by using nickel sensitivity assays, measurements of intracellular...
Nickel content, expression of a nickel-responsive reporter gene, and direct measurement of $^{63}$Ni(II) transport kinetics, in single and double mutants deficient in NixA and/or Niu proteins. All of these experiments demonstrated that Niu functions as a genuine nickel transporter in H. pylori. The fact that the phenotypes of the $\Delta$nixA and $\Delta$niu mutations are additive strongly suggests that the two systems function independently of each other. Strikingly, mutants in which both NixA and Niu systems are inactivated displayed non-measurable nickel uptake rates and very low to undetectable intracellular nickel levels, indicating that they are most likely the two sole inner membrane nickel importers in H. pylori.

Transport of other metals in H. pylori was also indirectly examined by sensitivity tests. Cobalt is a known cofactor of several proteins (e.g., arginase in H. pylori [63]). We showed that the Niu transporter is additionally involved in cobalt uptake, while the NixA permease is not, although the latter belongs to the NiCoT family. Bismuth, a metal that is not a natural protein cofactor, is a component of the new Pylera medication, in combination with two antibiotics and a proton pump inhibitor [64]. Pylera has proven to be more efficient in eradicating H. pylori infections than the classical triple therapy but the precise mode of action of bismuth and the nature of its toxicity in H. pylori remains unclear. Our experiments indicate that NiuBDE, but not NixA, participate in bismuth substrate potassium uptake in H. pylori. Thus, in vivo bismuth toxicity in H. pylori might, at least in part, result from its interference with the essential Niu nickel transporter.

We showed that NiuB constitutes the corresponding high-affinity solute binding protein of the Niu transport system. Genomics reveal that ~50% of the sequenced H. pylori strains, including the B128 strain, possess two closely related paralogous genes, that we designated niuB1 and niuB2, and that are likely the result of a recent duplication. The structure of the NiuB protein from H. pylori strain G27, which corresponds to the NiuB1 paralogue, has been determined [56]. This protein belongs to the class III periplasmic substrate-binding protein family and binds nickel as a Ni(II)-(L-His)$_2$ complex. Binding of such complexes seem to be a common feature for many periplasmic binding proteins of nickel ABC transporters such as NikA in E. coli or NikZ in Campylobacter jejuni [29,34,65]. Our results reveal that NiuB1 is more efficient than NiuB2 in nickel, cobalt and bismuth uptake. Sequence alignments and comparison of B128 NiuB1 and NiuB2 with NiuB from strain G27 indicate that both proteins share all the structural determinants required for the binding of Ni(II)-(L-His)$_2$ complex and for the predicted interaction with NiuD [56] (S8 Fig), and only minor differences. Comparison of the putative binding sites reveals only a few substitutions within the binding pocket that could interfere with or influence ligand binding (S8 Fig). The difference in NiuB1 and NiuB2 efficiencies thus remains elusive and future experiments will be needed to understand the precise contribution of each NiuB and to define whether the paralogues underwent functional subspecialization.
In a previous study, we demonstrated that two essential nickel-binding proteins (Hpn and Hpn-2) emerged exclusively in gastric Helicobacter species and postulated that this constituted a decisive evolutionary event to allow Helicobacter to colonize the hostile gastric environment [22]. Because nickel is necessary to activate urease and therefore to colonize the stomach, we hypothesized that acquisition of nickel transport systems may have followed a similar pattern. We thus analyzed the distribution of all known inner membrane nickel transporters of Helicobacter species, and reconstructed the phylogeny of NixA, NiuB and NiuD. Results clearly show that EH species predominantly possess the NikABCDE ABC transport system, as was demonstrated in H. hepaticus [48]. The nixA gene is specific to the gastric Helicobacter species and was likely acquired via HGT by the LCA of gastric species probably from species closely related to Paenibacilli. NiuBDE was probably acquired by the LCA of gastric species via HGT from species related to H. fennelliae. NiuBDE was then inherited vertically by other gastric species and conserved its function as in H. mustelae, where the corresponding homolog was shown to contribute to nickel acquisition [66]. Within the heilmannii-like branch, NiuBDE is absent, and has been functionally replaced by another transporter, comprising NikZ, suggesting that the common ancestor of heilmannii-like species may have acquired this NikZYXWV-like system via HGT from Firmicutes (as also suggested in [34]). In all cases, unlike EH species, gastric Helicobacter species acquired and retained two different nickel transport machineries, NixA and an ABC transporter.

