Solitary BioY Proteins Mediate Biotin Transport into Recombinant *Escherichia coli*

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Energy-coupling factor (ECF) transporters form a large group of vitamin uptake systems in prokaryotes. They are composed of highly diverse, substrate-specific, transmembrane proteins (S units), a ubiquitous transmembrane protein (T unit), and homo- or hetero-oligomeric ABC ATPases. Biotin transporters represent a special case of ECF-type systems. The majority of the biotin-specific S units (BioY) is known or predicted to interact with T units and ABC ATPases. About one-third of BioY proteins, however, are encoded in organisms lacking any recognizable T unit. This finding raises the question of whether these BioYs function as transporters in a solitary state, a feature ascribed to certain BioYs in the past. To address this question in living cells, an *Escherichia coli* K-12 derivative deficient in biotin synthesis and devoid of its endogenous high-affinity biotin transporter was constructed as a reference strain. This organism is particularly suited for this purpose because components of ECF transporters do not naturally occur in *E. coli* K-12. The double mutant was viable in media containing either high levels of biotin or a precursor of the downstream biosynthetic path. Importantly, it was nonviable on trace levels of biotin. Eight solitary *bioY* genes of proteobacterial origin were individually expressed in the reference strain. Each of the BioYs conferred biotin uptake activity on the recombinants, which was inferred from uptake assays with [³H]biotin and growth of the cells on trace levels of biotin. The results underscore that solitary BioY transports biotin across the cytoplasmic membrane.

Energy-coupling factor (ECF) transporters are a large group of ABC transporters for trace nutrients and intermediates of salvage pathways in prokaryotes. They consist of a transmembrane high-affinity substrate-binding protein (S unit), a moderately conserved but ubiquitous transmembrane protein (T unit), and homo- or hetero-oligomeric ABC ATPases. In contrast to canonical ABC importers, ECF transporters do not rely on extracytoplasmic soluble substrate-binding proteins (1–3).

Shortly after the initial discovery of ECF-type importers for cobalt and nickel ions in 2006 (4), ECF systems were described in 2009 (2) as a novel group of modular ABC uptake transporters for highly diverse substrates. As originally proposed by functional genomics and then confirmed by biochemical analyses, ECF transporters fall into two subgroups. Subgroup I systems are encoded by operons containing genes for all subunits. Each S unit interacts with a dedicated A₂T or A1A2T module. In contrast, subgroup II transporters share the copies of a single A1A2T module, and the genes for the S units are scattered around the genome. A significant body of work on the structure of individual subunits (5–8) and the mode of subunit interactions (8–10) was published recently. The stoichiometry of subunits in the holotransporters, however, is controversial. A 1:1:1:1 stoichiometry (S:T:A1:A2) was reported for the subgroup II ECF transporters of *Lactococcus lactis* (11) and for the ECF transporters for hydroxymethylpyrimidine (12) and folate (13) of *Lactobacillus brevis*. In contrast, a 2:2:1:1 (S:T:A1:A2) stoichiometry was proposed for the subgroup II riboflavin transporters of *Thermotoga maritima* and *Streptococcus thermophilus* (8). Oligomeric S (14, 15) and T (10) units were also found in the case of the subgroup I biotin transporter of *Rhodobacter capsulatus*.

Biotin-specific S units (BioY) are the most widespread S components among prokaryotes. About one-third of *bioY* genes are located in *bioMNY* operons encoding subgroup I biotin transporters. One-third of BioY is known or predicted to interact with a shared A1A2T module in subgroup II ECF transporters. A signif-

