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Nickel recognition by bacterial importer proteins

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Nickel supports the growth of microbes from a variety of very different growth environments that affect nickel speciation. The mechanisms of nickel uptake and the molecular bases for the selectivity of this process are emerging. The recent surge of Ni-importer protein structures provides an understanding of Ni-recognition in the initial binding step of the import process. This review compares the structural basis for Ni-recognition in the complexes (ABC and ECF-type) that dominate primary (ATP-dependent) transport, with a focus on how the structures suggest mechanisms for Ni selectivity. The structures raise key questions about the mechanisms of nickel-transfer reactions involved in import. There is also a discussion of key experimental approaches necessary to help establish the physiological importance of these structures.

Introduction

Nickel is a critical nutrient for microbes found in a broad range of growth environments. Its various roles as an enzyme cofactor in microbes and plants have been described elsewhere.[1] Human-associated pathogenic and non-pathogenic microbes require Ni for a small number of enzymes, predominantly urease, [NiFe]-hydrogenase, and methyl CoM reductase. Terrestrial and marine microbes can require nickel to metabolize urea in the soil, generate methane, produce H_2 to get rid of excess reducing equivalents from photosynthesis, or to defend against reactive oxygen species. The proteins required for Ni–enzyme assembly and Ni-homeostasis are also well studied,[2] with a particular focus on their metal coordination properties.[3,4] This latter aspect is important for understanding metal selective function, but also bears on mechanisms of metal transfer reactions, an emerging area of research activity for Ni-assembly proteins. Key structural and functional insights have recently been obtained into the first step of cellular nickel recognition, acquisition *via* ATP-driven transmembrane importer proteins. This review summarizes these observations and analyses their significance, with a focus on important questions for future study.

Divalent nickel is present in various environments either as Ni–$(H_2O)_6$ or in complex with various organic and inorganic ligands, the latter of which may be poorly soluble. Ni(II) is stable to oxidation unless coordinated by several thiolate ligands, as seen in various redox active Ni-enzymes. The position of nickel in the Irving–Williams series means that nickel–ligand complexes will be relatively stable. Thus, metal import is potentially a competition between extracellular speciation and protein affinity.

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Ni-importers have been identified in a variety of bacteria by different methods including genetic screens,[5,6] gene neighbourhood analysis,[7] analysis of microarray experiments,[8] and bioinformatics surveys of candidate nickel-regulated operons.[9] Experimental evidence has been provided for ATP-dependent Ni-uptake by two classes of multiprotein complex (primary transport) as well as single transmembrane component co-transport systems that depend on chemical potential (secondary transport). Structural breakthroughs in Ni-recognition have recently come from the ATP-dependent systems and they are the focus of this review.

Ni-recognition by binding proteins of ATP-dependent transporters

ATP-dependent Ni-transport has been demonstrated for two different classes of transporter complex. The well-known ABC-type transporters,[10] which use a soluble periplasmic binding protein for solute delivery, and the more recently identified Energy Coupling Factor (ECF) transporter,[11] which uses a membrane embedded solute binding protein. Both interact, *via* distinct interfaces, with membrane subunits that are coupled to cytoplasmic ATP-hydrolyzing subunits necessary to drive the import cycle. The distinct structures of the binding proteins involved in ABC- *versus* ECF-dependent nickel uptake are accompanied by distinct modes of nickel recognition.

ABC-type transporters

The first bacterial nickel ABC-transporter was identified in *E. coli* in a genetic screen for loss of [NiFe]-hydrogenase-3 activity.[5,12] The *nikABCDE* operon sequence revealed significant homology with the di- and oligopeptide transporter family.[13] The recent

structures of *E. coli* NikA and NikA-like proteins from other bacteria have revealed a common general mechanism for Ni-import by means of recognition of a Ni–(L-His)$_n$ complex.[14]

E. coli NikA

Evidence that NikA recognized a nickel-complex was first provided by a crystal structure of a NikA:Fe–EDTA complex,[15] and a subsequent NikA:Ni(II)–polycarboxylate ligand complex structure,[16] which was not studied for activity in Ni(II) import. The implied hypothesis that *E. coli* synthesizes a secreted ligand for nickel-complex formation and subsequent binding by NikA, analogous to iron uptake strategies, has never been tested.