The genomes of gastric Helicobacter species are small (1.6 Mb for H. pylori) suggesting reduced functional redundancy, which has been documented in the case of H. pylori [67]. Therefore, the existence of two nickel transporters in these organisms is intriguing. Our results indicate that, in H. pylori, the two transporters differ in their uptake properties as a function of pH and of nickel concentration. Therefore, they might be involved at different stages of the infection in agreement with the differences that we observed during the in vivo experiments. At pH 5, the ΔnixA mutant displays a much stronger decrease in $^{65}$Ni(II) uptake rates than the ΔniuD mutant, suggesting that NixA is, kinetically, the major contributor under these conditions (Fig 2). However, since urease activity in single ΔnixA or Δniu mutants does not decrease when compared to the wild type, we conclude that, at pH 5, accumulation of nickel is not significantly affected (Fig 4). Indeed, as shown in Fig 5, individually NixA and Niu can transport sufficient nickel to activate urease and thereby allow survival of H. pylori to exposure to highly acidic conditions. In contrast, urease activity of the double mutant is very low confirming that these transporters are the two sole nickel uptake systems in H. pylori.

At pH 7, $^{65}$Ni(II) uptake cannot be measured possibly because the outer membrane transporter FrpB4 requires acidity to be activated [26]. Under neutral conditions, ΔnixA mutants are more tolerant to nickel overload than Δniu mutants suggesting again a predominant role of NixA in nickel uptake. In the ΔnixA mutant, in which only the Niu transport system is active, intracellular nickel content measured by ICP-OES, remains constant when extracellular Ni(II) is increased from 10 to 100 μM. In contrast, nickel content doubles in the Δniu mutant in which NixA is the only transporter. Thus, the Niu system may already be saturated at low nickel concentrations in contrast to the situation of the NixA transporter. This saturation is likely the basis of higher tolerance to nickel overload in the nixA mutant (Fig 1). At pH 7, deletion of the genes encoding the Niu system strongly affects urease activity, while the ΔnixA strain displays wild type activity. This suggests that, under these conditions of low nickel availability, NixA is poorly efficient in fueling urease with nickel. We conclude that NixA is required for rapid nickel acquisition at acidic pH, while the Niu system is active under both neutral and acidic conditions even at low nickel concentrations (such as those in the urease activity test). Given that FrpB4 is acid-activated [26], we propose that under neutral conditions, less nickel enters the periplasm thereby providing an advantage to the Niu ABC transporter.
over the NixA permease, since it is equipped with a high affinity periplasmic binding protein, NiuB.

The in vivo function of the two H. pylori nickel transporters was evaluated by measuring their importance in colonization of a mouse model. The Niu transporter was found to be strictly essential in vivo. The ΔnixA mutant reproductively presented a bimodal colonization profile, with about half of the mice that were not colonized and the other half that presented normal colonization. This profile is consistent with a bottleneck event in the first steps of colonization, as suggested by previous results reporting that a nixA mutant alone can colonize but is outcompeted by a wild type strain during mixed colonization [43]. The non-essentiality of the NixA transporter in the mouse model seems surprising given its conservation in all gastric Helicobacter. However, our data suggest that NixA and Niu have different transport properties, the Niu system being more adaptable and capable of acquiring nickel at low concentrations under moderately acidic or neutral conditions like those encountered at its persistent colonization site, the epithelial cell surface. It could also be that during in vivo colonization, Niu uses nickel chelated to a nickelophore (as reported for the NikABC transporter), and that this complexed form is not efficiently recognized by NixA. Thus, H. pylori has two versatile systems that are required to allow efficient and persistent colonization of a hostile environment.

