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# Comparative and Functional Genomic Analysis of Prokaryotic Nickel and Cobalt Uptake Transporters: Evidence for a Novel Group of ATP-Binding Cassette Transporters†

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**The transition metals nickel and cobalt, essential components of many enzymes, are taken up by specific transport systems of several different types. We integrated in silico and in vivo methods for the analysis of various protein families containing both nickel and cobalt transport systems in prokaryotes. For functional annotation of genes, we used two comparative genomic approaches: identification of regulatory signals and analysis of the genomic positions of genes encoding candidate nickel/cobalt transporters. The nickel-responsive repressor NikR regulates many nickel uptake systems, though the NikR-binding signal is divergent in various taxonomic groups of bacteria and archaea. $B_{12}$ riboswitches regulate most of the candidate cobalt transporters in bacteria. The nickel/cobalt transporter genes are often colocalized with genes for nickel-dependent or coenzyme $B_{12}$ biosynthesis enzymes. Nickel/cobalt transporters of different families, including the previously known NiCoT, UreH, and HupE/UreJ families of secondary systems and the NikABCDE ABC-type transporters, showed a mosaic distribution in prokaryotic genomes. In silico analyses identified CbiMNQO and NikMNQO as the most widespread groups of microbial transporters for cobalt and nickel ions. These unusual uptake systems contain an ABC protein (CbiO or NikO) but lack an extracytoplasmic solute-binding protein. Experimental analysis confirmed metal transport activity for three members of this family and demonstrated significant activity for a basic module (CbiMN) of the *Salmonella enterica* serovar Typhimurium transporter.**

The transition metals nickel and cobalt are essential cofactors for a number of prokaryotic enzymes involved in a variety of metabolic processes (36, 41). Among the known nickel-dependent enzymes are urease (8), [NiFe] hydrogenase, carbon monoxide dehydrogenase (Ni-CODH) (35), acetyl-coenzyme A decarbonylase/synthase (21), superoxide dismutase SodN (22), methyl-coenzyme M reductase (20), and glyoxylase I (50). In contrast to the diverse roles of nickel in microbial metabolism, cobalt is mainly found in the corrin ring of coenzyme $B_{12}$, a cofactor involved in methyl group transfer and in rearrangement reactions (36). Since in natural environments, soluble $Ni^{2+}$ and $Co^{2+}$ are usually present only in trace amounts, the synthesis of the respective metalloenzymes requires high-affinity uptake of metal ions. Until recently, two major types of microbial high-affinity nickel and cobalt transporters were known: ATP-binding cassette (ABC) systems and secondary permeases of the NiCoT family (reviewed in reference 24).

The NikABCDE system of *Escherichia coli* belongs to the nickel/peptide/opine ABC transporter family and is composed of the periplasmic binding protein NikA, two integral membrane components (NikB and -C), and two ATPases (NikD and -E) (43). The molecular basis of selective high-affinity binding of $Ni^{2+}$ remains elusive, although crystal structures of *E. coli* NikA have been determined by two approaches (10, 31). The two studies uncovered that NikA does not coordinate $Ni^{2+}$ directly but argue for the requirement of a metallophore. Though distantly related ABC transporter systems from pathogenic *Yersinia pseudotuberculosis* and *Brucella suis* are also implicated in the high-affinity nickel uptake (33, 49), many other representatives of this ABC transporter family are involved in uptake of other compounds, i.e., dipeptides and oligopeptides (1, 37).

Nickel/cobalt permeases of the NiCoT family are widely distributed in bacteria and are also present in some archaea and fungi. The substrate preferences of many representatives have been analyzed in detail (17, 18, 30). The NiCoT family includes at least one nickel-specific permease and many proteins with mixed metal ion specificities that have a preference for either nickel or cobalt ions.

Two other families of putative secondary metal transporters, HupE/UreJ and UreH, are distantly related to NiCoTs, and certain members of these families have recently been shown to mediate nickel transport (24). HupE/UreJ proteins are widespread among bacteria and often encoded within [NiFe] hydrogenase (HupE) and urease (UreJ) gene clusters. Subgroups of UreH proteins are found in marine cyanobacteria and in plants. The cyanobacterial variants are encoded adjacent to [Ni] superoxide dismutase genes predicting a role in nickel uptake.

Several hypothetical transporters (CbiMNQO) with some similarity to ABC transporters have been annotated as cobalt uptake systems based on their genomic colocalization with $B_{12}$ biosynthesis genes or on the presence of the regulatory $B_{12}$ elements in their upstream regions (46, 47). In two studies, *cbiM*, *cbiQ*, and *cbiO* genes were identified adjacent to bacte-

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† Supplemental material for this article may be found at http://jb.asm.org/.

rial urease genes (7, 9) and were shown to be important for the urease activity in cells grown under nickel limitation.

Despite the importance of $Ni^{2+}$and $Co^{2+}$ for bacterial metabolism, their uptake must be tightly regulated to avoid toxic effects. In *E. coli*, nickel overload is avoided via the repressor NikR, which binds to the promoter region of the *nikABCDE* operon when nickel is present (11, 19). NikR has both strong (in the pM range) and weak (nM) Ni-binding sites, allowing sensing of nickel at concentrations corresponding to the range from 1 to 100 molecules per cell (6, 12). Recently determined crystal structures of NikR from *E. coli* and *Pyrococcus horikoshii* reveal a plausible mechanism of the Ni-dependent promoter recognition (13, 48). Known cobalt-transporting NiCoTs are controlled on the level of translation initiation by $B_{12}$ riboswitch elements (46). These RNA regulatory elements encoded in the leader regions of bacterial $B_{12}$ biosynthesis genes are able to selectively bind coenzyme $B_{12}$ and repress expression of target genes (42).

The mechanism of nickel and cobalt uptake in many bacteria and most archaea is not known, although, for instance in methanogenes, Ni- and Co-containing enzymes are essential for energy metabolism and anabolism. Comparative analysis of binding sites for transcriptional regulators is a powerful approach to the gene annotation. Here, we analyzed prokaryotic genomes for the presence of candidate NikR-binding sites and $B_{12}$ riboswitches. We combined these data with additional comparative genomics techniques to gain comprehensive insight into the mechanism of nickel and cobalt uptake. This analysis demonstrated that variants of the CbiMNQO-type transporters are the most widespread uptake system for the two metals. We propose the designations "Cbi" and "Nik" for systems related to cobalt and nickel homeostasis, respectively. Heterologous expression of the respective gene cassettes from *Salmonella enterica* serovar Typhimurium and *Rhodobacter capsulatus* in *E. coli* and metal accumulation assays confirmed the substrate preferences of these transporters, as initially predicted by genomic analyses. The CbiMNQO systems of both organisms are transporters that have a strong preference for cobalt ions, whereas the Nik(MN)QO system of *R. capsulatus*, in which the M and N components are fused and form a single protein, is a high-affinity nickel transporter.

## MATERIALS AND METHODS

**Identification of nickel and cobalt transporter genes and their genomic locations.** Complete and partial bacterial genomes were downloaded from GenBank (5), the Institute for Genomic Research (http://www.tigr.org/), and the DOE Joint Genome Institute (http://jgi.doe.gov/). Homologs of previously described nickel and cobalt transporters (see the introduction) were identified in microbial genomes using the Smith-Waterman algorithm implemented in the GenomeExplorer software (40). Orthologous proteins were defined as bidirectional best hits (51). Distant homologs were identified using PSI-BLAST and confirmed by construction of phylogenetic trees (2). Furthermore, we analyzed positional gene clustering of identified candidate nickel/cobalt transporters with known Ni- and Co-containing enzymes using the SEED tool for comparative analysis and annotation of multiple microbial genomes (44) (http://theseed.uchicago.edu/FIG/index.cgi; see the "Transport of Nickel and Cobalt" subsystem). To do that, we first identified homologs of known Ni-dependent enzymes in microbial genomes and also noted the presence or absence of the $B_{12}$ biosynthesis pathways. The obtained distribution of nickel/cobalt transporters and Ni/Co-dependent enzymes in microbial genomes and their positional clusters are described in Table S1 in the supplemental material. Genes encoding proteins from the HupE/UreJ family were called *ureJ* if they were located within urease gene clusters (also indicated by "*U*" in parentheses) and *hupE* otherwise. Likewise, for genes encoding members of the UreH/SodT family, *ureH* was the default name and *sodT* was used only for those genes that clustered with *sodN* (encoding [Ni] superoxide dismutase). Finally, the CbiMNQO-related genes were named using either the *cbi* or *nik* prefix, depending on the predicted specificity. Candidate nickel- and $B_{12}$-responsive regulatory elements are also described in Table S1 in the supplemental material (see below).