Experiments using wild-type and mutant *E. coli* strains showed that exogenous L-histidine was the best candidate for the nickel-complexing ligand.[17] No other molecule used, including polycarboxylic acids, provided even a modest fraction of the Ni(II)-uptake rate observed with L-His. The concentration dependence of Ni-uptake as well as binding to purified NikA both supported a ternary complex with a stoichiometry of Ni:2L-His:NikA. D-Histidine was not a substrate for transport, and was in fact inhibitory. L-Histidine is present in the intestine in amounts consistent with its role in *E. coli* nickel-uptake.[18]

A structure of the Ni–(L-His)$_2$:NikA complex followed shortly thereafter.[19] In addition to confirming the experimentally determined stoichiometry, several interesting features of Ni-recognition were revealed. First, the Ni(II) atom remains almost fully coordinated to the two L-His molecules (Fig. 1a and Table 1), maintaining five of six contacts. NikA His$_{416}$, previously shown to be important for Ni-uptake,[20] displaces a COO^- ligand to complete the six-coordinate complex. The structure also accounts for the unexpectedly high conservation of Arg residues in a presumptive cation binding pocket, as they interact with the $-COO^-$ groups of the L-His molecules (Fig. 1a). The Arg residues also enforce a third interesting feature of the structure – a previously unobserved stereoisomer configuration of the Ni–(L-His)$_2$ complex (Fig. 1a and Table 1). By itself, Ni(II)–(L-His)$_2$ crystallizes as the *trans*-imidazole configuration.[21] However, when the similar Co(II)–(His)$_2$ was oxidized to the exchange inert Co(III) complex, three isomers were isolated (*trans*-COO^-, *trans*-NH_2, and *trans*-imidazole) in a non-equivalent ratio, albeit strongly favouring *trans*-imidazole.[22–24] The Ni–(L-His)$_2$:NikA structure stereoisomer configuration most closely resembles the *trans*-COO^- species, less the displaced $-COO^-$ ligand. The functional significance of the recognition of this isomer is not known, nor is it known if the protein initially binds the *trans*-imidazole isomer, which then undergoes His$_{416}$-dependent ligand rearrangement to the observed arrangement. Further changes in complex stereochemistry may occur upon transfer from NikA to the NikBC transmembrane subunits.

A His$_{416}$–Ni(II) interaction is present in a non-cognate Ni-complex bound to NikA.[16] The significance of the His$_{416}$ interaction in the discrimination of Ni(II) *versus* other metals for transport has not been studied. Both Cu(II)–(His)$_2$ and Zn(II)–(His)$_2$ display lower number coordination numbers,[25,26] and are consequently unable to satisfy the observed interactions between *Ec*NikA residues and the L-His molecules. Co is the only divalent metal tested that shows any inhibition of Ni-accumulation,[17] but, as predicted by the Irving–Williams series, the weaker association with L-His[27] means that higher concentrations of Co(II) will be required to attain Co(II)–(L-His)$_2$ concentrations required for binding. Thus, selectivity of Ni(II) over Co(II) in this instance is due, at least in part, to the relative abundance of the metal–(L-His)$_2$ complexes for binding to NikA.

Y. pestis YntA

The YntABCDE (*Yersinia* nickel transporter) ABC transporter was identified by gene neighbourhood analysis and the subsequent demonstration of reduced Ni-uptake and loss of urease activity in a deletion mutant.[28] YntA is distinct from *E. coli* NikA in the sequence and position of residues in the binding pocket,[19] and has been previously denoted as NikA2.[9] Consequently, the orientation of the complex in the binding pocket and the protein–complex interactions are quite clearly different. Here, YntA His$_{482}$ displaces an L-His $-NH_2$ ligand (Fig. 1b), although the same *trans*-COO^- stereoisomer configuration is seen. Only one Arg interaction is observed, however, likely because the two COO^- groups are coordinating the Ni(II) and so charge balance is maintained in the binding pocket.