In conclusion, we have identified a novel H. pylori nickel transporter that is essential for colonization and together with NixA represent the two sole nickel transporters in H. pylori. These transporters have been acquired by gastric Helicobacter species through horizontal transfer. Together with the acquisition of the Ni(II)-binding proteins, Hpn and Hpn-2, this further highlights how important it has been during evolution of gastric Helicobacter species to increase their capacity to accumulate intracellular nickel without toxicity and thereby optimize urease activity required for persistent colonization of the acidic stomach.

Materials and Methods

Ethics statement

Experiments in mice were carried out in strict accordance with the recommendations in the Specific Guide for the Care and the Use of Laboratory Animals of the Institut Pasteur, according to the European Directive (2010/63/UE) and the corresponding French law on animal experimentation (Arrêtés de 1988). The protocol has been approved by the Committee of Central Animal Facility Board of the Institut Pasteur. To follow the new European directives, the project was approved by the CETEA, Comité d’éthique en Expérimentation Animale of the Institut Pasteur (#2013–0051) and by the Ministère de l’Enseignement Supérieur et de la recherche (#751501).

Bacterial strains and growth conditions

All plasmid constructions of this work were made in the E. coli strain BTH101 [68] grown on solid or liquid Luria-Bertani medium [69] supplemented with spectinomycin 100 μg.mL⁻¹, chloramphenicol 30 μg.mL⁻¹, ampicillin 100 μg.mL⁻¹ or kanamycin 40 μg.mL⁻¹, when required.

The H. pylori strains used in this study (S1 Table) are G27 [50], B128 [70,71], B128-S (this study), SS1 [72] and 26695 [73] H. pylori strains were grown on Blood Agar Base 2 (Oxoid) plates supplemented with 10% defibrinated horse blood and with the following antibiotics-antifungal cocktail: amphotericin B 2.5 μg.mL⁻¹, polymyxin B 0.31 μg.mL⁻¹, trimethoprim 6.25 μg.mL⁻¹ and vancomycin 12.5 μg.mL⁻¹. For liquid cultures, we used Brucella broth, designated BB (BD Difco) supplemented with 10% fetal calf serum (FCS, Eurobio) or with 0.2% β-cyclodextrin (Sigma) (designated here BBβ), with the antibiotics-antifungal cocktail and the selective
antibiotic when required. Selection and growth of \textit{H. pylori} mutants and transformants were performed using kanamycin 20 \(\mu\)g.mL\(^{-1}\), chloramphenicol 6 \(\mu\)g.mL\(^{-1}\) or streptomycin 10 \(\mu\)g.mL\(^{-1}\).

**Molecular techniques**

Molecular biology experiments were performed according to standard procedures [74] and the supplier (Fermentas) recommendations. NucleoBond Xtra Midi Kit (Macherey-Nagel) and QIAamp DNA Mini Kit (Qiagen) were used for plasmid preparations and \textit{H. pylori} genomic DNA extractions, respectively. PCR were performed either with Taq Core DNA polymerase (MP Biomedicals), or with Phusion Hot Start DNA polymerase (Finnzymes) when the product required high fidelity polymerase. The pGEMT vector (Promega) was used to construct in \textit{E. coli} the suicide plasmids that served for mutagenesis in \textit{H. pylori}.