icant fraction of BioY, however, exists in organisms lacking recognizable T units, and these “solitary” BioYs cannot be distinguished from their homologs on the sequence level. This finding suggests that the latter BioY proteins function independently of dedicated or shared A₂T or A1A2T modules. Biotin transport activity was reported for BioY of *R. capsulatus* in the absence of its A₂T module (15, 16) and for the solitary BioY of *Chlamydia* spp. that lack any other components of ECF transporters (17). In contrast, the role of BioY proteins as transporters in the absence of cognate T and A units was questioned in a combined structural and biochemical analysis of detergent-solubilized and purified BioY from *L. lactis* and *R. capsulatus* (7). In that study, the high-resolution structure of L1BioY was determined and the binding of biotin to L1BioY and RcBioY was characterized using proteoliposomes. The authors of that study determined 300 pM biotin to be the dissociation constant (*K_d*) for L1BioY and, likewise, an extremely low *K_d* value that could not be quantified exactly for RcBioY. Neither biotin accumulation by the proteoliposomes nor counterflow activity with biotin-charged vesicles was observed. Thus, it was concluded that in the *in vitro* system, BioY acts as a high-affinity biotin-binding protein but does not transport its substrate across the membrane (7).

These conflicting results left questions regarding the precise role of BioY in living cells open. This uncertainty prompted us to investigate the function of selected BioY proteins in biotin-auxotrophic recombinant *Escherichia coli* cells. We show here that

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solitary BioY proteins from various sources not only bind biotin molecules on the cell surface but also transport the vitamin into the cytoplasm of the recombinants.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains JW3375-1 ($\Delta bioH::Km$) and JW3803-2 ($\Delta yigM::Km$) are contained in the Keio collection (18) and, together with strains BT340(pCP20) and BW25113, were obtained from the *E. coli* Genetic Stock Center at Yale University (New Haven, CT). Plasmid pCP20 is an ampicillin and chloramphenicol resistance-conferring temperature-sensitive pSC101 replicon containing the *FLP* yeast recombinase gene under the control of a λ p_R promoter, and a λ cI857 allele (19).

Plasmid pSIM6 (20), a temperature-sensitive, ampicillin resistance-conferring pSC101 replicon encoding the bacteriophage λ *exo bet gam* gene functions under the control of a λ p_L promoter, and a λ cI857 allele, was kindly provided by Donald L. Court (Frederick, MD).

For construction of plasmid pPimA, the *pimA* gene from *Rhodopseudomonas palustris* CGA009 encoding a pimelic acid:coenzyme A ligase (21) was amplified using genomic DNA as the template and primers that added restriction endonuclease recognition sites to the 5' (PciI) and 3' (BglII) ends. The digested amplicon was inserted between the NcoI/BglII sites of a streptomycin resistance-conferring expression plasmid, derived from a ColE1 replicon (14).

For amplification of *E. coli* *yigM* and *bioH*, genomic DNA from strain BW25113 (parent strain of the Keio collection) was used as the template. The primers added PciI/XbaI (*bioH*) and NcoI/XbaI (*yigM*) sites during PCR. The digested and purified amplicons were inserted between the NcoI/XbaI sites of an ampicillin resistance-conferring expression plasmid (15) to give pBioH and pYigM, respectively.

Plasmids encoding BioY proteins (the identifiers from the SEED database are given in parentheses) from *Bradyrhizobium japonicum* (224911.1.peg.4297), *Oceanicola batsensis* (252305.3.peg.1979, ObBioY1; 252305.3.peg.3712, ObBioY2), *Rhodopseudomonas palustris* (258594.1.peg.2432), *Roseobacter denitrificans* (375451.6.peg.2382) and *Silicibacter pomeroyi* (246200.3.peg.2614, SpBioY1; 246200.3.peg.3204; SpBioY2) were described previously (15). The plasmid encoding BioY of *Roseovarius nubinhibens* (89187.3.peg.3420) is a derivative of the former plasmids. Expression of the *bioY* genes in recombinant *E. coli* is controlled by a Lac repressor encoded by plasmid pLacI-Rare2.

Mutations in *bioY* genes were introduced by two rounds of PCR. In the first round, a mutagenic forward primer and a reverse primer were used to create an amplicon of ~100 bp. The amplicon was purified and used as a primer, together with an upstream forward primer in the second round. The digested products of the second round were used to replace the wild-type *bioY* segment. All synthetic DNAs were verified by nucleotide sequencing.

