Biotin uptake in prokaryotes by solute transporters with an optional ATP-binding cassette-containing module

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BioMNY proteins are considered to constitute tripartite biotin transporters in prokaryotes. Recent comparative genomic and experimental analyses pointed to the similarity of BioMN to homologous modules of prokaryotic transporters mediating uptake of metals, amino acids, and vitamins. These systems resemble ATP-binding cassette-containing transporters and include typical ATPases (e.g., BioM). Absence of extracytoplasmic solute-binding proteins among the members of this group, however, is a distinctive feature. Genome context analyses uncovered that only one-third of the widespread *bioY* genes are linked to *bioMN*. Many *bioY* genes are located at loci encoding biotin biosynthesis, and others are unlinked to biotin metabolic or transport genes. Heterologous expression of the *bioMNY* operon and of the single *bioY* of the α-proteobacterium *Rhodobacter capsulatus* conferred biotin-transport activity on recombinant *Escherichia coli* cells. Kinetic analyses identified BioY as a high-capacity transporter that was converted into a high-affinity system in the presence of BioMN. BioMNY-mediated biotin uptake was severely impaired by replacement of the Walker A lysine residue in BioM, demonstrating dependency of high-affinity transport on a functional ATPase. Biochemical assays revealed that BioM, BioN, and BioY proteins form stable complexes in membranes of the heterologous host. Expression of truncated *bio* transport operons, each with one gene deleted, resulted in stable BioMN complexes but revealed only low amounts of BioMY and BioNY aggregates in the absence of the respective third partner. The results substantiate our earlier suggestion of a mechanistically novel group of membrane transporters.

biotin transporter | functional genomics | transport systems

Biotin (vitamin H) is an essential cofactor in carboxylation, decarboxylation, and transcarboxylation reactions in both prokaryotes and eukaryotes. Recent studies suggest additional roles for this vitamin in cell signaling, gene expression, and chromatin structure in mammalian cells (reviewed in ref. 1). Biotin is synthesized by many bacteria, certain archaea, fungi, and plants (reviewed in ref. 2). Several metabolic routes seem to exist for the synthesis of the intermediate pimeloyl-CoA, which then is converted into biotin in a four-step path encoded by the universal genes *bioF*, *bioA*, *bioD*, and *bioB* (3, 4). In plants, the pathway is distributed between the cytosol and the mitochondria. At least the final step, catalyzed by biotin synthase, occurs in mitochondria. This enzyme, a member of the radical SAM enzyme family, inserts a sulfur atom into dethiobiotin in a complex reaction that is linked to mitochondrial iron/sulfur metabolism (2).

Biotin uptake has been analyzed in eukaryotes. In mammalian cells, the vitamin is transported across the plasma membrane by a sodium-dependent multivitamin transporter and, at least in certain tissues, by monocarboxylate transporter 1 (1). In the naturally biotin-auxotrophic yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, biotin uptake is mediated by unrelated proton symporters (5). Surprisingly little is known on the mechanisms behind biotin transport into prokaryotic cells, and multiple systems seem to exist. Active transport was observed for *Escherichia coli* K-12 >30 years ago (6). Despite extensive experimental work, knowledge of the complete genome sequence, and assignment of the biotin-transport locus to the 75-min genomic region, the gene(s) for the biotin transporter has not yet been identified. Recent studies by Walker and Altman (7) suggest that this system in *E. coli* and related Gram-negative bacteria not only transports the small vitamin, but in addition facilitates the uptake of biotinylated peptides with chain lengths up to 31 amino acid residues.

In 2002, Entcheva *et al.* (8) reported that mutations in *bioM* and *bioN* lead to reduced biotin uptake in *Sinorhizobium meliloti*. Because the products of these two genes share distinct similarity with CbiO and CbiQ, which are components of prokaryotic cobalt transporters, it has been proposed that *bioMN* may encode a biotin transporter (8). Comparative genomic analysis suggested, however, that *bioY*, located adjacent to *bioMN* in *S. meliloti*, is more likely to encode this transporter. *bioY* genes are widespread among bacterial and archaeal genomes but are absent from many β-, γ-, and ε-proteobacteria, including *E. coli*. Only a minority of *bioY* genes is linked to *bioMN* (3). Subsequently, Guillén-Navarro *et al.* (9) demonstrated that mutations in *bioY* and *bioM* in *Rhizobium etli* result in reduced biotin uptake and diminished capacity to form nodules on bean plants, respectively.

In a recent functional genomic and experimental investigation (10), we presented initial evidence that homologs of CbiO and CbiQ interact with unrelated membrane proteins to form a huge group of high-affinity transporters in prokaryotes for the transition metals cobalt and nickel, and for organic solutes like biotin. These systems are exceptional in many respects. On one hand, they contain a typical ATPase (e.g., CbiO and BioM) and thus resemble ATP-binding cassette (ABC) transport systems. On the other hand, among several hundred members of this transporter group, we were unable to identify cognate extracytoplasmic binding proteins, which are considered essential for prokaryotic ABC transporters involved in solute uptake (11). Moreover, our analysis showed that the CbiMN module of a bacterial CbiMNQO system has basal cobalt-transport activity, suggesting a secondary active transport mechanism for CbiMN in the absence of the ATPase-containing module CbiQO (10).