**Construction of unmarked \textit{H. pylori} mutants**

Unmarked \textit{niuD} and \textit{niuB} deletion mutants of \textit{H. pylori} were constructed by allelic exchange as described [53] using \textit{H. pylori} suicide plasmids derived from pGEMT, in which about 500 bp of the 5’-end and the 3’-end regions immediately flanking the open reading frame of the target gene were cloned using PCR fragments generated with the primers indicated in S2 Table on each side of a difH-cat-rpsL-difH cassette amplified with primers difHrpsLcat-1 and difHrpsLcat-2. The \textit{H. pylori} mutants were obtained by natural transformation of B128-S, a streptomycin resistant derivative of B128, with 1 \(\mu\)g of a preparation of the suicide plasmid DNA and selection on blood agar plates containing chloramphenicol 6 \(\mu\)g.mL\(^{-1}\) (for G27 and B128-S) or 10 \(\mu\)g.mL\(^{-1}\) (for SS1) as described previously [75]. Removal of the cassette was achieved by plating the Cm\(^R\) clones on blood agar plates containing streptomycin 10 \(\mu\)g.mL\(^{-1}\). Deletion of the gene of interest was verified by PCR and sequencing of the gene region.

**Construction of plasmids and complemented mutants**

The mutants were complemented through two strategies. One was by introducing the wild type copy of the gene to be complemented under the control of the \textit{ureI} promoter on the chromosome, at a neutral site that was shown to affect neither \textit{in vitro} growth nor mouse colonization as described in [22,54]. The corresponding constructs were designated “c-X” for chromosomally inserted, X being the name of the gene. The second one was by cloning the gene under the control of an IPTG inducible promoter in the pILL2157 \textit{E. coli}/\textit{H. pylori} shuttle vector [76], the corresponding constructs were designated “p-X” for inserted on a plasmid, X being the name of the gene. For the chromosomal complementation, the \textit{niuDE}, \textit{niuB1} and \textit{niuB2} genes were PCR-amplified from the B128 chromosome and cloned into the EcoRV sites of pIRC plasmid in which we had introduced the \textit{P}_{ureI} promoter [22] (PCR using the couple of primer IR-UP/IR-DO), allowing the transcription of the genes under the control of \textit{P}_{ureI} promoter. The pIRC(\textit{P}_{ureI}) derivatives were used to transform strain 26695 selecting for chloramphenicol resistance. We verified the presence of the wild type copy of the genes at the correct location by sequencing PCR amplification products made with primers hp203/hp204. The chromosomal DNAs from correct chloramphenicol resistant clones (i.e. where the wild type genes and the \textit{cat} cassette integrated by homologous recombination between genes \textit{hp0203} and \textit{hp0204}) were then used to introduce the \textit{niuD}, \textit{niuB1} or \textit{niuB2} genes into various strains by natural transformation and selection for chloramphenicol resistance. For cloning into pILL2157, the genes were PCR-amplified from the B128 chromosome using the couple of primers pil-UP/pil-DO and cloned into the \textit{NdeI}/EcoRV sites (for \textit{niuD} and \textit{niuDE}) or the \textit{NdeI}/\textit{BamHI} sites (for \textit{niuB1} and \textit{niuB2}) of pILL2157. To construct the \textit{P}_{\textit{fecA3}}:\textit{lacZ} reporter, we PCR-amplified the promoter sequence from the B128 chromosome using the couple of
primers PfecA3-UP/ PfecA3-DO and cloned it into the BglII/SpeI sites of pILL2157. β-galactosidase activities of strains carrying this fusion were measured with the classical E. coli protocol [69] adapted to H. pylori as described in [22].

Mouse model of colonization

NMRI-specific pathogen-free mice (Charles River Laboratories) were orogastrically inoculated with 10⁹ CFU of H. pylori strains prepared in 100 μL of peptone broth. One month after inoculation, mice were sacrificed and stomachs were crushed in peptone broth (as described in [58]). Viable H. pylori colonizing the stomach were enumerated by culture of serial dilutions of homogenized tissue on blood agar plates containing in addition bacitracin (200 μg.mL⁻¹) and nalidixic acid (10 μg.mL⁻¹).