S. aureus NikA

An unpublished structure of *S. aureus* NikA with Ni–(L-His)$_2$ bound (Fig. 1e and Table 1) was initially overlooked in both the *E. coli* NikA experimental and structure work described above.[17,19] This transporter, initially annotated as Opp5A, has been shown to be important in *S. aureus*-linked urinary tract infections.[29] The Opp5A structure can be distinguished from all the others described here because there is no His residue in the protein binding pocket to displace an L-His ligand atom. The stereoisomer configuration of the Ni–(L-His)$_2$ complex is *trans*-NH_2.

C. jejuni NikZ

The NikZYXWV transporter is required for hydrogenase activity in the foodborne pathogen *Campylobacter jejuni*.[30] *C. jejuni* NikZ closely resembles YntA except that two additional protein His residues (His$_{26}$ and His$_{481}$) are present in the binding pocket (Fig. 1d, compare with Fig. 1b). This eliminates the need for one of the L-His ligands. The Arg–His–COO^- interaction seen in YntA is preserved. The structure suggests that either NikZ binding by Ni–(L-His)$_2$ displaces one His molecule, or Ni–(L-His) is relatively abundant in the *C. jejuni* growth niche when hydrogenase activity is required.

A NikZ structure with a different bound Ni-complex supports an exogenous ligand-requirement for Ni-binding to NikZ (Fig. 1f). The ligand is suggested to be oxalate but its absence in the purification and crystallization buffers raises questions as to its identity. There is little to structurally distinguish this complex from the Ni–L-His complex.

H. pylori CeuE

CeuE belongs to a different class of solute binding protein (Group II) compared to NikA. They are well known to bind metal-complexes, but are roughly half the size of the NikA-type proteins.[31]