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Abbreviations: ABC, ATP-binding cassette; DDM, dodecyl β-D-maltoside.

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In the present study we attempted to analyze the contributions of BioM, BioN, and BioY to biotin transport using both *in silico* and experimental approaches. The BioMNY system of the α-proteobacterium *Rhodobacter capsulatus* was chosen for biochemical experiments. Evidence is presented that the single BioY is a high-capacity biotin transporter in the absence of BioMN. BioM-mediated ATPase activity, however, is needed to convert the system into a high-affinity transporter.

Results

Comparative Genomics of Biotin Uptake in Prokaryotes. Homologs of BioY are widely distributed among bacteria and archaea and form a unique protein family (pfam02632). Scanning of a nonredundant set of the sequenced prokaryotic genomes identified 150 orthologs of *bioY* in 127 genomes [for groups of BioYs with >98% amino acid sequence identity, only one ortholog was included in the set; see supporting information (SI) Table 1 and subsystem "Biotin biosynthesis" in the SEED database, www.nmpdr.org/FIG/subsys.cgi]. Among taxonomic groups with the largest number of organisms possessing at least one *bioY* ortholog are firmicutes (34 species), α-proteobacteria (28 species), cyanobacteria (15 species), actinobacteria (15 species), and archaea (13 species). Also, *bioY* was found in several small taxonomic groups including *Chlamydia*, chloroflexi, *Thermus*/*Deinococcus*, *Treponema*, and *Thermotoga*. In contrast to α-proteobacteria, *bioY* genes are absent in most analyzed species from other subdivisions of proteobacteria (β, γ, δ, and ε). We noted that many firmicutes and α-proteobacteria possess two (or even three in *Clostridium acetobutylicum*) paralogs of *bioY* (SI Table 1). The phylogenetic analysis of the BioY protein family showed its considerable heterogeneity. A phylogenetic tree showing the various subgroups of BioY proteins and including information about colocalization of their genes and the presence of putative transcription factor-binding sites is presented in SI Fig. 6. The proteins from various α-proteobacteria are not clustered together but dispersed between two very diverged branches of the tree. The same heterogeneity is observed for BioY proteins in some other taxonomic groups (firmicutes, actinobacteria, and *Thermus*/*Deinococcus*).

Genome context analysis revealed many cases of chromosomal clustering of *bioY* genes with additional components of the biotin transporter (*bioM* and *bioN*), as well as with genes involved in biotin biosynthesis (*bioA*, *bioD*, and *bioB* in firmicutes and α-proteobacteria), a hypothetical signal peptidase (*lpsA* in cyanobacteria), hypothetical fatty acid synthesis genes (*yhfT-yhfS* in firmicutes), and the *fab* genes in *Thermotoga* spp. (Fig. 1). Previous comparative analyses of transcription factor binding sites (3, 10) proposed that many of these *bioY*-containing loci are members of biotin regulons.

Candidate biotin transport operons containing the *bioY*, *bioM*, and *bioN* genes were mostly found in the groups of actinobacteria, archaea, and α-proteobacteria, and in some other species including *Deinococcus radiodurans*, *Syntrophobacter fumaroxidans*, *Treponema*, *Bordetella*, and *Photorhabdus* spp. However, some species among these groups (*Pyrococcus* spp., *Tropheryma whipplei*, and *Sulfitobacter* sp.) have *bioMN* genes that are located separately from *bioY*. On the phylogenetic tree of the BioY proteins, the BioMN-associated paralogs in α-proteobacteria and actinobacteria form a separate branch, very diverged from the *bioY*-encoded copies that are unlinked to *bioMN*. Interestingly, candidate biotin transport operons in *Methanosarcina* spp. and *Fusobacterium nucleatum* (*bioYMMN*) contain tandem copies of the ATPase (*bioM*) genes. In *Desulfovibrio* spp., the candidate biotin transport operon *bioYM* is incomplete and *bioN* is absent from the genome. All other BioY-encoding genomes lack obvious orthologs of *bioMN* genes. Overall, the genome context analysis suggested that only approximately one-third of