Measurement of metal sensitivity

To test metal sensitivity during growth in liquid, H. pylori cells were inoculated at OD₆₀₀ 0.05 in 10 mL liquid Brucella-Broth containing 10% FCS either without or with 0.5, 1.5 or 2 mM NiCl₂, with 1 or 2 mM for ZnCl₂, CuCl₂, MnSO₄, with 0.01 mM CoCl₂ and 0.1 mM of a colloidal preparation of Bismuth subcitrate potassium. Bacterial growth was monitored 24 hours later by measuring OD₆₀₀. The data correspond to at least three independent experiments with two replicates per experiment.

Nickel content measurements by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

Overnight liquid cultures of H. pylori strain were grown until OD₆₀₀ 0.9 at 37˚C in 6 ml Brucella-Broth with FCS, then 10 μM or 100 μM NiCl₂ were added and the cultures were left to grow until OD₆₀₀ 6. Then, the 6 ml of culture were centrifuged at 4000 g at 4˚C for 25 min through 400 μL of a 1:2 mixture of the silicone oils AR20/AR200 (Wacker) in order to separate the cells from the medium. Cells were lysed with 400 μL 0.2 M NaOH/1% SDS for 60 min at 95˚C. Samples were calibrated by protein concentration measurements with the DC Protein Assay kit (BioRad). Then, the samples were mineralized overnight at 80˚C with 300 μL of ultrapure 70% nitric acid (JT Baker) and diluted to 1/20 in ultrapure water. Nickel contents were measured by ICP-OES using a Vista MPX spectrometer (Varian). The content of Ni(II) was determined using a curve established with certified ICP grade standards. The measurement of each strain in each condition was performed in triplicates in at least two independent experiments.

Transport of radiolabelled nickel

The procedure was adapted from our previously published protocol [26]. As shown in that paper, this test is only functioning at pH 5, presumably because the outer membrane transporter FrpB4 is acid-activated [26]. Overnight 10 mL precultures of H. pylori B128-S wild-type and mutant cells were diluted to OD₆₀₀ 0.25 into 10 mL fresh BB medium supplemented with fetal calf serum and incubated in microaerophily with shaking at 37˚C. When the cultures reached OD₆₀₀ 0.5, cells were harvested, washed twice in fresh BB medium with β-cyclodextrin (BBβ), resuspended in the same volume of BBβ, that was adjusted to pH 5, and shaken during 20 minutes in microaerophily, at 37˚C. Radiolabelled ⁶³NiCl₂ (3.71 mCi/mL, 16.3 mCi/mg), was isotopically diluted 10-fold with cold NiCl₂ and added to each culture to a final concentration of 10 μM. ⁶³NiCl₂ was supplied by the United States Department of Energy Office of Science by the Isotope Program in the Office of Nuclear Physics. Kinetics were performed during 45 minutes with a time point before nickel addition, and time points at 5, 20, 30 and 45
minutes after nickel addition. Aliquots of 1 mL were withdrawn, immediately vacuum filtered through 0.45 μm pore-size cm filters (ø = 2.5; Millipore) and washed with 10 mL of 50 mM Tris-HCl (pH 7.0) containing 1 mM cold NiCl₂ to avoid unspecific binding. Two series of experiments were performed and each time point was measured in duplicates. Filters were dried and radioactivity was counted. Controls comprised counting of the filters after incubation in the absence of bacteria or with lysed cells and background values were subtracted. Uptake rates were calculated as cpm of accumulated ⁶³Ni as a function of time.