**Identification of NikR-binding sites and $B_{12}$ regulatory elements.** For identification of candidate NikR-binding sites, we started from sets of upstream regions of potentially coregulated genes that are homologs of known nickel transporters. A 28-bp inverted repeat, GTATGA-(16 bp)-TCATAC, is known to serve as the NikR-binding site upstream of the *nikA* gene in *E. coli* (11). An iterative signal detection procedure implemented in the program SIGNALX (27) was used for construction of common NikR-binding signals in sets of upstream gene fragments. Each NikR-encoding genome was scanned with the constructed profile using the GenomeExplorer software, and additional genes with candidate regulatory sites in the upstream regions were selected. Sequence logos for derived regulatory signals were drawn using the WebLogo package v. 2.6 (15) (http://weblogo.berkeley.edu/). For group I NikRs, we identified similar sites upstream of orthologous *nikA* genes in eight proteobacterial genomes. A recognition profile was constructed based on these eight NikR-binding sites. The profile is highly selective: there are only one to three candidate sites with a score of >5.00 per genome. The procedure for identification of the signal for group II NikRs in δ-proteobacteria was described previously (45). A recognition profile of group III NikRs in ε-proteobacteria was constructed using the sites from upstream regions of putative nickel transporters from *Wolinella succinogenes* and two *Helicobacter* species. It was less selective than other NikR profiles, since it selected up to 10 other candidate sites with comparable scores per genome. However, this analysis is supported by an experiment in which one NikR-binding site has been shown to be involved in the negative regulation of the divergently transcribed *nikR* and *exbBD-tonB* genes in *Helicobacter pylori* 26695 (14). To identify a common signal of group IV NikRs, we collected the upstream regions of the *nikMNQO* operons from *Pyrococcus furiousus*, *Thermococcus kodakarensis*, and *Thermoanaerobacter tengcongensis*. To detect the candidate NikR signal in methanogenic archaea (group VI), we collected upstream regions of the *nikMNQO*, *nikABCDE*, and *nikR* operons and used the same procedure. In some archaeal genomes, we failed to identify candidate NikR-binding sites. Surprisingly, two other *Pyrococcus* species (*P. abyssi* and *P. horikoshii*) lack homologs of known nickel transporters, and the search for similar NikR sites could not identify candidate NikR targets in their genomes. Though the structure of *Pyrococcus horikoshii* NikR protein has been determined (13), its in vivo target genes are not known.

The RNA-PATTERN program and the *B12*-element RNA pattern were used to search for additional $B_{12}$ riboswitch regulatory elements in bacterial genomes as previously described (46).

**Other bioinformatics programs.** The phylogenetic trees were constructed by the maximum likelihood method implemented in PHYLIP (26) using multiple sequence alignments of protein sequences produced by CLUSTALX (52). Gene identifiers from GenBank are used throughout in the phylogenetic trees. The SIGNALP 3.0 (www.cbs.dtu.dk/services/SignalP/) and TMPRED (www.ch.embnet.org/software/TMPRED_form.html) servers were used to predict signal peptides and transmembrane helices, respectively (4).

**Plasmid constructions.** The *cbi* and *nik* genes were amplified by PCR using genomic DNA from *Salmonella enterica* serovar Typhimurium lysotype 2 strain SGSC1412 (kindly provided by Steffen Porwollik, Sidney Kimmel Cancer Center, San Diego, CA) and *Rhodobacter capsulatus* strain SB1003 (kindly provided by Gabriele Klug, Universität Gießen, Gießen, Germany) as the templates, Platinum *Pfx* DNA polymerase (Invitrogen), and a "touchdown" protocol. For construction of plasmid pSt*cbiMNQO* (containing *cbiMNQO* of *S. enterica* serovar Typhimurium under control of a *lac* promoter and a ribosomal binding site), primers were used that introduced a PciI site at the 5′ end of *cbiM* and a BamHI site at the 3′ end of *cbiO*. The amplicon was treated with PciI and BamHI, purified, inserted between the NcoI and BglII sites of plasmid pCH675-RP (30), and introduced into *E. coli* XL1-Blue. Deletions within the cloned St*cbiMNQO* operon were constructed by inverse PCR using pSt*cbiMNQO* as the template, reverse primers that inserted a BglII site immediately downstream of the last codon of *cbiQ*, *cbiN*, and *cbiM*, respectively, and a forward primer that generated a BglII site and was directed to the sequence adjacent to the 3′ end of *cbiO* containing the stop codon. The products were digested with BglII, religated to give plasmids pSt*cbiMNQ*, pSt*cbiMN*, and pSt*cbiM*, and transformed into *E. coli* XL1-Blue. Deletions were verified by PCR techniques. pRc*cbiMNQO* was constructed in two steps. First, the gene cluster was amplified with primers introducing NcoI and BglII sites to the 5′ and 3′ ends, respectively, and the NcoI/BglII-treated amplicon was introduced into pCH675-RP to give pRc*cbiMNQO*

TABLE 1. Number of nickel and cobalt transporters predicted from genomic localization and regulatory characteristics

| Characteristic (total) | No. of transporters with characteristic | | | | |
|---|---|---|---|---|---|
| | CbiMNQO/NikMNQO | NikABCDE | NiCoT | HupE/UreJ | UreH/SodT |
| Predicted nickel transporters (96) | 23 | 25 | 16 | 23 | 9 |
| Regulation by Ni repressor NikR (42) | 16 | 20 | 7 | 0 | 0 |
| Genomic linkage with Ni-dependent enzymes | | | | | |
| Total (57) | 9 | 6 | 10 | 23 | 9 |
| Urease (30) | 5 | 5 | 4 | 12 | 4 |
| Ni-hydrogenase (19) | 3 | 1 | 6 | 10 | 1 |
| Ni-CODH (2) | 1 | 0 | 0 | 1 | 0 |
| Ni-superoxide dismutase (4) | 0 | 0 | 0 | 0 | 4 |
| Predicted cobalt transporters (44) | 28 | 4 | 8 | 6 | 0 |
| Regulation by $B_{12}$ riboswitch (40) | 22 | 4 | 8 | 6 | 0 |
| Genomic linkage with $B_{12}$ biosynthesis genes (32) | 24 | 0 | 8 | 0 | 0 |

($M_{His2Asp}$). The His-to-Asp exchange arose from the generation of the NcoI site. Since position 2 in all mature CbiM and NikM proteins is occupied by a His residue (see Fig. S1a in the supplemental material), the Asp codon was replaced by a His codon in the second step. For this purpose, a PCR product was generated with a forward primer that introduced a HindIII site at the 5′ end, overlapped the start codon of *cbiM*, and replaced the GAT (Asp) codon with a CAT (His) codon. The amplicon was treated with HindIII and BglII and used to replace the respective fragment of pR*cbiMNQO*($M_{His2Asp}$) to give pR*cbiMNQO*. The exchange was verified by restriction analysis with NsiI because the G-to-C nucleotide replacement resulted in a recognition site (ATGCAT) for this enzyme. Likewise, pR*nik*(*MN*)*QO*—the parentheses indicate that *M* and *N* are fused to a single open reading frame in the *R. capsulatus nik*(*MN*)*QO* cluster—was constructed in two steps. First, *nik*(*MN*)*QO* was amplified with primers that generated AflIII and BamHI sites at the 5′ and 3′ ends, respectively, and the digested product was inserted between the NcoI and BglII sites of pCH675-RP to give pR*nik*(*MN*)*QO*($M_{His2Tyr}$). pR*nik*(*MN*)*QO* with a His-2 codon was generated after a second round of PCR.