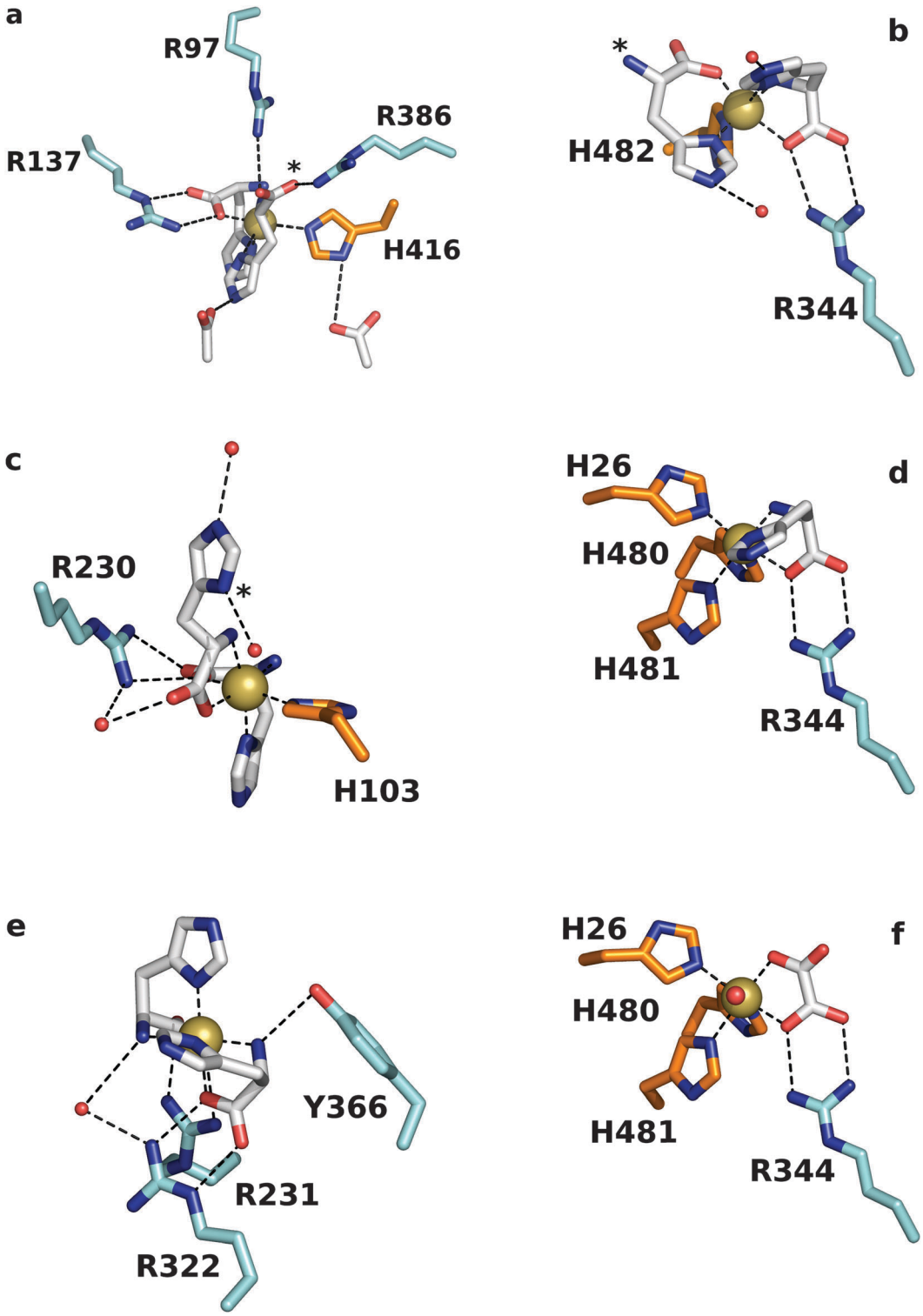

Fig. 1 Active site features of Ni–(L-His)$_n$:NikA ortholog complex structures. Structures were aligned in PyMOL, using EcNikA as the template. (a) EcNikA; (b) YpYntA; (c) HpCeuE; (d) CjNikZ; (e) SaNikA(Opp5A); (f) CjNikZ; CjNikZ+"oxalate". *, indicates the displaced L-His (gray) liganding atom in structures where this is observed. Protein His residues are coloured orange, and Arg residues coloured cyan. Small red spheres are waters. Two acetate molecules are present in the EcNikA site. Only key contacts described in the text are shown here, and described in Table 1. Additional analysis of the structures can be found in the original papers.

Deletion of *ceuE* in *H. mustelae* results in reduced Ni accumulation.[32] *ceuE* (HPG27_1499) transcription is repressed by nickel and NikR and activated by iron (this issue).[33] Nonetheless, the CeuE structure reveals a Ni–(L-His)$_2$ complex bound in a manner similar to the other structures described here (Fig. 1d).[34] In this case, CeuE His$_{103}$ displaces one His imidazole Nδ. The L-His ligand

Table 1 Coordination of features of Ni–(L-His)$_n$:NikA homolog protein structures

Protein	Ni–(L-His)$_2$ stereoisomer	Ni(II)–protein contact	L-His atom displaced	L-His interactions	PDBID (res., Å)
*Ec*NikA	*trans*-COO^-	His_{416}	COO^-	COO^-:Arg_{97}; Arg_{137}; Arg_{386} Imid:Trp_{398}; acetate	4I8C (2.50)
*Hp*CeuE	All *cis*	His_{103}	Nδ	COO^-:Arg_{230}; H_2O Imid:Phe_{123}, Trp_{249}; $2H_2O$	4LS3 (1.70)
*Sa*NikA (Opp5A)	*trans*-NH_2	None	None	COO^-:Arg_{231}; Arg_{322}; NH_2:Tyr_{366}; H_2O	3RQT (1.50)
*Cj*NikZ	None	His_{26}; His_{480}; His_{481}	None	COO^-:Arg_{344}	4OEU (2.20)
*Yp*YntA	*trans*-COO^-	His_{482}	NH_2	COO^-:Arg_{344} Imid:Trp_{369}; $2H_2O$	4OFL (2.70)

atoms are in the all *cis* configuration, and the bound complex is virtually identical in structure to the five-coordinate free Cu(II)–(L-His)$_2$ complex.[26] The completion of the coordination sphere by His_{103} exemplifies the manner in which a protein His residue could be used to discriminate metals (divalent Ni *versus* divalent Cu) based on coordination number, if not stereoisomer preference. A single Arg residue interacts with both COO^- groups, which are proximal in the all *cis* stereoisomer.