**Urease activity measurements and acid resistance assays**

Urease activity of whole *H. pylori* cells was assayed by measuring ammonia production using the Ammonia-Assay Kit (Sigma) as described before [77]. *H. pylori* bacteria grown on blood agar plates for 24 hours were inoculated at an OD₆₀₀ 0.05 in BB liquid medium with fetal calf serum (10% v/v) and grown overnight. This preculture was used to inoculate the bacteria at an OD₆₀₀ 0.25 in liquid BB with 10% (v/v) fetal calf serum without added nickel. When an OD₆₀₀ of 0.5 was reached, cells were washed once in phosphate buffer saline (Sigma) and resuspended to the same OD₆₀₀ in BB pH 5 or pH 7 and incubated 1 hour at 37°C under microaerophily with shaking. From this culture, log-phase bacteria (0.5 OD₆₀₀) were harvested, washed once with phosphate-buffered saline and resuspended in 1 ml of buffer (citric acid, 0.1 M; Na₂HPO₄, 0.2 M at pH 5) containing 6 mM urea. The data correspond to at least three independent experiments with two technical replicates each time. Aliquots were taken after 8 min of incubation at 37°C and centrifuged to pellet the bacteria. The NH₃ concentration in the supernatant was measured with the ammonia-assay kit according to the manufacturer’s (SIGMA) instructions. One unit (U) of urease activity was defined as the amount of enzyme that generates 1 μmol ammonia per min per mg of total proteins. For clarity, results were represented in the graphs after normalizing absolute values relative to the wild type strain.

For acid resistance assays, *H. pylori* bacteria were grown as described for urease assays. When an OD₆₀₀ of 0.5 was reached, cells were washed once in phosphate buffer saline (Sigma) and resuspended to the same OD₆₀₀ in BB pH 5 and incubated 1 hour at 37°C under microaerophily with shaking. From this culture, log-phase bacteria (0.5 OD₆₀₀) were harvested and washed once with phosphate-buffered saline and resuspended in phosphate-buffered saline pH 7 (control) or pH 2 (assays). Cells exposed to acidic conditions (pH 2) were supplemented or not with 6 mM urea. After 40 min exposure, an aliquot of 0.2 mL of each bacterial suspension was centrifuged (3 min, 8000 x g), and 10 μL of supernatant was applied onto pH-paper. Ten μL aliquots of standard solutions of known pH were used to produce a control pH scale (pH 2, 4, 5, 7 and 10). Aliquots of 20 μL of each strain was taken in parallel and added into individual wells of a 96-well plate each containing 80 μL of BB medium and 25 μL of Alamar Blue reagent to test for viability (Thermo Scientific) and grown at 37°C under microaerophily until staining developed (typically 1 to 2 hours). The Alamar Blue test uses the cell-permeable resazurin blue molecule and measures the capacity of viable cells to keep an intracellular reducing environment where resazurin is converted to a red resorufin derivative [59].

**Statistical analysis**

The Student’s t test was used to determine significance of the means of the data. The Mann-Whitney test was used for mouse colonization assay to compare colonization loads.

**Genomic analyses**

To determine the distribution of nickel transporter proteins, hydrogenase and urease among *Helicobacter* species, we used MycoHIT as previously described [60,61]. Briefly, we performed
an alignment search with the StandAlone TBLASTN program [78], using the proteins sequences of HydA, UreB, UreI, NixA, NiuB, NiuD, NiuE from H. pylori B128, NikA, NikB, NikCD, NikE from H. hepaticus ATCC51478, NikZ from H. felis ATCC 49179 and OppB-, OppC-, OppD- and OppE-like proteins from H. felis ATCC 49179, genes that are situated downstream of NikZ (operon nikZ-oppBCDE) as the query sequences to search for matches in the genomic DNA of other organisms. To categorically assign that there was no hit, we employed the default E-value (or Expectation value) of e-10 which is the default value provided at NCBI and has been used in a similar study [60,61]. Thus, if the statistical significance ascribed to a comparison is greater than this E value, we assigned a percentage of similarity and identity of 0% to that comparison. As a database, we used the genomes of H. canadensis str. Sheeba (NC_008229.1), H. bilis ATCC 43879 (NZ_KI392032.1), H. hepaticus ATCC 51478 (NZ_KI669458.1), H. etorum MIT 00–7128 (NC_017735.1), H. pylori B128-SΔhpn parental strain and isogenic mutants. The results are presented as % of growth in the presence of nickel relative to growth without nickel after 24h incubation. The data correspond to the mean value of three independent experiments and error bars represent the standard deviation. ** indicates that the mean value is significantly different from that of the wild type strain (P ≤ 0.001).