**Metal uptake assays.** $^{63}Ni^{2+}$ and $^{57}Co^{2+}$ uptake of recombinant *E. coli* XL1-Blue strains was analyzed as previously described (17, 18, 25, 30, 54). Cells were grown in LB medium supplemented with the radiolabeled ions at concentrations between 100 and 500 nM, ampicillin (100 μg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM). Cells were harvested in the early stationary phase, washed in 50 mM Tris-hydrochloride, pH 7.5, and concentrated. Radioactivity of aliquots was quantitated in a Canberra-Packard Tri-Carb 2900 TR liquid scintillation counter. Metal accumulation is expressed as pmol × (mg protein)$^{-1}$. Each data point in Fig. 5 and bar in Fig. 6 represents the mean value of double assays using independent cultures grown in the same lot of medium.

## RESULTS

**Genomic analysis of nickel/cobalt transporters in microbial genomes.** Initially, orthologs of known nickel and cobalt transporter genes in available prokaryotic genomes were identified by similarity search (see Table S1 in the supplemental material; also see the supplemental material for genomic identification numbers). We searched the genomic databases for the presence of secondary transporters of the NiCoT, HupE/UreJ, and UreH families and the ABC transport systems of the NikABCDE and CbiMNQO families. All five families of the nickel/cobalt transporters showed a mosaic distribution along bacterial lineages (see Table S1 in the supplemental material). Many bacterial species possess transporters from only one family, and few species have a redundant set of nickel/cobalt transporters from different families. For example, the *Salmonella enterica* serovar Typhimurium genome contains *cbiMNQO*, *nikABCDE*, and the gene for an NiCoT, whereas *Rhodopseudomonas palustris* has *hupE*, *ureH*, and an NiCoT gene. Overall, we have not found any candidate nickel/cobalt transporter gene in approximately one-third of 200 analyzed microbial genomes.

To predict the substrate preferences of identified nickel/cobalt transporters, we analyzed the genomic localization of their genes. In Table S1 in the supplemental material, we marked in parentheses the cases when a nickel/cobalt transporter gene is located adjacent to genes encoding Ni-dependent enzymes or enzymes involved in the coenzyme $B_{12}$ biosynthesis. We observed 89 cases of such genomic linkages (Table 1). At that, 32 transport systems are encoded by genes associated with $B_{12}$ biosynthesis gene clusters, allowing one to annotate them tentatively as cobalt transporters. Genes for 57 candidate nickel transport systems are located adjacent to genes encoding Ni-dependent enzymes. In most of these cases, the predicted transporter genes form clusters with either urease or [NiFe] hydrogenase genes.

**NikR regulons in bacteria and archaea.** Orthologs of the *E. coli* repressor NikR were found in the genomes of 23 proteobacteria and 14 archaea (see Table S1 in the supplemental material). Genomes of methanogenic archaea contain up to four copies of *nikR* and multiple copies of candidate nickel transporters, suggesting that the maintenance of Ni homeostasis is of great importance for these organisms. The phylogenetic tree of the NikR proteins has several major branches (Fig. 1): two large groups including proteins of most proteobacteria and archaea; three smaller groups, consisting of the NikR factors of ε- and δ-proteobacteria and *Thermococcales*; and a diverged branch of pseudo-NikR factors from some proteobacteria. The *Thermococcales* branch includes the NikR proteins from hyperthermophilic archaea (*Pyrococcus* spp. and *Thermococcus kodakarensis*), and a single extremely thermophilic bacterium, *Thermoanaerobacter tengcongensis* (Fig. 1). In order to identify nickel-regulated transporters, we analyzed the NikR-binding signal in all of these groups separately (see Materials and Methods for details). Candidate NikR-binding sites identified in the analyzed microbial genomes are listed in Table S2 in the supplemental material.

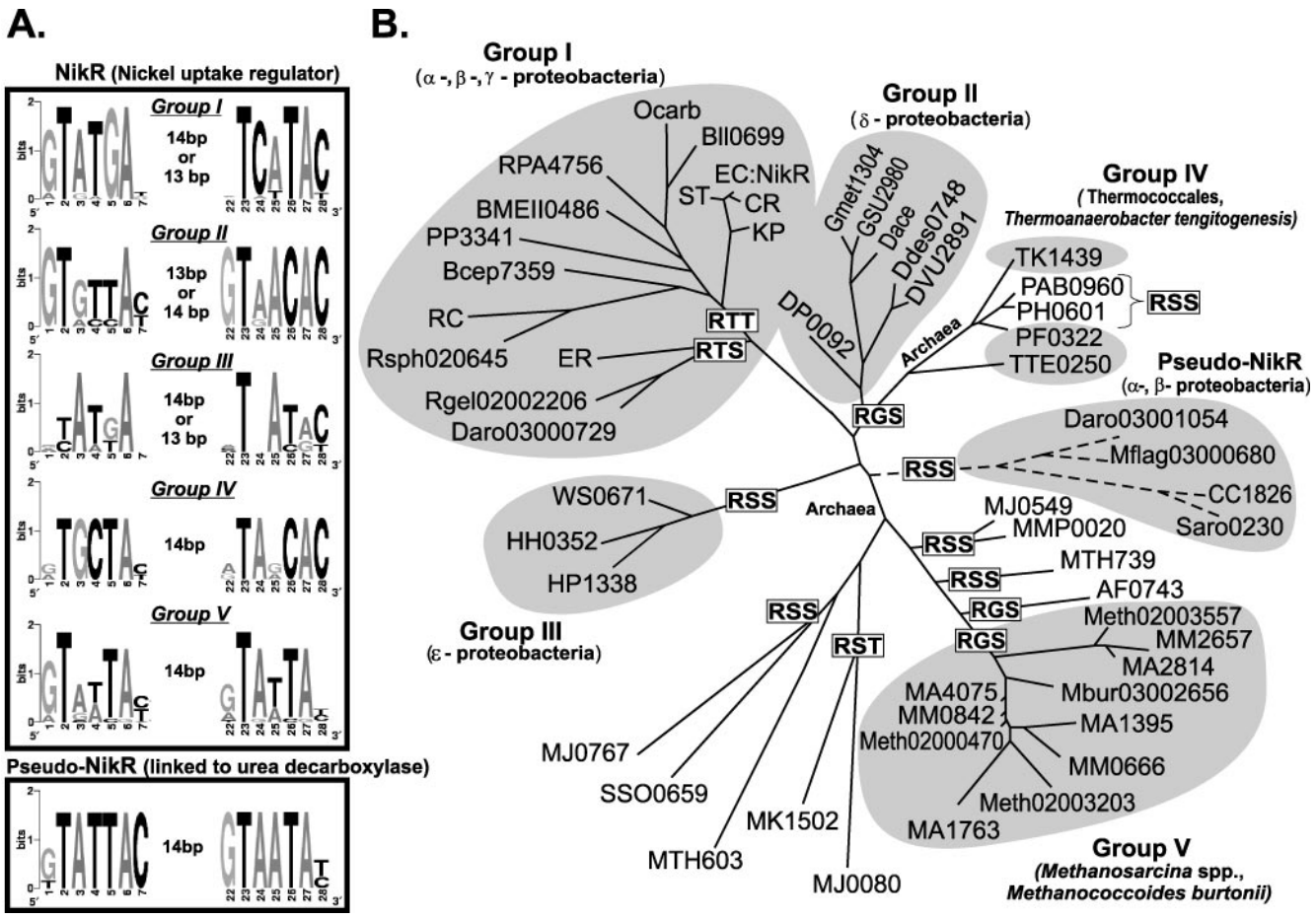


FIG. 1. Conservation of the NikR regulon in bacteria and archaea. (A) Sequence logos for the predicted NikR-binding signals in various taxonomic groups. (B) Maximum likelihood phylogenetic tree of the NikR proteins. All NikR-binding sites identified in the genomes that encode NikR from a certain group (marked by background gray) were used for construction of the sequence logo for the corresponding group. Candidate NikR-binding sites were not identified in only a few archaeal genomes encoding NikR (unmarked proteins on the phylogenetic tree). Three surface residues of the N-terminal beta sheets involved in DNA recognition are shown in boxes.