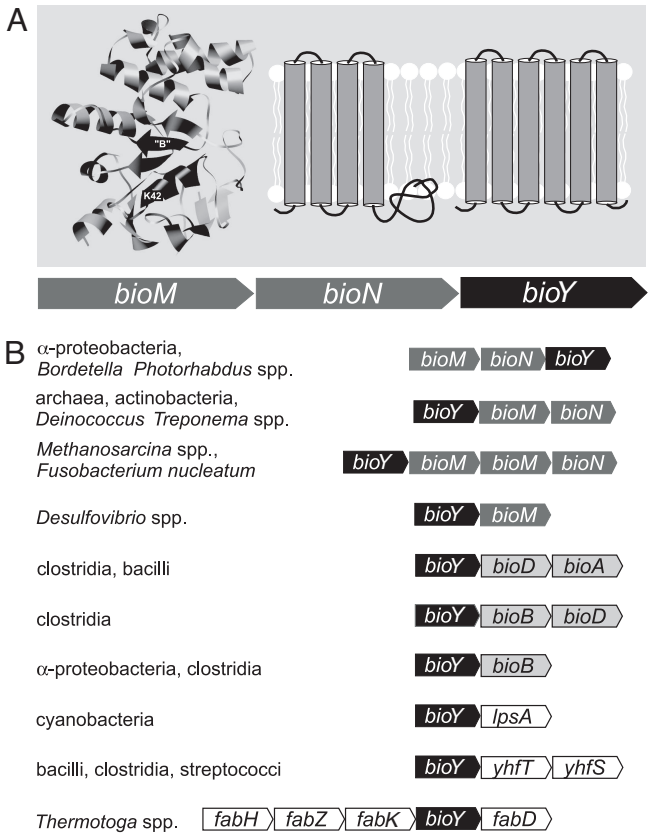

Fig. 1. Bioinformatics-based analysis of biotin-transport proteins. (*A*) Structure model for BioM (modeled by the 3D-JIGSAW server, based on similarity with other ABC proteins, and modified with CHIMERA) and topological models for BioN and BioY (based on PONGO and PREDICTPROTEIN predictions and multiple hydropathy profile alignments using PEPWINDOWALL). K42 and "B" indicate a Lys residue in the Walker A region and the Walker B region, respectively. (*B*) Genomic context of *bioY* genes. The most representative cases of positional clusters with genes encoding components of biotin transporters (*bioM* and *bioN*), biotin synthase (*bioB*), and other biotin biosynthesis genes (*bioD* and *bioA*), hypothetical lipoprotein signal peptidase (*lpsA*), and hypothetical fatty acid synthesis genes (*yhfTS*, *fab*) are shown.

BioY proteins is associated with BioMN components (Fig. 1, SI Fig. 6, and SI Table 1).

Based on these bioinformatic data, previous work on biotin uptake in rhizobia (8, 9), and the distinct similarity of BioMN to the CbiOQ and NikOQ modules of prokaryotic cobalt and nickel transporters (10), we hypothesized that BioM, BioN, and BioY may encode a tripartite, modular biotin-uptake system. *bioM* genes encode standard ATPases with the typical Walker A, Q loop, signature (LSGGQ), Walker B, and H motifs (see ref. 11 for a review on ABC proteins). *In silico* topological analyses of a multitude of BioN and BioY proteins using the algorithms on the PONGO (12) and PREDICTPROTEIN servers and PEPWINDOWALL for multiple hydropathy profile alignments resulted in the models shown in Fig. 1. A four-transmembrane-helix architecture with a C-terminal extension in the cytoplasm is predicted for most bacterial BioN proteins. This extension contains two amphipathic helices and the signature (**E**/D)**A**(**Q**/X)(**R**/K)**ARG**(X_9)(V/I)**P,** which resembles the "EAA" loop in the transmembrane proteins of classical ABC transporters (SI Fig. 7). Because the EAA loop is in close contact with the ATPase subunits (13), this signature in BioN is a candidate site for interactions with BioM. EAA loop motifs are apparently absent from BioY proteins. For archaeal BioNs, a fifth trans-

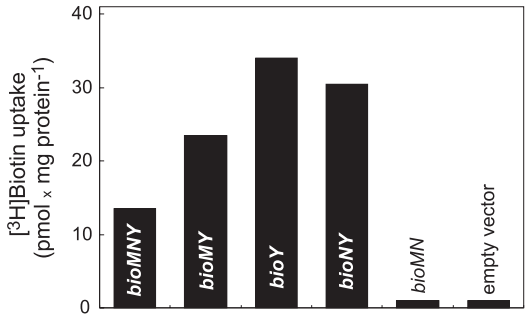

Fig. 2. Biotin uptake of recombinant *E. coli*. *E. coli* cells containing pRcBioMNY, pRcBioMY, pRcBioY, pRcBioNY, pRcBioMN, or an empty vector were incubated in glucose-containing phosphate buffer in the presence of 4 nM [^{3}H]biotin. Cellular biotin contents were determined after 3.5 h. The values represent the means of double or triple independent assays.

membrane domain C-terminal to the amphipathic helices and the EAA loop is predicted.

Heterologous Expression of Biotin-Transport Genes. For experimental analysis we chose the *bioMNY* operon from the α-proteobacterium *R. capsulatus*. Expression of these genes from pRcBioMNY conferred significant biotin-uptake activity on cells of the biotin transport-deficient *E. coli* strain S1039 (Fig. 2). Subsequently, the deletion derivatives pRcBioMY, pRcBioY, pRcBioNY, and pRcBioMN were tested for biotin-transport encoding capacity. The data shown in Fig. 2 demonstrate that BioY is the central unit of the biotin transporter. At a substrate concentration of 4 nM and in the absence of BioMN, BioY-containing cells accumulated the vitamin in long-term assays $\approx$1,000-fold, suggesting an active uptake mechanism. No activity was observed for cells producing BioMN in the absence of BioY.

Kinetic Analysis of Biotin Transport into Recombinant *E. coli* Producing Holo-Transporters (BioMNY) or Core (BioY) Transporters. The aforementioned results raised questions about the contributions of each of the BioMNY proteins to biotin uptake. In particular, we were curious to learn about the role of BioMN because these components seemed to be dispensable in the initial accumulation assays. This view changed, however, when kinetic parameters of BioY- and BioMNY-mediated transport were determined and compared. These results are illustrated in Fig. 3. Transport rates showed concentration-dependent saturation kinetics. BioY-containing cells took up biotin with a maximal velocity of 60 pmol $\times$ min^{-1} $\times$ (mg protein)$^{-1}$, and this process was apparently half-saturated at a substrate concentration of 250 nM. Markedly altered values were observed for BioMNY-producing cells. The maximal velocity of the tripartite system was lower by an order of magnitude but noticeable; the affinity for biotin was significantly enhanced as indicated by a 50-fold-lower apparent half-saturation constant.