Construction of a $\Delta bioH \Delta yigM::Km$ double mutant. The biotin-auxotrophic and biotin uptake-deficient double mutant was isolated in a multistep process by deleting *yigM* in the $\Delta bioH$ strain using a λ Red-based recombineering protocol. First, the kanamycin resistance cassette in strain JW3375-1 ($\Delta bioH::Km$) was removed upon introduction of plasmid pCP20 and a heat shock as described previously (22). The heat shock induces expression of the *FLP* recombinase gene and subsequently leads to loss of the plasmid. Kanamycin-sensitive colonies in which the resistance cassette originally located between two FRT sites was removed, were selected and subsequently verified by PCR. Second, plasmid pPimA was transformed into the unmarked $\Delta bioH$ strain. Addition of pimelate to the growth medium was expected to feed a pimeloyl thioester into the biotin synthesis path in the resulting strain (Fig. 1). Third, plasmid pSIM6 providing the λ *exo bet gam* gene functions was transformed into the precursor strain. Fourthly, the $\Delta yigM::Km$ allele was amplified using genomic DNA of strain JW3803-2 ($\Delta yigM::Km$) as the template and the amplicon was electroporated into the $\Delta bioH$ (pPimA, pSIM6) strain as recommended (18, 20, 22). Kanamycin-resistant colonies were selected on ly-

sogeny broth agar containing 3 mM pimelate. Replacement of *yigM* by $\Delta yigM::Km$ was verified by PCR analysis.

Growth assays. The growth of *E. coli* $\Delta bioH \Delta yigM::Km$ and recombinants producing BioY variants was analyzed on solid or liquid GN mineral salts medium (35 mM sodium/potassium phosphate buffer [pH 7.0], 20 mM D-glucose, 37.5 mM NH₄Cl, 810 μ M MgSO₄, 68 μ M CaCl₂, and 18.5 μ M FeCl₃, plus 100 μ g of ampicillin/ml where appropriate) containing supplements as indicated. Cells were grown in liquid GN medium supplemented with 1 μ M biotin for ~20 h, washed three times with phosphate buffer, diluted 1:200 in biotin-free GN medium, and incubated under shaking at 37°C for another 24 h. The resulting biotin-deficient cultures were diluted with GN medium to an optical density at 578 nm (OD₅₇₈) of 0.1, and 12 μ l of this suspension and of serial 10-fold dilutions thereof were spotted onto agar plates with the indicated compositions and 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside). For growth analysis in liquid medium, the biotin-deficient cultures were diluted in parallel to an OD₅₇₈ of 0.05 in GN medium with the indicated supplements plus 0.5 mM IPTG. Growth was analyzed as OD₆₀₀ with duplicate samples (200 μ l per well) in a microtiter plate reader (SpectraMax M2; Molecular Devices) at 37°C. Each growth experiment was performed at least three times and representative results, i.e., mean values of two technical replicates, are shown.

Biotin uptake. Biotin uptake of recombinant *E. coli* $\Delta bioH \Delta yigM::Km$ strains expressing wild-type and mutant *bioY* genes was analyzed as described previously (15). Briefly, the cells were grown overnight in lysogeny broth supplemented with 100 μ g of ampicillin/ml. The cells were diluted 1:100 in lysogeny broth supplemented with ampicillin and 1 mM IPTG, incubated for 3 h at 37°C under shaking, washed, and resuspended in buffer (35 mM sodium/potassium phosphate [pH 7.0]). The cell suspensions were diluted in the same buffer to give an OD₅₇₈ of ~0.2. Upon the addition of [³H]biotin to a final concentration of 4 nM, the suspensions were incubated for 3.5 h at 37°C under shaking. Samples (0.5 ml) were filtered through nitrocellulose membranes, the filters were washed, and filter-bound radioactivity was quantified in a Packard Tri-Carb 2900 TR liquid scintillation counter.