Sequence logos for the obtained group-specific NikR signals are shown in Fig. 1.

In α-, β-, and γ-proteobacteria possessing group I NikR proteins, the signal is conserved on the sequence level and is similar to the previously known NikR-binding site in *E. coli*. However, in three genomes the space between two dyad-symmetric consensus sequences is 13 bp instead of usual 14 bp. The NikR proteins from these three organisms have an Arg-Thr-Ser pattern of surface residues of the N-terminal beta-sheet involved in DNA recognition, whereas all the other group I proteins have an Arg-Thr-Thr pattern (see "RTS" and "RTT" boxes in Fig. 1). Although the identified binding signals of the NikR proteins from four other groups differ significantly from the consensus of the group I NikR signal, they retain the same palindromic structure and the distance between the two half-sites (Fig. 1). The observed degeneracy in the recognition sequences could be explained by differences in DNA-contacting surface residues of the NikR proteins (Fig. 1 and see Fig. S7 in the supplemental material).

A diverged branch of pseudo-NikR proteins from five proteobacteria seems not to be involved in the regulation of nickel uptake systems. Instead, we have observed that these pseudo-*nikR* genes are located within a conserved cluster for an alternative pathway of urea degradation: i.e., urea amidolyase consisting of biotin carboxylase, urea carboxylase, and allophanate hydrolase activities (29, 34). The predicted binding sites of pseudo-NikR regulators are present upstream of these gene clusters but are absent upstream of the nickel transport genes. Interestingly, *Dechloromonas aromotica* has two diverged copies of NikR: group I NikR is predicted to regulate adjacent nickel uptake genes, whereas the pseudo-NikR regulon contains solely urea amidolyase genes. In confirmation of our hypothesis, the amino acids H76, H87, H89, and C95, corresponding to the high-affinity Ni-binding site in the *E. coli* NikR structure, are not conserved in the pseudo-NikR proteins (see Fig. S7 in the supplemental material).

Tentative identification of NikR-binding sites in prokaryotic genomes possessing the nickel repressor genes allowed us to assign nickel specificity to many previously uncharacterized transporters. Overall, we found 28 bacterial and 14 archaeal transport systems under predicted NikR regulation (Table 1). The majority of them are ABC-type transporters (19 NikABCDE and 16 NikMNQO systems), whereas only seven NiCoT transporters were predicted to belong to NikR regulons.

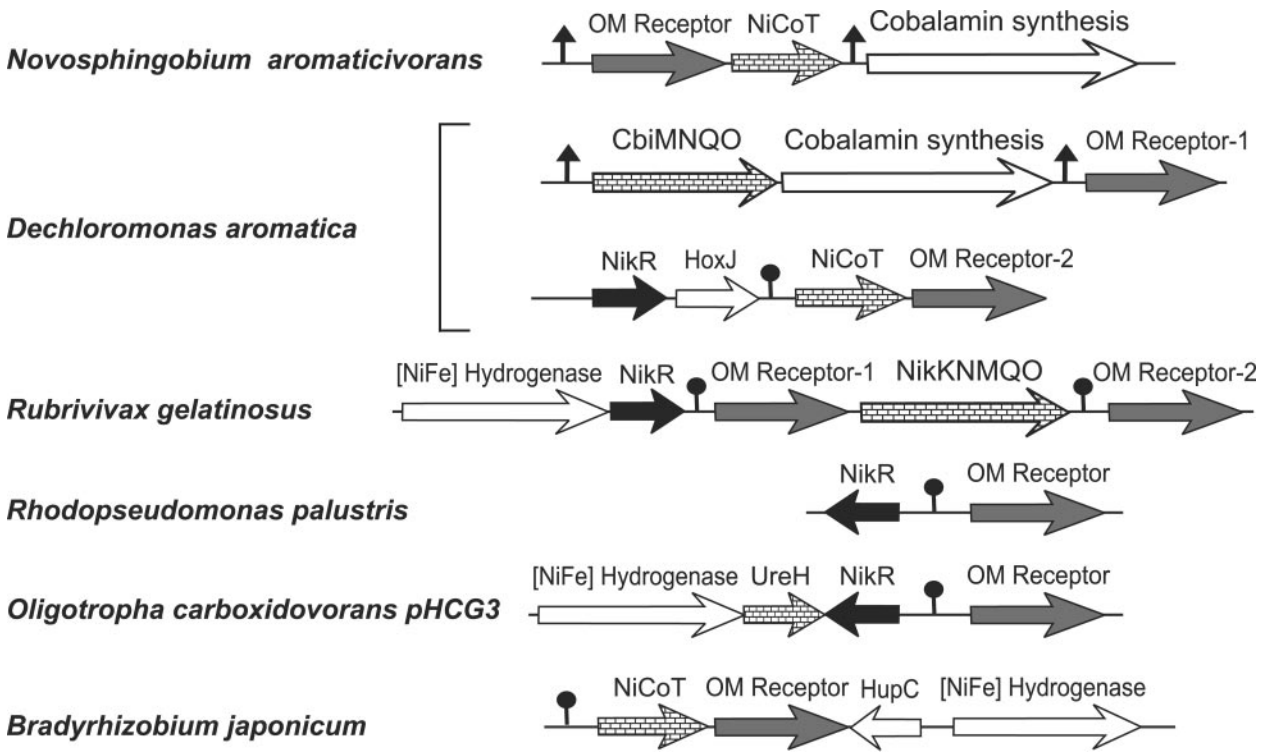


FIG. 2. Genomic organization of bacterial genes encoding nickel- or coenzyme $B_{12}$-regulated outer membrane receptors. Black circles and triangles indicate candidate NikR-binding sites and $B_{12}$ riboswitch regulatory elements, respectively.

**$B_{12}$ regulons in bacteria.** $B_{12}$ riboswitches are widespread regulatory RNA elements modulating gene expression in bacteria in response to changing coenzyme $B_{12}$ concentrations (42, 46). The presence of a $B_{12}$ riboswitch upstream of a putative nickel/cobalt transporter gene is a strong indication that the transporter would prefer the cobalt ion. We analyzed regulatory regions of identified transporter genes for the presence of $B_{12}$ riboswitches (see Table S1 in the supplemental material). Among analyzed protein families, we tentatively assigned cobalt preference to 40 transport systems that were preceded by $B_{12}$ regulatory elements on the DNA level (Table 1). The CbiMNQO transporters constitute the largest and most diverse family of transporters regulated by the $B_{12}$ riboswitches.

**Novel outer membrane proteins for Ni and Co transport?** Based on genomic linkage to NiCoT genes and on the presence of regulatory elements, we assign a function in nickel and cobalt transport across the outer membrane to a group of TonB-dependent receptors in certain gram-negative bacteria. Candidate NikR sites were identified at *RPA4757* in *R. palustris* and its orthologs in *Rubrivivax gelatinosus* and *Oligotropha carboxidovorans* (see Table S2 in the supplemental material). Products of these genes are weakly homologous to TonB-dependent outer membrane receptors for coenzyme $B_{12}$ and ferric siderophores but lack a typical TonB box on their N-terminal end. Orthologs of RPA4757 from some proteobacteria constitute a unique family of putative outer membrane receptors.