High-Affinity Biotin Uptake Depends on the Functional BioM ATPase. Kinetic analyses suggested that BioMN are required for efficient transport at very low biotin concentrations, conditions that resemble many natural environments. To investigate this assumption biotin-uptake rates of recombinant *E. coli* S1039 cells producing combinations of BioM, BioN, and BioY were compared over a range of biotin concentrations between 100 pM and 200 nM. In addition, a variant BioMNY holotransporter with a K42N exchange within the Walker A motif in BioM was included in this series of experiments. Replacements of this conserved residue in ATPases are known to cause strongly reduced activity of ABC transporters (e.g., 99% inhibition of the *E. coli* maltose transporter by the

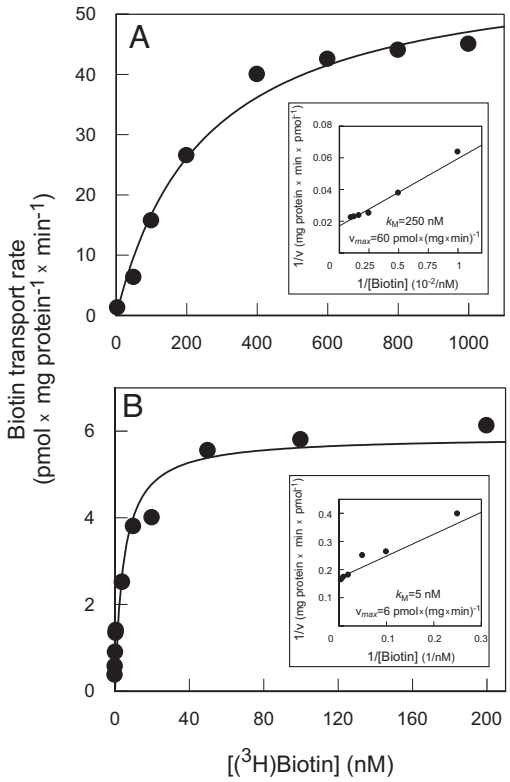

Fig. 3. Kinetics of biotin uptake. Initial velocities of biotin uptake into intact *E. coli* containing pRcBioY (*A*) or pRcBioMNY (*B*) were determined at various substrate concentrations. Apparent half saturation (k_M) values and maximal velocities (v_{max}) of transport were calculated with the help of GRAFIT.

MalK$_{K42N}$ exchange; ref. 14). The results of these biotin-uptake experiments are summarized in Fig. 4. Comparison of BioMNY with BioY (Fig. 4*A*) revealed significantly higher activity for the holotransporter under low-substrate conditions but the exact opposite at elevated concentrations. In Fig. 4*B*, wild-type (BioMNY) and mutant (BioM$_{K42N}$NY) holotransporters are compared. It is evident that inactivation of the ATPase resulted in clearly diminished activity under any condition tested. Finally, Fig. 4*C* illustrates biotin uptake of *E. coli* producing BioMY and BioNY subcomplexes. BioMY-containing cells transported biotin essentially as their BioMNY-containing counterparts under both substrate limitation and excess. This result suggested functional interaction of BioM and BioY in the absence of BioN. Biotin transport of BioNY-producing recombinants resembled the pattern of BioY-harboring rather than BioM$_{K42N}$NY-harboring cells. Interpretation of this result is difficult. It may be explained by a limited tendency of BioY and BioN to form complexes in the absence of BioM. Alternatively, it may indicate that BioNY complexes have similar activity as the single BioY but are inhibited if a defective BioM is included in the complex. Taken together, these experiments demonstrated that a functional BioM is required for efficient transport at low biotin concentrations.

BioM, BioN, and BioY Proteins Form Stable Complexes. Membranes of *E. coli* cells producing various combinations of tagged BioM, BioN, and BioY were analyzed for the presence of complexes between these proteins. After treatment with the nondenaturing detergent dodecyl β-D-maltoside (DDM), solubilized membrane protein was subjected to affinity chromatography. Fig. 5 demonstrates that BioMNY complexes could be purified via the His-tag on BioM or the FLAG epitope on BioY. Likewise, tripartite complexes were detectable in membranes of cells

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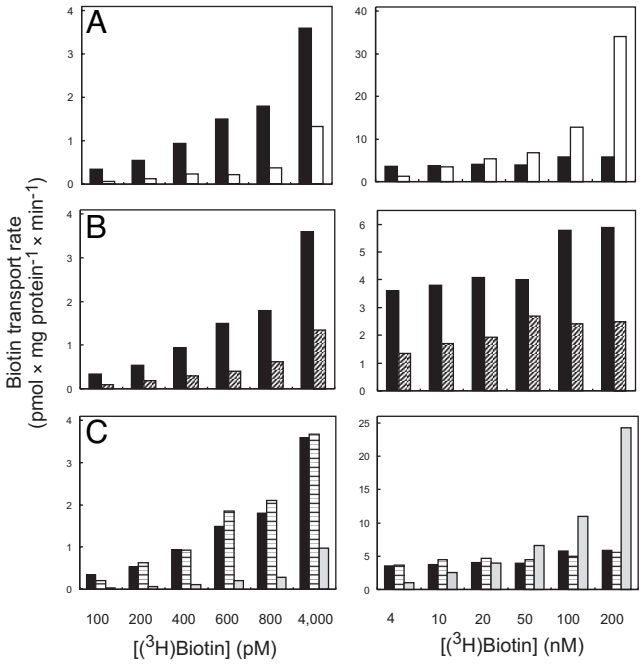