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S2 Fig. NiuD and NixA control the intracellular nickel content in H. pylori in a wild type and B128-S Δhpn context. Nickel amounts measured by Inductively Coupled Plasma Optical

Phylogenetic analyses

We retrieved sequences of NixA (HPB8_1196), NiuB (HPB8_1657), and NiuD (HPB8_663) from H. pylori B128 that were annotated in the KEGG database (http://www.genome.jp/kegg/). These were used as queries to search for homologs in complete proteomes (non redundant protein database) at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) using the BlastP program [78] (default parameters). For each protein, homologs with amino acid identity >35% were extracted. Protein sequences were aligned with the Muscle program [79] and the resulting multiple alignments were trimmed with the BMGE software [80] with a BLOSUM30 substitution matrix. Maximum likelihood (ML) trees of NixA, NiuB and NiuD were then reconstructed using IQ-TREE [81] and the LG+Γ4 model. The branch robustness of the ML trees was estimated with the nonparametric bootstrap procedure (100 replicates of the original datasets). Trees were drawn and annotated with the FigTree (v1.4.2) program (http://www.webcitation.org/getfile?fileid=27177ee8dd2f34cfd254b9c5ec6c6dfb65329f6).

Supporting Information

S1 Fig. NiuD and NixA mediate H. pylori sensitivity to high nickel concentrations in a B128-S Δhpn context. Effect of 0.5 and 1 mM NiCl₂ on growth of H. pylori B128-S Δhpn parental strain and isogenic mutants. The results are presented as % of growth in the presence of nickel relative to growth without nickel after 24h incubation. The data correspond to the mean value of three independent experiments and error bars represent the standard deviation. ** indicates that the mean value is significantly different from that of the wild type strain (P ≤ 0.001).

(TIF)
Emission Spectrometry (ICP-OES) and expressed as μg of nickel. g⁻¹ of proteins. Strains were grown either without added nickel, with 10 μM or with 100 μM nickel. The data correspond to the mean value of three independent experiments and error bars represent the standard deviation. *** indicates that the mean value is significantly different from that of the wild type strain (\( P \leq 0.001 \)).

(TIF)

S3 Fig. Distribution of NixA, NiuB, NiuD, NiuE, UreI (urea channel) and subunits of urease (UreB), hydrogenase (HydA), NikABCDE and NikZOppBCDE throughout the Helicobacter genus. Protein homologs of each query protein were detected with the MycoHIT program and the table represents the resulting hits with details on sequence identities. The lower panel presents examples of the organization of genes encoding NiuB, NiuD, NiuE, NixA (H. pylori 26695, H. cetorum MIT 00–7128, H. mustelae), NikABCDE (H. hepaticus), NikZOppBCDE (H. felis, H. suis). NixA is present only in gastric species, while niuBDE is found as a putative operon (in H. fennelliae, an EH species), or as an niuDE operon separated from niuB gene(s) (H. pylori, H. cetorum, H. acinonychis, H. mustelae). NikZ, a periplasmic nickel binding protein, is always encoded by a gene lying within a putative 5-genes operon, annotated nikZOppBCDE, where oppBCDE encodes components of an ABC transport system (oppBC encoding permease components and oppDE ATPase subunits). Interestingly, in addition to this ABC transport system, H. felis possesses two nixA genes. The nikZOppBCDE operon is found in all heliimmelani-like species (H. suis, H. heliimmelii, H. bizzozeronii, H. felis and H. ailurogastricus). In H. hepaticus, the nikABCDE operon encoding a nickel-specific ABC transporter is found downstream the ureAB-ureEFGH cluster encoding urease subunits UreA and UreB and accessory proteins necessary for urease maturation (UreE, F, G and H). This nikABCDE operon is found in several EH species.