*R. gelatinosus* has two genes for this type of outer membrane protein, both of which are preceded by the candidate NikR-binding sites. Also it has an additional gene cluster preceded by a NikR site, *exbBD-tonB*, which encodes energizing components of outer membrane transporters. In *Bradyrhizobium japonicum* and *Dechloromonas aromatica*, the candidate NikR site precedes a two-gene cluster encoding the NiCoT transporter and the outer membrane receptor (Fig. 2). These findings are in agreement with the hypothesis of TonB-dependent nickel transport across the outer membrane. On the other hand, in *N. aromaticivorans* and *D. aromatica*, homologs of these receptors are preceded by $B_{12}$ riboswitch elements. Thus, this family seems to comprise both nickel and cobalt transporters.

**Phylogenetic and functional analysis of nickel/cobalt transporters. (i) CbiMQO/NikMQO family.** Based on genomic localization, regulatory characteristics, and phylogenetic analysis, we divided the CbiMNQO-like systems into putative nickel and cobalt transporters. The attribution to one of these two groups is indicated by using the *nik* and *cbi* prefixes. Comparison of operon structures revealed several variations in bacterial and archaeal genomes. M, Q, and O are universal components, which are present in all predicted transport systems, whereas the transmembrane proteins CbiN, NikN, and NikL and the bitopic transmembrane or periplasmic protein NikK are additional components (Fig. 3). The N and L components are not similar to each other on the sequence level but are predicted to have the same topology with two membrane-spanning segments flanking an extracytoplasmic loop.

The CbiM/NikM proteins constitute a unique family of transmembrane proteins with seven predicted transmembrane segments. We constructed a maximum likelihood phylogenetic tree based on multiple alignment of 86 bacterial and archaeal

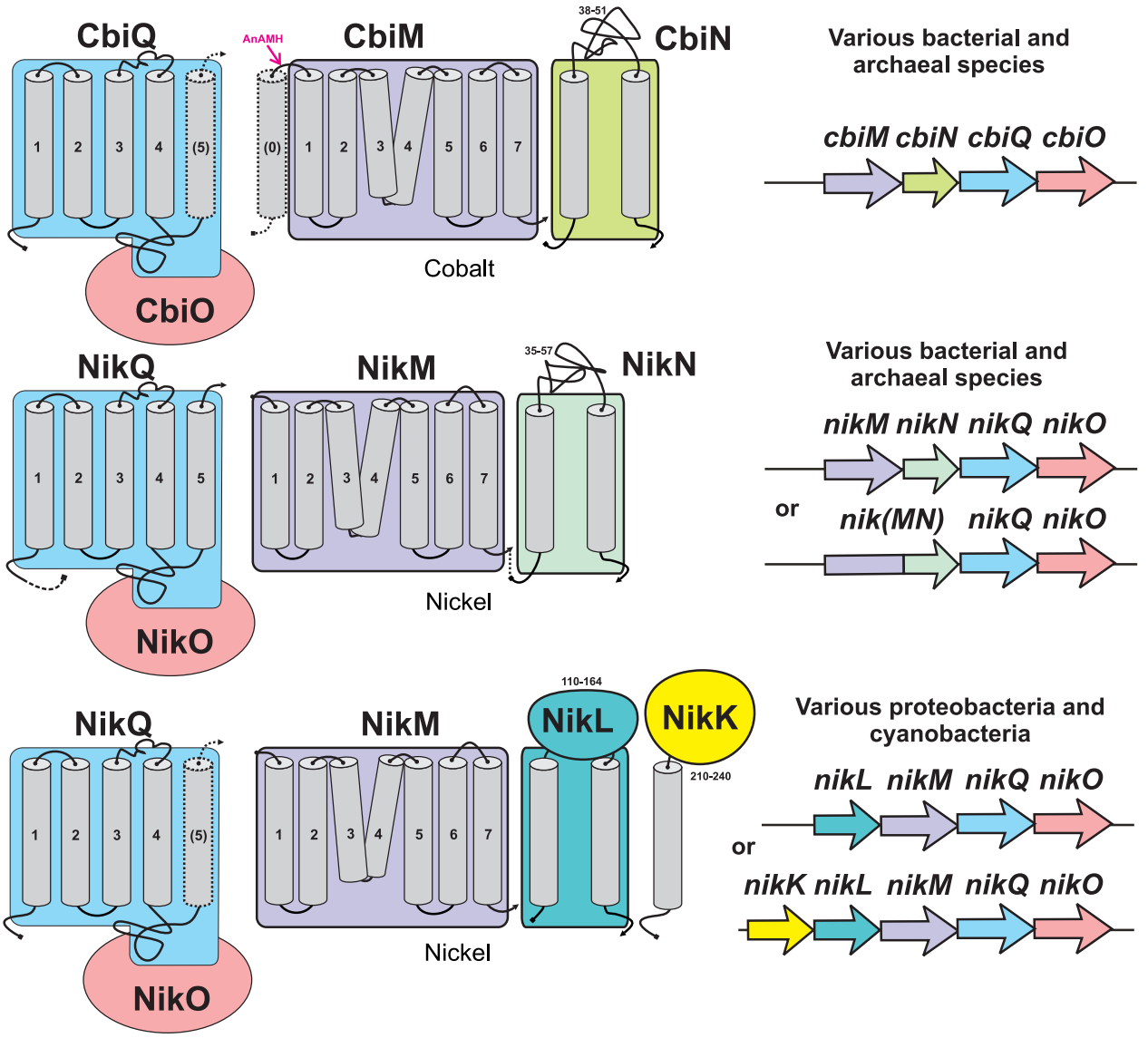


FIG. 3. Topology prediction for nickel and cobalt ABC transporters of the CbiMNQO/NikMNQO family. Homologous protein components of different transport systems are colored in the same way. Membrane-bound components are shown by rectangles with predicted transmembrane helices shown by cylinders. Putative ATPase components are shown by pink ovals. Periplasmic domains of NikL and NikK are shown by turquoise and yellow ovals, respectively. The fifth transmembrane helix is not conserved in some CbiQ/NikQ proteins and is shown in dotted lines. NikM and NikN components are fused to form a single protein in many cases. The illustrated physical interaction between O and Q components is speculative. The red arrow indicates a putative cleavage site (AnAMH) by bacterial signal peptidase I in all CbiM proteins that contain transmembrane helix 0.

CbiM/NikM proteins (Fig. 4). In general, the tree is subdivided into two large branches, which correspond to 36 predicted cobalt transporters and 50 candidate nickel transporters. Some of the strongly conserved positions in the CbiM/NikM proteins, including two histidines and several negatively charged amino acids, could be involved in metal ion recognition (Fig. 3; and see Fig. S1a in the supplemental material). Furthermore, there are several distinctive positions within the CbiM/NikM proteins which are conserved within each subgroup but differ between CbiM and NikM and thus could be involved in $Ni^{2+}$ or $Co^{2+}$ selectivity (Fig. 3). For example, the CbiM proteins have the signature motif Leu-Ala-His-Gly-Gly in the extracytoplasmic loop between transmembrane helices 4 and 5 and a conserved Gln residue at the beginning of helix 7, whereas the NikM proteins have the signature motif Phe-Ala-Asp-Gly-Gly and a conserved His residue at the respective positions. Another distinctive feature of many CbiM proteins, an additional N-terminal signal peptide with a conserved cleavage site, was detected in 21 out of 36 proteins (see Fig. S1a in the supplemental material). This observation agrees with the predicted orientation of the mature CbiM/NikM proteins, with their N-terminal Met-His motif located outside (Fig. 3).