Fig. 4. Biotin-transport rates of recombinant *E. coli*. Initial velocities were calculated at biotin concentrations in the picomolar (*Left*) and nanomolar (*Right*) ranges. Transport rates of cells producing *R. capsulatus* BioMNY (black bars) were compared with those containing BioY (white bars in *A*), BioM$_{K42N}$NY (hatched bars in *B*), BioMY (horizontally hatched bars in *C*), and BioNY (gray bars in *C*). The values represent the means of at least two independent assays.

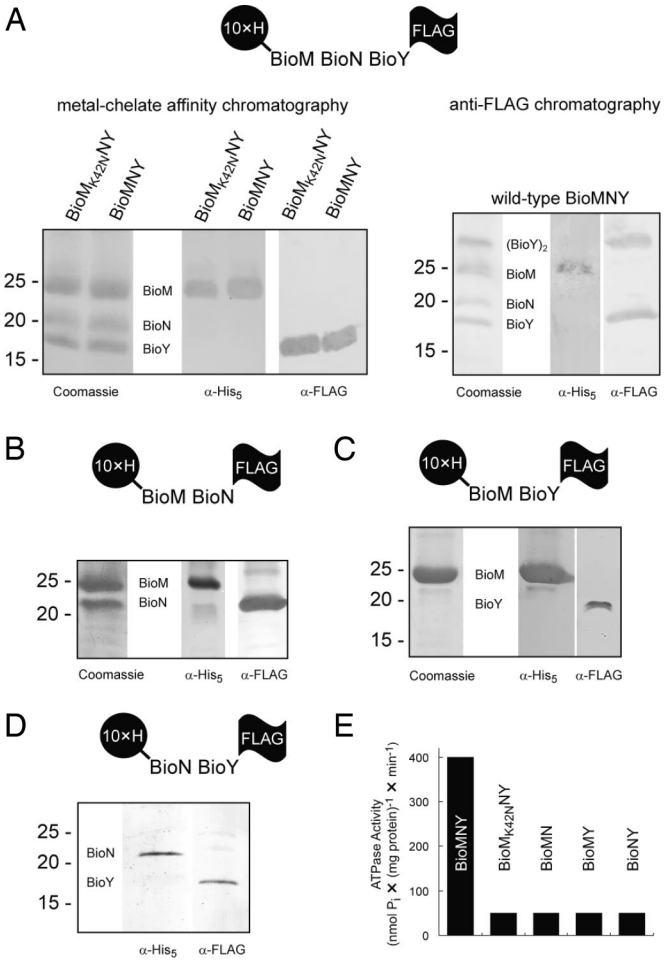

Fig. 5. Complexes of biotin-transport proteins. Membranes of *E. coli* cells producing *R. capsulatus* BioM, BioN, and BioY proteins in combinations and with tags as indicated were solubilized by treatment with DDM. Proteins purified by anti-FLAG affinity chromatography (*A*) or metal-chelate affinity chromatography (*A–D*) were separated by SDS/PAGE and subsequently stained (Coomassie) or blotted onto nitrocellulose membranes that were treated with anti-penta-His (anti-His$_5$; Qiagen) or anti-FLAG (Sigma) antibodies. Numbers (in kDa) represent the positions of standard proteins. (*E*) Approximately 1 μg of purified protein was subjected to ATPase assays. ATPase activities represent the means of multiple assays with independently purified samples. BioMNY-containing samples yielded ≈400 nmol P_i × (mg protein)$^{-1}$ × min^{-1}. ATPase activity of all other samples was <50 nmol P_i × (mg protein)$^{-1}$ × min^{-1}.

expressing the mutant *bioM*(K42N)*NY* operon. These results indicate that the three proteins form stable aggregates in the membrane independent of ATPase activity. Some preparations contained significant amounts of a putative BioY dimer, which was not resolved by SDS, as an additional species (Fig. 5*A*).

Bioinformatic analyses have suggested that BioMN and their homologs specifically interact with core transporters to yield a huge group of modular solute-uptake systems. We wondered whether BioMN would form a complex in the absence of the BioY transporter. Fig. 5*B* illustrates that large quantities of the two proteins copurified, an observation compatible with the view of a separate BioMN module. On the other hand, ATPase assays repeatedly failed to detect ATP hydrolysis of BioMN and other subcomplexes whereas significant activity was found for the tripartite BioMNY (Fig. 5*E*).