Western blotting. For immunological detection of C-terminally Flag-tagged BioY proteins in recombinant *E. coli*, the cells were grown in lysogeny broth with 100 μ g of ampicillin/ml and 1 mM IPTG to the late exponential phase. The cells were harvested, washed twice with phosphate buffer (35 mM sodium/potassium phosphate [pH 7.0]), and resuspended to an OD₅₇₈ of 5 in phosphate buffer containing DNase I and protease inhibitor cocktail as recommended by the manufacturer (Roche). Cells were lysed by sonication, and cell debris was removed by centrifugation at 3,300 $\times$ g for 10 min at 4°C. Membranes in the supernatants were pelleted in a desktop centrifuge at 21,000 $\times$ g and 4°C. The membranes were solubilized in sodium dodecyl sulfate (SDS)-containing buffer (50 mM Tris-HCl [pH 6.8], 1.7% [wt/vol] SDS, 6% [vol/vol] glycerol, 0.8% [vol/vol] 2-mercaptoethanol, 0.002% [wt/vol] bromophenol blue). Approximately 10 μ g of solubilized membrane protein was subjected to polyacrylamide gel electrophoresis. Upon blotting onto a nitrocellulose membrane, FLAG-tagged proteins were visualized with a monoclonal anti-Flag antibody-alkaline phosphatase conjugate (Sigma).

RESULTS

Reference strain for biotin transport activity. To address the question of whether solitary BioY proteins not only bind biotin on the cell surface but also transport the vitamin into the cytoplasm of living cells, we attempted to isolate a biotin-deficient *E. coli* reference strain. In this strain, both biotin biosynthesis and biotin uptake should be interrupted, resulting in a conditionally lethal phenotype. Biotin synthesis in *E. coli* can be divided into several stages. It is initiated by BioC that methylates malonyl-acyl carrier protein (malonyl-ACP) to target it to the biotin synthesis pathway. Upon two rounds of chain elongation reactions catalyzed by the fatty acid synthesis machinery, further elongation is prohib-

FIG 1 Biotin synthesis and uptake in *E. coli* wild-type and $\Delta bioH \Delta yigM::Km$ strains. (A) Wild type. BioC targets a portion of malonyl-ACP to biotin synthesis by converting it to malonyl-ACP methyl ester (33). Two rounds (condensation with malonyl-ACP and decarboxylation, reduction, dehydration, and reduction) of the fatty acid biosynthesis pathway result in pimeloyl-ACP methyl ester. The latter compound is converted into pimeloyl-ACP by the BioH methyl esterase. Biotin is produced from that intermediate in a universal four-step path via 7-oxo-8-aminononanic acid (7-keto-8-aminopelargonic acid [KAPA]), 7,8-diaminononanic acid (7,8-diaminopelargonic acid [DAPA]) and dethiobiotin. See reference 23 for a review of biotin biosynthesis. YigM has recently been identified as the long-sought-after high-affinity biotin transporter (24). (B) The $\Delta bioH \Delta yigM::Km$ strain can neither produce nor take up biotin since *bioH* and *yigM* are deleted. PimA-producing recombinants are predicted to grow on pimelic acid provided that this compound enters the cells and pimeloyl-CoA (the product of the PimA-catalyzed pimelic acid:coenzyme A ligase reaction) serves as a substrate for BioF.

ited by BioH-mediated demethylation of the intermediate pimeloyl-ACP methyl ester to give pimeloyl-ACP. The latter compound is converted into biotin in a universal four-step path (Fig. 1A) (see reference 23 for a review).

It has long been known that *E. coli* K-12 strains can take up biotin from the environment by a high-affinity transport system. Despite extensive research by various groups, it took about 40 years until this transporter (YigM) was identified by J. Stolz's group (24). YigM is not related to ECF transporters. Rather, it is a membrane protein with 10 transmembrane helices and belongs to the carboxylate/amino acid/amine family of secondary transporters (TC 2.A.78).

Biotin is an essential cofactor for fatty acid biosynthesis. We hypothesized that the elimination of both YigM and an enzyme of the biosynthetic route would only be tolerated if an intermediate of the biotin synthesis path downstream of the genetic block was available and utilized. To test this assumption, we chose an *E. coli* $\Delta bioH$ strain and introduced the *pimA* gene of *Rhodopseudomonas palustris* encoding a pimelic acid:coenzyme A ligase (21) into the mutant. As shown in Fig. 1B, exogenous pimelate was predicted to result in intracellular pimeloyl-CoA, which may serve as a precursor of biotin in the recombinant (25). This assumption was confirmed by the growth assay shown in Fig. 2. The *pimA*-expressing $\Delta bioH$ strain grew well in biotin-free mineral salts medium supplemented with 3 mM pimelate, whereas the plasmid-free strain without *pimA* did not grow. These results are in agreement with

earlier work in which the expression of the *Bacillus subtilis* *bioW* (encoding a pimeloyl-CoA synthase) allowed *E. coli* *bioH* mutants to utilize exogenous pimelate for biotin synthesis (26).