Two other universal components of the CbiMNQO/NikMNQO family are a transmembrane protein (CbiQ/NikQ) and a cytoplasmic ATP-binding protein (CbiO/NikO). CbiO/NikO proteins have the linker peptide and the Walker A and

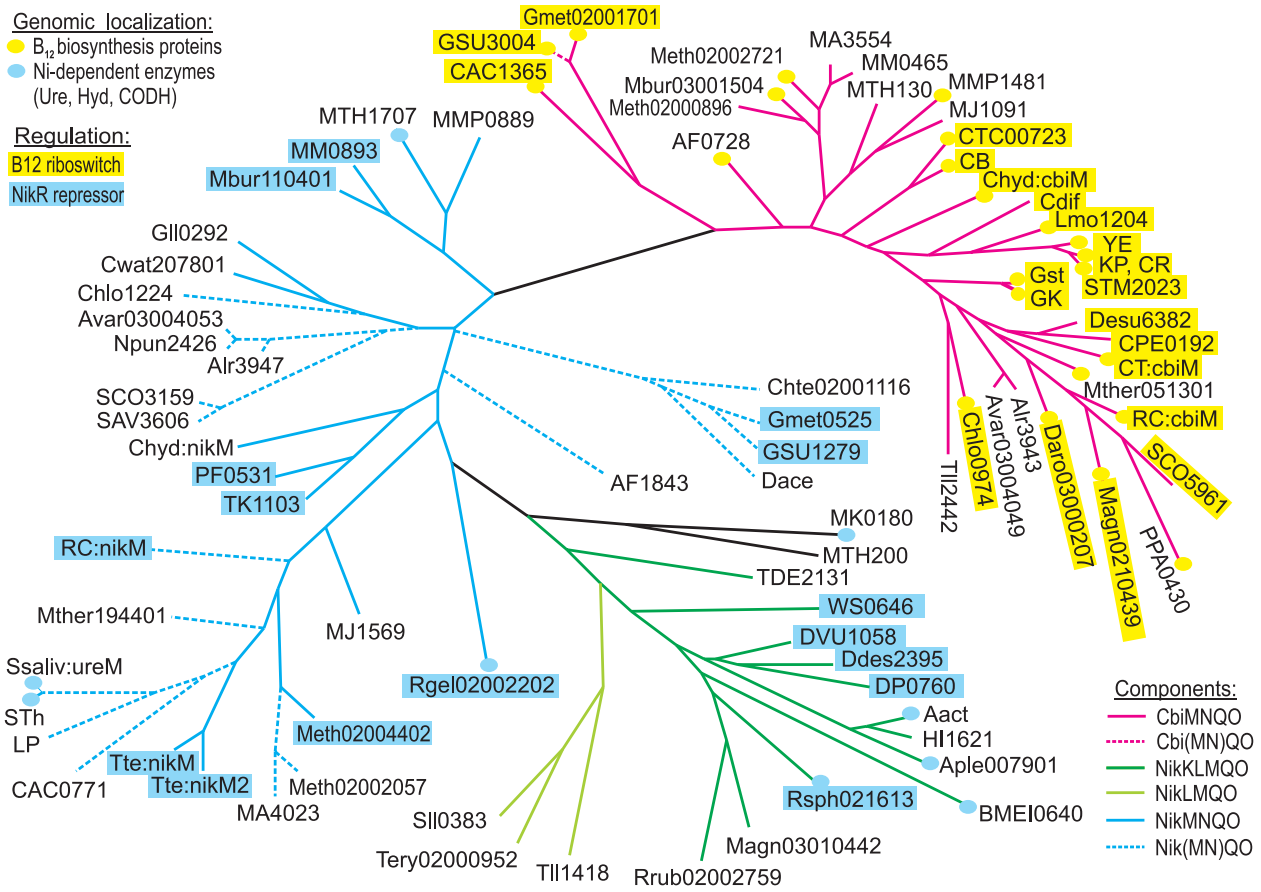


FIG. 4. Maximum likelihood phylogenetic tree of the M components of Cbi/Nik transport systems. Genes predicted to be regulated by the nickel repressor NikR and coenzyme $B_{12}$ riboswitch are in blue and yellow background colors, respectively. Genomic colocalizations of *cbi*/*nik* genes with genes encoding Ni-dependent enzymes and $B_{12}$ biosynthesis proteins are indicated by blue and yellow dots, respectively. The color of each of the lines indicates the subunit composition of the respective nickel/cobalt transport system. Dashed lines indicate transporters containing fused M and N subunits.

B motifs commonly found in the ATPase components of classical ABC-type transport systems (16). The topological model for CbiQ/NikQ proteins predicts the presence of four conserved transmembrane helices, a periplasmic domain between the third and fourth transmembrane helices, and an optional C-terminal transmembrane helix (Fig. 3). A cytoplasmic domain of CbiQ/NikQ, although lacking the conserved EAA motif, is likely to correspond to the cytoplasmic loop in other membrane-spanning domains of ABC systems, which has been identified previously as a contact site of ATP-binding proteins with membrane-spanning proteins in ABC transporters (16). Additional similarity searches identified a huge number of gene pairs weakly homologous to CbiQ/NikQ and CbiO/NikO (data not shown). However, the genomic analysis showed that all of these *cbiQO*-like genes do not seem to be implicated in nickel or cobalt homeostasis.

Almost all predicted Cbi transport systems possess a separate CbiN component and are encoded by conserved gene cluster *cbiMNQO* (Fig. 4). As an exception, the CbiM and CbiN components in *Geobacter sulfurreducens* are fused together, encoded by a single gene, designated *cbi*(*MN*). The predicted nickel transport systems may be subdivided into two subclasses, dependent on the presence of one of two different additional components, NikN or NikL (Fig. 2). The NikMNQO system is widely distributed in bacteria and archaea, whereas NikMLQO shows a mosaic distribution in several groups of proteobacteria and in cyanobacteria (Fig. 4). Almost all NikMLQO systems have an NikK component. In 19 out of 33 NikMNQO cassettes, the NikM and NikN components are fused together and encoded by a single gene, named *nik*(*MN*).

To analyze the functionality of these novel transport systems in nickel and cobalt transport and to validate the predicted substrate preferences, we selected CbiMNQO of *S. enterica* serovar Typhimurium and *R. capsulatus* and Nik(MN)QO of *R. capsulatus* for an initial experimental analysis. Plasmids encoding these transport systems were constructed and introduced into *E. coli*, a bacterium that intrinsically does not produce high-affinity nickel and cobalt transporters under aerobic conditions. The results of metal uptake assays with the recombinants are presented in Fig. 5 and indicate clearly that the three plasmids encode functional metal transporters. CbiMNQO of *S. enterica* serovar Typhimurium and *R. capsulatus* transport both nickel and cobalt ions, but in accordance with the bioinformatic data have a strong preference for cobalt. The differences in transport capacity between cells containing StCbiMNQO and RcCbiMNQO may be explained by differences in heterol-