Based on the outcome of the biotin-uptake experiments we expected complex formation at least between BioM and BioY and perhaps BioN and BioY in the absence of the respective third partner. Fig. 5*C* shows that expression of *bioMY* and nickel-chelate affinity chromatography yielded large quantities of BioM but only traces of BioY, detection of which depended on immunoblotting. Membrane fractions of BioMY- and BioMNY-producing cells contained comparable amounts of BioY (data not shown). Ternary BioMNY complexes are stable, but binary BioMY aggregates are obviously unstable, resulting in loss of most of the BioY during attempts to purify BioMY. It is conceivable that BioN is important for stabilizing the complex. Likewise, attempts to purify BioNY complexes via the His tag on BioN resulted in very low amounts of the two proteins (Fig. 5*D*). These data may be interpreted in a sense that, although formed in the membrane, BioMY and BioNY dimers or oligomers are much less stable than tripartite aggregates containing all three subunits.

Discussion

The majority of prokaryotic inner-membrane transporters involved in active solute uptake belongs to one of the following four groups (15): (*i*) ABC transporters containing extracellular solute-binding proteins, (*ii*) secondary active transporters, (*iii*) binding protein-dependent secondary active transporters, and (*iv*) phosphoenolpyruvate-dependent phosphotransferase systems. Binding proteins of ABC transporters play dual crucial roles (reviewed in ref. 11). They are not only responsible for efficient substrate recognition in the extracytoplasmic space resulting in low k_M values of the transport processes, but they are also involved in signaling substrate availability to the core transporters and triggering ATP hydrolysis of the ATPase domains. Therefore, our recent observation that a huge group of bacterial and archaeal transporters containing typical ABC proteins seems to be independent of solute-binding proteins was surprising (ref. 10 and subsystem ''CbiQO-type ABC transporter systems'' in the SEED database, www.nmpdr.org/FIG/subsys.cgi). This group comprises experimentally verified transporters (CbiMNQO and NikMNQO) for the transition-metal

ions cobalt and nickel and predicted systems for amino acids, vitamin precursors, and vitamins including biotin. Originally, our focus was on the metal transporters. During recent investigation of bacterial Cbi and Nik systems, we made the surprising observation that single CbiMN components have Co^{2+}-transport activity in the absence of CbiQO (ref. 10 and P.H. and T.E., unpublished results). These data led us conclude that CbiMN acts as a core transporter, and CbiQO, consisting of an integral membrane protein (CbiQ) and an ATPase (CbiO), functions as an optional module. This hypothesis is corroborated by the fact that CbiQO homologs are linked to unrelated membrane proteins to yield binding protein-independent ABC transporters for various substrates. The modular architecture becomes especially obvious in the case of biotin transporters. BioM and BioN are homologs of CbiO and CbiQ, respectively. In contrast to most other CbiQO-associated systems, which are encoded by *cbiQO*-like genes and adjacent gene(s) for a transporter module, approximately two-thirds of the *bioY* genes are not linked to *bioMN*. Many firmicutes including streptococci, on the other hand, contain *cbiQOO* operons located adjacent to genes encoding essential cellular functions (e.g., ribosomal proteins, RNA polymerase subunits, and pseudouridylate synthase). Linkage of CbiQOO to specific transport processes is not obvious in these organisms. Because streptococci have limited biosynthetic capacities, the products of these operons may be involved in the uptake of essential nutrients. Indeed, it has been shown that one of the *cbiO* orthologs (*ybaE*) is an essential gene in *Streptococcus pneumoniae* (16).

It was tempting to speculate that single *bioY* genes encode functional biotin transporters. Results of the present investigation clearly confirm this hypothesis. Heterologous expression of both a *bioMNY* operon and the single *bioY* gene conferred biotin-transport activity on a biotin transport-deficient *E. coli* strain. Nevertheless, kinetic considerations strongly argue against the notion that BioMN are superfluous. BioM-mediated ATPase activity was essential for efficient transport at low biotin concentrations resembling natural conditions. Transport assays and the fact that strains of *Desulfovibrio* contain *bioYM* operons but lack *bioN* genes suggested functional interaction between BioM and BioY in the absence of BioN. Biochemical analyses showed, however, that stable complexes were dependent on the tripartite system. Because the BioYs constitute a diverse protein family, it cannot be excluded that dependency on BioMN differs among the various members.

Although mitochondria and plastids play important roles in biotin metabolism in plants, the mechanisms of biotin transport across the organellar envelopes have not yet been unraveled. The final step of biotin synthesis is catalyzed in mitochondria. Biotin-dependent carboxylation of acetyl-CoA in the course of fatty acid biosynthesis is a central reaction in chloroplasts. It is conceivable that mitochondria and chloroplasts contain export and import systems for biotin, respectively. It was interesting to note in this context that the chloroplast genome of *Mesostigma viride*, a member of the earliest lineage of green plants (17), encodes a BioY homolog (YCX1-MESVI).

This study has assigned clear-cut roles to the components of prokaryotic biotin transporters whose functions were previously ambiguous. The results are in favor of our recent suggestion of a mechanistically novel group of prokaryotic membrane transporters that combines features of both secondary and ABC transporters. Clear evidence for the modular composition has been provided, and we are convinced that this is a solid basis for elucidating the molecular interactions among the subunits of this type of transporters.