Comparison of the five structures containing a bound Ni–(L-His)$_n$ complex reveals clear similarities. In four structures, a protein His residue is part of the primary coordination sphere. One or more Arg residues interact with L-His COO^- groups. These interactions serve to select against D-His, for example, and also specify a single stereoisomer configuration in the Ni–(L-His)$_2$ complexes. Whether this selects for a particular nickel complex or against other divalent transition metal–ligand complexes is not known. Such non-cognate complexes would likely exist in the complex growth environments of the organisms in which the proteins are found, but are likely to vary in different growth environments.

The requirement for L-His in Ni-uptake must still be demonstrated for YntA, NikZ, SaNikA, and CeuE. In the ideal case, growth media can be manipulated to control for the presence or absence of transporter expression and any available exogenous ligands. This is easily done for *Yersinia* species, which grow readily in a minimal medium in which the L-His concentration can be varied, as has been done for *E. coli*.[17] Bacteria such as *H. pylori*, for which defined growth medium studies have shown an L-His requirement,[35] present a significant challenge. Further, the use of undefined medium may result in underestimation of the efficiency of Ni transport because of the presence of nickel complexes that are not recognized by the transporter at all, or bind but inhibit transport. It is likely that heterologous expression in a host, such as *E. coli*, will be useful in aiding studies to quantitatively determine the L-His requirements.

Similarly, titration studies using purified NikA and homologs with a range of independently varied nickel and histidine concentrations will provide information about binding stoichiometry and affinity. Currently, studies have been done using different assay methods and buffer conditions,[14,17] so the reported affinities are not comparable. The choice of assay conditions may also prevent observation of binding of lower stoichiometry complexes, such as observed for NikZ.

B. ECF type transporters

This class of transporter was discovered relatively recently, partly through identification of candidate genes regulated by the nickel-responsive NikR repressor,[9] and are found in a variety of microbes from disparate growth environments (*e.g.*, *Streptococcus salivarius* in the oral cavity).[7] The initial solute-binding step involves a membrane embedded protein that contrasts with the largely soluble or membrane-tethered solute binding proteins of the ABC transporter. The proposed mechanism involves tipping of the binding protein after solute binding to transfer the ligand from the outer to the inner face of the membrane.

T. tengcongensis NikM2

The recently reported NikM2–Ni(II) protein structure[36] uses a well-known four-coordinate planar N-terminal nickel-coordination motif (Fig. 2).[37] Although, like the NikA proteins, NikM2 is homologous to transporters that bind vitamins, no exogenous ligand is necessary. However, the NikM2 binding pocket is filled with the N-terminal Ni-binding motif, here acting in *cis*, to allow binding in the ordinarily larger pocket. Other Ni(II)-binding proteins that use an N-terminal Xaa_1–His_2 coordination motif include NmtR,[38] RcnR,[39] and HypA,[40] all of which are six-coordinate. Currently, NikM2 the motif is the first Ni-binding protein with an NH_2–Met_1–His_2 N-terminal sequence, rather than a Met_1–Xaa_2–His_3 sequence that must be processed to Xaa_1–His_2 by methionine aminopeptidase.

Mechanistic considerations of Ni-binding site features

Nickel recognition by different transporters most likely reflects its speciation in the immediate environment of the binding protein. The coordination features will also affect the mechanisms of metal association and dissociation during transport. For ABC transporters in pathogenic bacteria the host environment is generally nutrient rich, with little competition for Ni with the host. For the ECF transporter, the physical location of the binding protein within the membrane suggests that nickel-binding could occur *via* direct exchange with Ni(II) ions that are associated with anionic lipid headgroups.

ABC transporters utilize a cycle of nucleotide binding, hydrolysis, and dissociation to drive association of the solute binding protein, *e.g.* NikA, and subsequent dissociation of the solute into the transmembrane channel. For the NikA homologs, the entire complex is most likely imported. The kinetics of transfer between the solute binding protein and the transmembrane channel will be influenced by the exchange of the protein–His ligand, *e.g.*, NikA His_{416}, with the displaced free