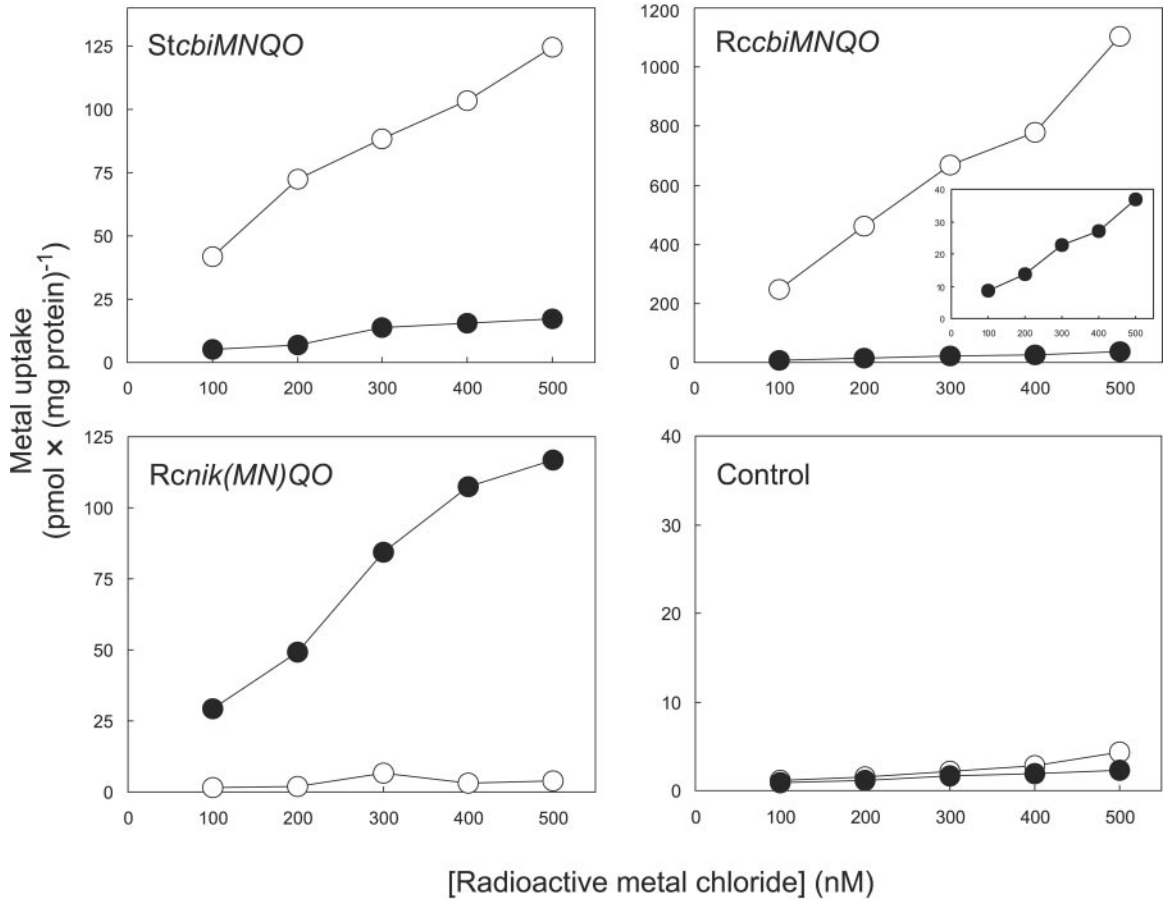


FIG. 5. $^{57}Co^{2+}$ (empty circles) and $^{63}Ni^{2+}$ (filled circles) uptake of recombinant *E. coli* cells expressing *cbiMNQO* from *Salmonella enterica* serovar Typhimurium (St) or *Rhodobacter capsulatus* (Rc) or *nik*(*MN*)*QO* from *R. capsulatus.* Control cells contained an empty pBluescript II KS+ vector. Cells were grown in the presence of either of the radiolabeled metal salts. The cellular metal content was determined by liquid scintillation counting. The inset in the upper right panel illustrates RcCbiMNQO-mediated nickel-uptake activity at a refined scale.

ogous expression efficiency and stability or by true differences in the transport mechanism. Similar results, i.e., apparent differences in transport velocity after heterologous production, had been obtained previously during characterization of various members of the NiCoT family (30). Rc*nik*(*MN*)*QO*, on the other hand, encodes a high-affinity nickel transporter. The cobalt-transport activity of *nik(MN)QO*-expressing cells was within the range of the background. Taken together, these data suggest that variants of *MNQO* genes in more than 50 prokaryotic genomes encode nickel or cobalt transporters. All of these operons encode an ABC protein (CbiO and NikO), but none contains a gene for a typical extracellular solute-binding protein. Since binding proteins are essential components of prokaryotic uptake ABC transporters (16), it was tempting to speculate that the mechanism of Cbi/NikMNQO is different from that of standard ABC systems. Experimental analysis of the *S. enterica* serovar Typhimurium CbiMNQO system confirmed this hypothesis. Results illustrated in Fig. 6 demonstrate that the basic modules CbiMN and CbiMNQ have significant Co uptake activity in the absence of the CbiO ABC protein, although these activities are considerably lower than those of the four-component (CbiMNQO) system. Deletion of *cbiN* abolished activity, suggesting that CbiM and CbiN are the minimal requirements for a functional transporter.

**(ii) NiCoT family.** Nickel/cobalt permeases of the NiCoT family were found in diverse taxonomic groups of bacteria, as well as in two archaeal genera and several species of fungi (23). The metal ion preferences of six NiCoTs extensively studied in metal accumulation assays correlate with the genomic localiza-

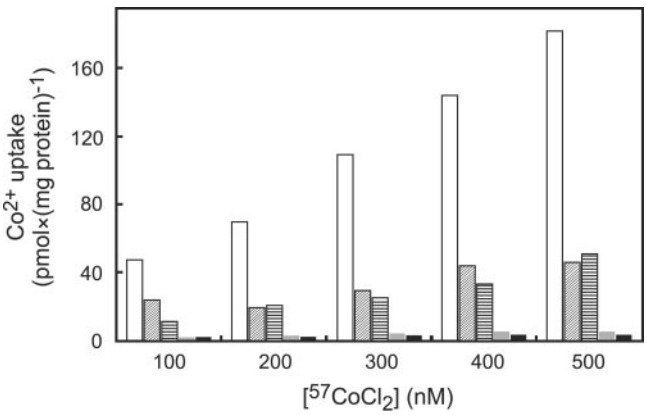


FIG. 6. Cobalt uptake of recombinant *E. coli* expressing *S. enterica* serovar Typhimurium *cbiMNQO* (white bars), *cbiMNQ* (diagonally hatched bars), *cbiMN* (horizontally lined bars), or *cbiM* (gray bars) or containing an empty vector (black bars).

tion of the transporter genes, which are often located adjacent to genes for Ni-dependent enzymes or coenzyme $B_{12}$ biosynthesis (30). In this study, we obtained additional indications of the metal ion preferences of NiCoTs. We found that seven NiCoT genes are preceded by candidate NikR binding sites, whereas eight genes are preceded by $B_{12}$ riboswitches (see Fig. S2 in the supplemental material). A known or predicted preference for either of the two metals does not reflect the phylogenetic neighborhood of individual NiCoTs, suggesting that this is a result of adaptation to specific $Ni^{2+}$ or $Co^{2+}$ requirements in various species.

**(iii) HupE/UreJ family.** Transporters of the HupE/UreJ family are widely distributed in proteobacteria and cyanobacteria. They contain an N-terminal signal peptide with a conserved cleavage site and six predicted transmembrane helices. The first helix has a conserved signature including two His residues and one Asp residue (24). In most proteobacteria, as well as in *Aquifex aeolicus* and *Deinococcus radiodurans*, the HupE/UreJ proteins are encoded within Ni-hydrogenase and urease gene clusters (see Fig. S3 in the supplemental material) and therefore named either HupE or UreJ, respectively (3, 32, 39). HupE from *R. palustris* and UreJ from *R. eutropha* have been heterologously produced in *E. coli* and shown to be involved in nickel transport (24). The *hupE*/*ureJ* genes in 6 out of 10 cyanobacteria are preceded by $B_{12}$ riboswitches and thus predicted to be involved in cobalt uptake linked to the biosynthesis of coenzyme $B_{12}$ (46) (see Table S1 and Fig. S3 in the supplemental material). Indeed, a very recent study showed that mutagenesis of *hupE* in *Synechocystis* sp. strain PCC 6803 results in $Co^{2+}$ deficiency (Jens Appel, Department of Botany, University of Kiel, Germany, personal communication).

**(iv) UreH/SodT family.** The *ureH* gene within the urease operon in *Bacillus* sp. strain TB-90 is required for full urease activity under $Ni^{2+}$ limitation (38). Orthologs of *ureH* are present in similar urease gene clusters in two *Geobacillus* species. The *ureH* genes were also identified in the genomes of some proteobacteria and other species (see Fig. S4 in the supplemental material). In *Microbulbifer degradans* and *Cytophaga hutchinsonii*, *ureH* is located adjacent to urease genes, whereas in *O. carboxidovorans* it is the promoter-distal gene in a putative [NiFe]-hydrogenase operon (see Table S1 in the supplemental material). Additional *ureH* homologs have been identified in marine cyanobacteria and many plants. The cyanobacterial homologs are located adjacent to the genes encoding Ni-dependent superoxide dismutase (24). There is no evidence for the existence of cobalt transporters within the UreH family.