Materials and Methods

Bacterial Strains. *R. capsulatus* strain SB1003 (kindly provided by Gabriele Klug, Universität Giessen, Giessen, Germany) was grown photoheterotrophically under white light. Genomic DNA was isolated as described by Egland *et al.* (18). For biotin-uptake assays, recombinant *E. coli* S1039 [*birB13*(Ts) Δ*bio-61 bioP98* (up promoter) *recA1 thi rpsL λb515 b519 galQ6 red-270 c*I857] containing plasmid pFDX500 (*lacI*^{q}) (19) and expressing *bioM*, *bioN*, and *bioY* genes of *R. capsulatus* strain SB1003 was used. *E. coli* S1039 (7, 20) lacks intrinsic high-affinity biotin-uptake activity and was kindly provided by Elliot Altman (University of Georgia, Athens, GA). BioMNY complexes were purified upon production in the OmpT$^-$ *E. coli* strain UT5600 (New England Biolabs, Ipswich, MA) harboring pFDX500 and a plasmid encoding the respective biotin-transport components.

Plasmids. The chromosomal *bioMNY* operon of *R. capsulatus* SB1003 was amplified by PCR using primers bioM+ and bioY−, which introduced an NcoI site at the 5′ end of *bioM* and a BglII site immediately downstream of the last codon of *bioY*, and *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). The purified amplicon was treated with NcoI and BglII and inserted into an expression plasmid essentially as described (10). In the resulting plasmid pRcBioMNY*, *bioMNY* expression is under control of a *lac* promoter and encodes a BioY variant with a C-terminal FLAG epitope. Because the native *bioY* contains a TTG initiation codon and initial biotin-uptake experiments with cells harboring pRcBioMNY* gave negative results, the TTG was replaced by an ATG by using the QuikChange protocol (Stratagene, La Jolla, CA). The replacement in the resulting pRcBioMNY was confirmed by treatment with BccI because a recognition site for this enzyme arises from the exchange. Likewise, a variant pRcBioMNY encoding a $BioM_{K42N}$ ABC protein with a Lys-to-Asn exchange in the Walker A site was constructed according to the QuikChange protocol. The modification was verified with restriction endonuclease BseRI. Then, a 470-bp *bioM* fragment comprising the mutated site was used to replace the corresponding fragment of pRcBioMNY to give pRcBioM(*K42N*)NY. Plasmids encoding BioY individually, BioMN, BioMY, and BioNY were constructed by inverse PCR using pRcBioMNY as the template and primer pairs as follows: an "upstream minus" primer complementary to the initiation codon and the upstream region of *bioM* and primer bioY+, which produced a product with NcoI sites at both ends (for pRcBioY), a bioN− primer and a "plus" primer, directed to the segment downstream of *bioY*, which introduced BglII sites (for pRcBioMN), a combination of EcoRI site-introducing bioM− and bioY+ primers (for pRcBioMY), and primers bioN+ and upstream minus (for pRcBioNY). The amplicons were treated with the appropriate restriction endonuclease, ligated, and transformed into *E. coli*. In the case of pRcBioMY the stop codon of *bioM* and the ATG initiation codon of *bioY* are separated by the hexameric EcoRI site. Plasmids encoding N-terminally 10×His-tagged BioM, BioN, or BioY proteins were constructed by inverse PCR using a minus primer that introduced 10 His codons, 1 Ser codon, an NcoI site including the initiation codon, and an appropriate (bioM+, bioN+, or bioY+) plus primer. The purified PCR products were digested with NcoI, ligated, and transformed.

Biotin-Uptake Assays. Recombinant *E. coli* S1039 strains were grown overnight at 37°C in supplemented mineral salts medium containing 35 mM sodium/potassium phosphate buffer (pH 7.0), 20 mM D-glucose, 37.5 mM NH_4Cl, 810 μM $MgSO_4$, 68 μM $CaCl_2$, 18.5 μM $FeCl_3$, 400 μg/ml casamino acids, 30 μM thiamin, 20 nM D-biotin, 100 μg/ml ampicillin, and 1 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested, washed twice, and resuspended in uptake buffer [35 mM sodium/potassium phosphate buffer (pH 7.0) containing 0.2% (wt/vol) glucose] to yield an OD_{578} of ≈0.2. For analysis of long-term accumulation, 4 nM D-[8,9-^{3}H]biotin [1.64 or 1.85 TBq/mmol;

purchased from PerkinElmer (Boston, MA)] was added, and the cells were incubated for 3.5 h at 37°C under shaking. A total of 0.5 ml of the cell suspensions was passed through cellulose nitrate filters (0.45-μm pore size), and the filters were washed three times with uptake buffer. Filter-bound radioactivity was quantitated in a Packard TriCarb 2900 TR liquid scintillation counter. For kinetic analysis of transport, cells were incubated in uptake buffer in the presence of [^{3}H]biotin at concentrations between 100 pM and 1 μM. Samples (0.5 ml) were taken at several time points within 3 min after addition of [^{3}H]biotin and processed as indicated above. Initial transport rates were calculated in the linear range of uptake.