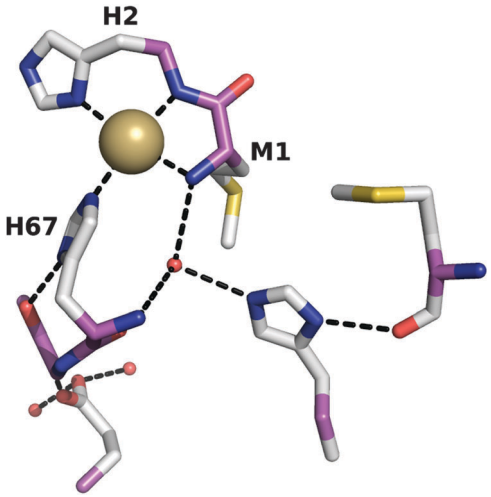

Fig. 2 Ni-coordination features of *T. tengcongensis* NikM2. Main chain carbons are coloured purple for emphasis. Residues in the hydrogen bond network stabilizing His_{67} are not labelled.

L-His ligand and by any rearrangements of the stereoisomer configuration of the L-His complex. Additional changes in the primary coordination sphere ligand composition may occur during transit through the transmembrane channel and could serve to facilitate the process and select against metals other than Ni(II).

The four-coordinate planar Ni(II) complex of NikM2 will require an external driving force for metal transfer, such as an associative ligand exchange reaction. Alternatively, the interaction of NikM2 with the transmembrane subunit of the transporter may cause perturbations to the H-bond network that stabilizes His_{67} in the primary coordination environment (Fig. 2).

Ligands other than L-His may be important for Ni-uptake in some microbial species. Nickel is known to form complexes with variety of organic ligands in plants, including citrate, as a means of detoxification.[41] In marine environments, metal availability can vary with the depth of the water column, thus providing different challenges for metal acquisition.[42] There is no evidence that bacteria or Archaea synthesize and excrete a nickelophore. Bioinformatics surveys of genes under Ni-responsive transcriptional regulatory control have not identified a gene or operon that correlates with a potential function in nickelophore synthesis,[9,43] although regulation of nickelophore synthesis by another physiological signal (*e.g.*, pH) cannot formally be ruled out. Further, siderophores are synthesized and excreted for the purpose of scavenging iron because of tight binding by mammalian host proteins, the insolubility of ferric iron, and competition with other microbes. Similar competition for Ni in humans is less likely, as the only defined nickel requirement is in human-associated bacteria and divalent nickel is stable to redox chemistry under ambient conditions. As importantly, the position of Ni(II) in the Irving–Williams series means that it will more readily bind organic ligands in the various nutrient-rich growth habitats in the human body. Thus, the greater challenge for Ni(II) acquisition by human-associated bacteria is likely not availability but speciation.

There are similarities in microbial metal uptake between the mechanisms of Ni-recognition and those for ferric ions. *E. coli* and other bacteria possess a citrate-dependent ferric iron ABC transporter (FecBCDE) and an outer membrane protein FecA, for which a structure of the complex has been determined.[46] Soluble Fe(III) transport can occur *via* ATP-dependent transport, and binding to FbpA requires an anion partner (phosphate), which is readily available.[44,45] Whether Ni import by some microbes uses similar complexes remains to be determined. Nickel transporters have been identified experimentally and *via* known mechanisms of nickel-dependent transcriptional regulation. Thus, little studied or hard-to-culture microbial species lacking known nickel-responsive regulators and known transporter homologs, potentially misannotated as iron importers, could be a source of new targets for study and subsequent expansion of the Ni-complexes identified as important for Ni-dependent growth.

Human associated gram-negative pathogens (*H. pylori*, *Yersiniae*, *Salmonella*) have both a NikABCDE-type transporter and a NixA-type secondary transporter.[43] The basis for this dual requirement is not clear. It may reflect changes in nickel speciation experienced by the pathogen traversing its route of infection. The activity and expression of the transporters may be different under different conditions. For example, pathogenic bacteria often face an acidic pH challenge that may reduce Ni-complex formation or influence the energetic coupling of transport. Further physiological and genetic studies will be help to inform how the two types of transporter contribute to survival.

Conclusions

The structures described here provide a host of exciting directions for future study to understand the detailed mechanistic basis of Ni import, mechanisms of metal selectivity,[47] and the biological roles of the transporters. These areas have been intensively investigated for microbial cytosolic proteins, but the interface between the membrane and the growth environment is chemically distinct and so mechanistic differences are likely to be present.

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