In the next step, *yigM* was deleted in the *PimA*-containing $\Delta bioH$ strain and the resulting recombinants ($\Delta bioH \Delta yigM::Km$

FIG 2 Viability of an *E. coli* *bioH* mutant on pimelate depends on the recombinant pimelic acid:coenzyme A ligase. The growth of the *PimA*-containing and *PimA*-free cells in biotin-free mineral salts medium supplemented with 3 mM pimelate was monitored in a microtiter plate reader.

FIG 3 Growth of *E. coli* strains with lesions in biotin synthesis and uptake. The $\Delta bioH \Delta yigM::Km$ strain and variants expressing plasmid-borne *pimA*, *bioH*, or *yigM* grown in biotin-containing medium were washed and subsequently starved for biotin in nonsupplemented mineral salts medium. Upon serial 10-fold dilutions, they were spotted onto agar plates containing lysogeny broth (complex medium) or mineral salts medium without a supplement, with biotin, or with pimelate.

PimA⁺) were selected on agar plates supplemented with kanamycin and pimelate. The properties of the double mutant are illustrated in Fig. 3. The presence of PimA allowed utilization of pimelate as a precursor of biotin. As expected, the strain did not grow in either unsupplemented mineral salts medium or in the presence of 4 nM biotin. The inability of the $\Delta bioH \Delta yigM::Km$ strain to utilize traces of biotin was attributable to the lack of YigM, since the parental strain ($\Delta bioH$ with a functional *yigM*) and the complemented mutant ($\Delta bioH \Delta yigM::Km$ YigM⁺, expressing cloned *yigM*) grew on this medium. Moreover, the growth in the absence of any supplement was restored by expression of plasmid-borne *bioH* ($\Delta bioH \Delta yigM::Km$ BioH⁺), excluding second-site effects as the basis for the phenotype.

The double mutant grew on complex medium containing large but undefined amounts of biotin. This finding suggested that *E. coli* can take up biotin by an alternative transporter, provided that the vitamin is present at a high concentration, and correlates with previous observations with less well-

defined biotin-deficient mutant strains (27). To investigate this possibility, we attempted to isolate a $\Delta bioH \Delta yigM::Km$ double mutant by deleting *yigM* in the $\Delta bioH$ strain in the absence of *pimA*/pimelate. Those recombinants were readily isolated on kanamycin-containing lysogeny broth plates. The dependency of growth of the $\Delta bioH \Delta yigM::Km$ strain on the availability of biotin in the medium was assayed in liquid mineral salts medium. As shown in Fig. 4, the strain grew well on 1 μ M biotin. Very slow growth and a low final optical density were observed on 10 nM biotin. In contrast to its *yigM*-containing parental strain, the $\Delta bioH \Delta yigM::Km$ recombinant was unable to grow on 1 nM biotin. We decided to use this isolate as the reference strain in the series of experiments described below.

Solitary BioY proteins mediate biotin transport into the reference strain. Plasmids encoding eight different solitary BioY proteins, i.e., BioY proteins that are not accompanied by any recognizable T unit in their natural host, were transformed into the $\Delta bioH \Delta yigM::Km$ reference strain. Four BioY variants with re-

FIG 4 Biotin-dependent growth of *E. coli* $\Delta bioH \Delta yigM::Km$ (solid symbols) and its parental strain ($\Delta bioH$; open symbols). Cultures in mineral salts medium were analyzed in a microtiter plate reader.