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S4 Fig. Maximum likelihood (ML) phylogeny of NixA. The tree was inferred with IQ-TREE and the LG+Γ4 model and rooted with sequences of Clostridia. It contains the 86 sequences closest to the NixA of H. pylori. Values at nodes represent statistical confidence based on 100 bootstrap replicates of the original dataset. The scale bar represents the average number of substitutions per site. Prokaryotic lineages are indicated on the right side. Important nodes are colored in red.

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S5 Fig. Maximum likelihood (ML) phylogeny of NiuB (unrooted). The tree was inferred with IQ-TREE and the LG+Γ4 model. Values at nodes represent statistical confidence based on 100 bootstrap replicates of the original dataset. The scale bar represents the average number of substitutions per site. Colors correspond to various prokaryotic lineages. Note that NiuB sequences from epsilon-proteobacteria (blue background) are scattered and that NiuB from gastric Helicobacter are clustered in a single clade with NiuB from C. sputorum and H. fennelliae (all epsilonproteobacteria, light pink background).

(TIF)

S6 Fig. Maximum likelihood (ML) phylogeny of NiuD (unrooted). The tree was inferred with IQ-TREE and the LG+Γ4 model. Values at nodes represent statistical confidence based on 100 bootstrap replicates of the original dataset. The scale bar represents the average number of substitutions per site. Colors correspond to various prokaryotic lineages. NiuD sequences from epsilon-proteobacteria (in blue) are also scattered. NiuB from gastric Helicobacter are, again, clustered in a single clade with NiuB from C. sputorum and H. fennelliae (pink background).

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S7 Fig. Maximum likelihood (ML) phylogeny of NiuB and NiuD (close-in views from S5 and S6). Colors correspond to various prokaryotic lineages that are indicated on the right side. Gastric Helicobacter species are highlighted with a light grey background. For each species, genetic organization of the niuB gene is indicated (associated or separated from niuD and niuE) in the upper tree, and information on the organization of niuD and niuE genes are indicated in the bottom tree. Cartoons codes are indicated at the bottom. Genes associated within a putative operon are highlighted with a light green background.

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S8 Fig. Structural analysis of NiuB. Upper panel–Multiple alignment of NiuB (CeuE) from H. pylori strain G27, NiuB1 and NiuB2 from strain B128. The putative signal peptide of the three proteins (predicted with SignalP) is indicated with their putative cleavage site. Alignments show that NiuB1 is closely related to NiuB (G27), while NiuB2 shows several differences, such as insertions and substitutions. This pattern is representative of the differences between the NiuB/NiuB1 proteins and NiuB2 proteins. Blue boxes indicate residues involved in Ni(II)-\((\text{L-His})_2\) binding and blue stars highlight the two key residues specifically involved in metal binding. Red boxes and red arrows indicate residues close to the binding site that are different between NiuB/NiuB1 and NiuB2. Black boxes emphasize the two residues (Glu110 and Glu236) that have been proposed to be involved in binding onto the NiuD permease. Green arrows show the differences between the two types of proteins. Middle panel–Representation of the 3D structure of NiuB (G27) (PDB code: 4LS3, [56]) with the ligand represented as solid spheres. Residues indicated by green arrows in the upper panel are represented here with green surfaces and show that they all lie on the surface of NiuB. Glu110 and Glu236 residues are conserved in both NiuB1 and NiuB2, indicating that they could bind NiuD similarly.

Lower panel–Close-in view of the binding site of NiuB (G27, [56]). Residues involved in direct nickel binding are indicated with a blue star and several other conserved residues are indicated in black. Residues from the binding site that change between NiuB1/NiuB1 (black) and NiuB2 (green) are indicated. The binding pocket is slightly different between both types of NiuB, with Val104, Ile208 and Asp209 in NiuB/NiuB1 being replaced by Thr106, Ile208 and Ser211 in NiuB2. These differences are putatively accounting for the differences observed in nickel uptake efficiencies between NiuB1 and NiuB2 in our experiments.

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S1 Table. Strains and plasmids used in this study.

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S2 Table. Oligonucleotides used in this study.

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