Purification of Biotin-Transport Protein Complexes. *E. coli* UT5600 containing pFDX500 and pRc10HBioMNY, pRc10HBioM(*K42N*)NY, pRc10HBioMN, pRc10HBioMY, or pRc10HBioNY was grown in Luria–Bertani broth supplemented with ampicillin (100 μg/ml) and isopropyl β-D-thiogalactopyranoside (1 mM) for ≈5 h at 37°C under shaking. Cells were harvested at an OD_{578} of ≈2, washed in 35 mM sodium/potassium phosphate buffer (pH 7.0), resuspended in the same buffer containing a mixture of protease inhibitors (Roche, Indianapolis, IN), and disrupted by three passages through a French pressure cell. Membranes were pelleted by ultracentrifugation in a Sorvall T-865 rotor (36,000 rpm for 30 min at 10°C), resuspended and homogenized in 50 mM Tris·HCl (pH 8.5), and solubilized by agitation for 90 min in the presence of 2% (wt/vol) DDM, 5% (vol/vol) glycerol, and 300 mM NaCl at 4°C. Nonsolubilized material was pelleted by ultracentrifugation. For metal-chelate affinity chromatography, imidazole was added to a final concentration of 20 mM and the solution was loaded onto a Ni-NTA spin column (Qiagen, Valencia, CA). The column was washed with 50 mM Tris·HCl (pH 7.5) containing 0.05% DDM, 5% glycerol, 300 mM NaCl, and imidazole up to 100 mM and eluted by increasing the imidazole concentration to 500 mM. The buffer was subsequently exchanged against 50 mM Tris·HCl (pH 7.5) containing 0.05% DDM, 5% glycerol, and 300 mM NaCl (TDGN) using a PD-10 column (GE Healthcare, Chalfont St. Giles, UK). For anti-FLAG affinity chromatography, solubilized membrane proteins were loaded onto an anti-FLAG M2 antibody-containing matrix (Sigma, St. Louis, MO), washed with TDGN, and eluted with 50 mM sodium citrate solution (pH 4.0) containing 0.05% DDM, 5% glycerol, and 500 mM NaCl. Purified protein was concentrated by ultrafiltration using Amicon concentrators.

ATPase Assay. ATPase activity of the concentrated biotin-transport proteins (≈1 μg of protein per assay) was determined by quantitating the release of inorganic phosphate from ATP through conversion of phosphate into phosphomolybdate as described (21, 22).

Bioinformatic Techniques. The comparative genomic analysis of the biotin transporters and biosynthesis genes, including protein similarity searches and analysis of chromosomal gene clusters, was performed by using the SEED genomic database and tools implemented therein (www.nmpdr.org/FIG/index.cgi) (23). Complete and nearly complete prokaryotic genomes were from the GenBank database (24) (www.ncbi.nlm.nih.gov/GenBank). Conserved functional domains were detected by the PFAM database search tool (25) (www.sanger.ac.uk/Software/Pfam). The PONGO (ref. 12; http://pongo.biocomp.unibo.it/pongo) and PREDICTPROTEIN (www.predictprotein.org) servers were used to predict the occurrence of transmembrane segments. Multiple hydropathy profile alignments were done by using PEPWINDOWALL (http://bioweb.pasteur.fr/seqanal/interfaces/pepwindowall.html).

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Correction

MICROBIOLOGY. For the article "Biotin uptake in prokaryotes by solute transporters with an optional ATP-binding cassette-containing module," by Peter Hebbeln, Dmitry A. Rodionov, Anja Alfandega, and Thomas Eitinger, which appeared in issue 8, February 20, 2007, of *Proc Natl Acad Sci USA* (104:2909–2914; first published February 14, 2007; 10.1073/pnas.0609905104), Fig. 4 appeared incorrectly, due to a printer's error. The online version has been corrected. The corrected figure and its legend appear below.

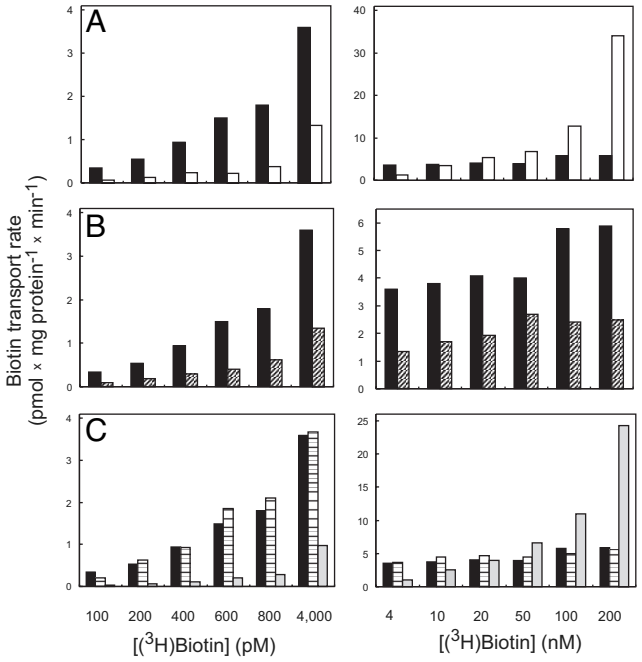

Fig. 4. Biotin-transport rates of recombinant *E. coli*. Initial velocities were calculated at biotin concentrations in the picomolar (*Left*) and nanomolar (*Right*) ranges. Transport rates of cells producing *R. capsulatus* BioMNY (black bars) were compared with those containing BioY (white bars in *A*), $BioM_{K42N}NY$ (hatched bars in *B*), BioMY (horizontally hatched bars in *C*), and BioNY (gray bars in *C*). The values represent the means of at least two independent assays.

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