FIG 5 $[^3\text{H}]$ biotin uptake of recombinant *E. coli* Δ *bioH* Δ *yigM::Km* cells expressing *bioY* alleles. (A) Buffered cell suspensions were incubated in the presence of 4 nM $[^3\text{H}]$ biotin for 3.5 h, cells were separated from the medium by filtration, and the filter-bound radioactivity was quantified by liquid scintillation counting. The values represent the means $\pm$ the standard deviations of three independent determinations with biological replicates, each analyzed in duplicate. Experiments with plasmid-free *E. coli* Δ *bioH* Δ *yigM::Km* cells resulted in a background of 0.8 ± 0.2 pmol of biotin per mg of protein. This value was subtracted from all other values. (B) The presence of wild-type and mutant BioY proteins was detected by immunoblotting with anti-FLAG antibodies directed against the C-terminal FLAG epitope of the proteins. Unusual electrophoretic mobility (e.g., caused by the formation of SDS-resistant oligomers) of BioY proteins has been observed before by us and by others (17).

placements of highly conserved charged residues in transmembrane helix VI were constructed. These residues, an aspartate and a lysine residue, were found in BioY crystals to serve as ligands to the ureido ring of biotin (7) and were identified in our recent work as essential for biotin transport activity (15). The activity of wild-type and mutant BioY proteins was first tested in biotin uptake assays with $[^3\text{H}]$ biotin. It was evident that all wild-type BioY proteins conferred biotin-uptake activity on the recombinant *E. coli* cells (Fig. 5A). The BjBioY_{K166R} and RdBioY_{K205R} variants were completely inactive. Surprisingly, the activity of the ObBioY2_{K181R} and SpBioY2_{K169R} variants was not completely abolished (data not shown). In these two cases, the conserved aspartate residue in transmembrane helix VI was replaced in addition to the conserved lysine residue to give the ObBioY2_{D178N/K181R} and SpBioY2_{D166N/K169R} variants. Cells containing one of these two variants did not accumulate radioactive biotin (Fig. 5A). As shown in Fig. 5B, all BioY variants were detectable in detergent-solubilized membranes. In SpBioY2 and BjBioY, the amino acid replacements had slight and prominent effects, respectively, on the protein's electrophoretic mobility.

We investigated whether BioY proteins can translocate biotin molecules across the cytoplasmic membrane and, hence, provide the vitamin for cellular metabolism. Recombinant Δ *bioH* Δ *yigM::Km* cells producing the various BioY variants were subjected to growth assays in liquid mineral salts medium under biotin limitation. Figure 6 clearly demonstrates that each of the eight wild-type BioYs allowed the cells to grow on 1 nM biotin, a phenotype that was correlated with a functional biotin transporter in the previous experiments (Fig. 4). Thus, the tested BioY proteins indeed transport biotin molecules into the cells. As shown in the right-hand panel of Fig. 6, mutant BioYs with amino acid replacements in the essential region within transmembrane helix VI were unable to carry out this function. This result excludes the possibility that the heterologous production of BioY proteins may render the membranes of the recombinants leaky, which could allow nonspecific entry of biotin to support growth.

DISCUSSION

Although the oligomeric architecture of ECF transporters is controversial, their overall composition of S, T, and A (ABC ATPase) units is undeniable in most cases. T and A units interact via two short amino acid motifs, each containing a central Arg residue, in a cytoplasmic helical segment of the transmembrane proteins (1, 6, 9, 10), and a groove in the ATPases formed by the Q-helix region (8, 10, 12, 13). Recent analysis of the crystal structures of two ECF transporters from *Lactobacillus brevis* identified the S units in a very unusual orientation almost parallel to the membrane. They are bound almost exclusively by the T units (12, 13).

Transport assays with reconstituted components and with whole cells, as well as growth experiments, demonstrated that the function of subgroup II transporters depends on both the S unit and the A1A2T module. Specifically, the dependency on the A1A2T components to give functional transporters for folate, pantothenate, riboflavin and thiamine was shown for the S units FolT (2, 9), PanT (9), RibU (2, 5, 8), and ThiT (2, 6), respectively. For many of these analyses, vitamin-auxotrophic plus vitamin transport-deficient *E. coli* strains were used as hosts for recombinant production of ECF transporter components. The growth of a recombinant on traces of the vitamin was considered a reliable indicator that the ECF system was able to transport the vitamin across the membrane. As mentioned above, these assays failed to demonstrate uptake activity for the solitary S units.