**(v) NikABCDE transporters of PepT family.** The NikABCDE system of *E. coli* belongs to a large family of ABC transporters, named the nickel/peptide/opine transporter family PepT. Based on genomic colocalization with genes encoding Ni-dependent enzymes and the presence of Ni- and $B_{12}$-responsive regulatory elements, we identified a set of candidate nickel/cobalt transporters within the PepT family (see Table S1 in the supplemental material). In the phylogenetic tree constructed for the substrate-binding components, 25 predicted nickel transporters fall into several separate branches. In particular, it contains two most diverse bacterial branches, named NikA1 and NikA2, and two additional clusters that include homologs from methanogenic archaea preceded by candidate NikR-binding sites (see Fig. S5 in the supplemental material). Strikingly, the predicted nickel transporters from proteobacteria show a mosaic distribution between the diverged NikA1 and NikA2 groups. For example, among the closely related taxonomic group of enterobacteria, the NikA1 branch is represented by proteins from *E. coli*, *Klebsiella pneumoniae*, *Erwinia chrysanthemi*, *Citrobacter rodentium*, and *Proteus mirabilis*, whereas the NikA2 branch contains proteins from *Yersinia* species, *S. enterica* serovar Typhimurium, and the second NikA paralog from *P. mirabilis*. In contrast, the predicted $B_{12}$-regulated transporters from the NikABCDE family are not closely related on the sequence level. Crystallographic analyses of *E. coli* NikA (10, 31) failed to uncover the molecular basis of selective $Ni^{2+}$ recognition. Comparative genomics allows predicting the physiological substrate of various members of the peptide/opine/nickel ABC transporter family.

## DISCUSSION

Transition metals nickel and cobalt are essential components of many enzymes and must be transported into the cell in appropriate amounts. In this study, we combined bioinformatics and experimental approaches to describe nickel and cobalt uptake transporters in prokaryotes. Genome context techniques including the analysis of nickel- and coenzyme $B_{12}$-specific regulatory elements allowed us to annotate 96 nickel and 44 cobalt transport systems belonging to five different types. Secondary nickel/cobalt transporters of the NiCoT, HupE/UreJ, and UreH families, as well as NikABCDE ABC-type nickel transporters, have been analyzed in previous experimental work (3, 17, 24, 25, 30–32, 38, 41, 43). In contrast, experimental studies of Cbi/NikMNQO-like transporters were very limited until this work, and involvement of any of these systems in nickel or cobalt uptake has not yet been shown directly. The *cbiMNQO* gene cassette in *S. enterica* serovar Typhimurium has been considered to encode a cobalt transporter because of its localization within the $B_{12}$ biosynthesis superoperon and since some $B_{12}$ biosynthetic mutations in this region were corrected by the addition of excess $Co^{2+}$ (47). Two "NikMQO"-like systems, Nik(MN)QO in *Streptococcus salivarius* (originally named UreMQO) (9) and NikKLMQO in *Actinobacillus pleuropneumoniae* (originally named CbiKLMQO) (7), have been considered to provide Ni for urease activation under Ni-limiting conditions. Insertional inactivation of NikM in *S. salivarius* completely abolishes the ability of the cells to accumulate $^{63}Ni^{2+}$ during growth (9). In the present study, we analyzed three different transport systems of this type in metal accumulation assays and proved their involvement in the cobalt or nickel uptake. The *cbiMNQO* gene cassettes from *S. enterica* serovar Typhimurium and *R. capsulatus* conferred high-affinity cobalt uptake on recombinant *E. coli* cells, whereas expression of the *R. capsulatus nik*(*MN*)*QO* genes selectively enhanced nickel uptake.

Cbi/NikMNQO transporters are present in diverse species of bacteria and archaea, where they are more widely distributed than nickel/cobalt transporters of other types. Based on genome context analyses, we were able to assign a function in nickel or cobalt transport to the majority of systems from this family. The subunit composition of these unusual ATP-binding cassette transporters is not uniform, but a typical solute-bind-

ing protein is not obvious in any case (Fig. 3). The cobalt transporters consist of two transmembrane components (CbiM and CbiQ), a small membrane-bound component (CbiN) and an ATP-binding protein (CbiO). Similar components constitute the nickel transporters with some variability in the small membrane-bound component, either NikN or NikL, which are not similar to CbiN on the sequence level. One-fourth of the nickel transporters are accompanied by an additional membrane-bound (or periplasmic) subunit (NikK), which could be involved in binding of $Ni^{2+}$, although NikK proteins are not homologous to any known solute-binding protein of ABC systems. The mechanism of metal ion translocation for the Cbi/NikMNQO transport systems is not clear. The presence of an ATPase subunit suggests that, like classical ABC transporters, these systems are energized by ATP hydrolysis. On the other hand, results of an initial analysis of the *S. enterica* serovar Typhimurium CbiMNQO system suggest that the transmembrane protein CbiQ and the ABC protein CbiO are at least not essential for function of CbiMN, which we consider the basic moiety of the cobalt transporter. These observations may be of general relevance. Several lines of evidence support the notion that the Cbi/NikMNQO members are representatives of a mechanistically novel type of membrane transporters. (i) None of about 50 "*MNQO*" operons identified by comparative genomics codes for a protein similar to known solute-binding proteins which are essential for activity of prokaryotic uptake ABC transporters. (ii) Homologs of *cbiQ* and *cbiO* are linked to genes for membrane proteins of unknown function in many prokaryotic genomes. In some cases, however, a function in methionine and thiamine metabolism and in biotin transport can be ascribed to these operons due to genomic colocalization and the presence of regulatory elements in the upstream regions (see Fig. S6 in the supplemental material). A very recent analysis of the *bioMNY* operon of *Rhizobium etli* (28), encoding a CbiO homolog (BioM), a CbiQ homolog (BioN), and the putative core biotin transporter (BioY), is in agreement with a function in biotin transport. (iii) *S. enterica* serovar Typhimurium CbiMN retains a residual activity in the absence of CbiQO. These observations lead us to speculate that CbiQ and CbiO homologs form energizing modules and specifically interact with different membrane transporters that are independent of a solute-binding protein. The combination of a secondary active transporter with an ABC domain is not unprecedented. LmrA, an ABC-type multidrug exporter in *Lactococcus lactis*, contains an N-terminal transmembrane domain and a C-terminal nucleotide-binding domain. Removing the nucleotide-binding domain results in a protein that functions as a proton/multidrug symporter (53). In vitro studies with reconstituted systems are required to elucidate the mechanism of transporters with CbiO- and CbiQ-like components. These studies are beyond the scope of the present investigation but are currently under way.

In this study, we attempted to reconstruct pathways of nickel and cobalt homeostasis in microbial genomes by identification of gene sets for candidate metal transporters and metal-dependent enzymes. Ni-dependent enzymes are absent from many bacterial and archaeal lineages, including opportunistic and obligate pathogens like chlamydia, rickettsia, and spirochetes. Overall, distributions of genes for transporters and utilizing enzymes coincide to a large extent, demonstrating that nickel- and cobalt-dependent pathways are completed by high-affinity metal uptake systems in most microorganisms (see Table S1 in the supplemental material). However, some bacterial genomes without any homolog of known nickel/cobalt transporters, including *Bacillus subtilis*, *Sinorhizobium meliloti*, *Campylobacter jejuni*, *Neisseria meningitidis*, and *Corynebacterium* spp., encode Ni-dependent enzymes. This may indicate that other, currently unknown, nickel transporters are present in these bacteria.

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