Comparable growth experiments have not yet been reported for recombinant *E. coli* producing subgroup I ECF transporter components. Transport assays with cells containing the ECF-type biotin or cobalt transporters from *R. capsulatus* using radioactive substrates demonstrated accumulation of the compounds by the holotransporters but also by the S units in the absence of their A and T components (4, 15, 16, 28). Likewise, expression of the solitary *bioY* from a *Chlamydia* species conferred biotin-uptake activity on *E. coli* (17). Nevertheless, the velocity of Co^{2+} transport and the affinity of the *R. capsulatus* biotin transporter for its substrate were clearly affected by the A and T units. Transport activity by a solitary S unit poses the question of how the substrate that is bound with subnanomolar affinity by those proteins can be released from the binding pocket. Oligomerization of the S unit which was observed for BioY proteins *in vivo* and *in vitro* (14, 15, 17) may provide the clue to this ability. *In vitro* analysis of biotin transport into proteoliposomes with *R. capsulatus* and *L. lactis* BioY showed binding of the vitamin to the vesicles but failed to

FIG 6 Stimulation of growth of the reference strain under biotin limitation by recombinant BioYs. $\Delta bioH \Delta yigM::Km$ cells expressing wild-type (left-hand panels) and mutant (right-hand panels) *bioY* alleles were grown in mineral salts medium with supplements as indicated. Cyan, empty vector control; black, BjBioY and BjBioY_{K166R}; dark blue, ObBioY1; light green, ObBioY2 and ObBioY2_{D178N/K181R}; red, RdBioY and RdBioY_{K205R}; brown, RnBioY; dark green, RpBioY; magenta, SpBioY1; ocher, SpBioY2 and SpBioY2_{D166N/K169R}.

demonstrate intravesicular accumulation. Hence, it was concluded that solitary BioY acts as a high-affinity binding protein that does not mediate transport (7).

In our present study, we attempted to clarify the physiological role of BioY and chose eight BioY proteins for this purpose from organisms that do not contain any other recognizable components of ECF transporters. We aimed at distinguishing between biotin binding and biotin transport by producing those BioYs in an *E. coli* strain deficient in both biotin synthesis and uptake. Since this type of strain was not available, a strategy for the selection of a

$\Delta bioH \Delta yigM::Km$ strain was developed. Biotin is required for fatty acid biosynthesis and, thus, it is an essential compound. Therefore, we considered that an intermediate of the synthesis pathway downstream of the block caused by the *bioH* deletion must be fed into such cells. As indicated in Fig. 1A, dethiobiotin, DAPA, KAPA, or a pimeloyl thioester were the candidates. Uptake of dethiobiotin may require the biotin transporter because significant inhibition of biotin uptake by dethiobiotin was observed in previous studies (29). KAPA and DAPA were identified as vitamins in cross-feeding experiments with biotin auxotrophs of *E. coli*

(30). These compounds are either not available commercially (DAPA) or are expensive (KAPA). Therefore, we decided to use the commodity chemical pimelic acid. Our results confirm earlier observations (26) that exogenous pimelate is used as a biotin precursor in *E. coli* provided that it can be converted to pimeloyl-CoA. In the presence of recombinant pimelic acid:coenzyme A ligase (PimA), the $\Delta bioH \Delta yigM::Km$ strain could be isolated without causing biotin starvation. Subsequent characterization of this strain uncovered that it can take up biotin by unknown alternative mechanisms, when the vitamin is present at a large concentration.

Uptake experiments with radioactive biotin and in particular the growth analyses presented here confirm the hypothesis that solitary BioY acts as a transporter in the absence of cognate A and T units. The transport activity may be low, but this is compatible with the organism's requirements. The biotin content of *E. coli* is as low as ~100 molecules per cell (31), and the vitamin is required for just one enzyme, the biotin carboxyl carrier protein of the multisubunit acetyl-CoA carboxylase (reviewed in reference 32).

Our results do not exclude that a significant fraction of biotin accumulated by the cells in the uptake assays with radioactive substrate remains bound to BioY on the cell surface. They clearly show, however, that in the absence of additional ECF components, BioY transports biotin molecules across the membrane in quantities sufficient for growth.

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