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A versatile *Escherichia coli* strain for identification of biotin transporters and for biotin quantification

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Biotin is an essential cofactor of carboxylase enzymes in all kingdoms of life. The vitamin is produced by many prokaryotes, certain fungi, and plants. Animals depend on biotin uptake from their diet and in humans lack of the vitamin is associated with serious disorders. Many aspects of biotin metabolism, uptake, and intracellular transport remain to be elucidated. In order to characterize the activity of novel biotin transporters by a sensitive assay, an *Escherichia coli* strain lacking both biotin synthesis and its endogenous high-affinity biotin importer was constructed. This strain requires artificially high biotin concentrations for growth. When only trace levels of biotin are available, it is viable only if equipped with a heterologous high-affinity biotin transporter. This feature was used to ascribe transport activity to members of the BioY protein family in previous work. Here we show that this strain together with its parent is also useful as a diagnostic tool for wide-concentration-range bioassays.

Keywords: biotin bioassay, ECF transporter, BioY, vitamins, ABC transporter

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Impact of Biotin

Biotin, also known as vitamin B_7 (or vitamin B_8 according to French nomenclature, or vitamin H based on the German term "Haut" for "skin") was identified as an essential nutritional component in the first half of the 20th century. It was first isolated in 1936 from egg yolk and later synthesized chemically as a racemic mixture in a multi-step process.[1,2] Chemical synthesis was optimized later to yield the biologically active D(+)-biotin enantioselectively.[3] Biotin is a cofactor of carboxylases in all kingdoms of life. Those enzymes include acetyl-CoA carboxylase (involved in fatty acid synthesis) and the mitochondrial enzymes propionyl-CoA carboxylase (involved in the degradation of odd-chain fatty acids, isoleucine, and valine), pyruvate carboxylase (involved in gluconeogenesis), and 3-methylcrotonyl-CoA carboxylase (involved in leucine degradation).[4] The vitamin is also attached to histone proteins in animals and thought to be involved in epigenetic processes and prevention of DNA damage.[5] Biotin is produced by plants and many microorganisms including fungi, bacteria, and archaea. Animals lack the biosynthetic route and depend on uptake of biotin in their diet. The latter fact makes biotin commercially important since it serves as a dietary supplement and a feed additive in livestock production. Moreover, biotin is contained in cosmetics for skin, nails, and hair. The annual worldwide production of biotin ranges in the hundreds of tons in feed, food and pharmaceutical purity with prices in the range of US$ 1000 per kg for the latter.

Biosynthesis

Biotin biosynthesis can be divided in an upper pathway resulting in a pimeloyl thioester and a universally conserved lower pathway that converts this intermediate in a four-step sequence into biotin.[6] At least three routes exist for synthesis of the pimeloyl thioester. *Bacillus subtilis* (and other bacteria) converts free pimelic acid into pimeloyl-CoA by means of a pimeloyl-CoA synthetase (BioW). Alternatively, *B. subtilis* BioI, an enzyme

of the cytochrome P450 family, produces pimeloyl-acyl carrier protein (pimeloyl-ACP) from long-chain acyl-ACPs through oxidative cleavage. A third pathway was analyzed in detail in *E. coli*. A portion of malonyl-ACP is channeled into biotin synthesis by BioC-catalyzed methylation. The resulting compound is elongated by addition of two C_2 units via the fatty acid synthesis machinery. Demethylation by BioH results in pimeloyl-ACP. Notably and curiously, fatty acid synthesis is instrumental in production of the vitamin that is essential for fatty acid synthesis. The upper-pathway reactions in eukaryotes have not been elucidated.[7] In all biotin-producing organisms the pimeloyl moiety is converted by BioF, BioA, BioD, and BioB (bacterial nomenclature) or their homologs into biotin via KAPA (7-keto-8-aminopelargonic acid), DAPA (7,8-diaminopelargonic acid), and dethiobiotin. The biotin synthase (BioB) is a member of the radical/S-adenosylmethionine enzyme family and donates a sulfur atom originating from an internal [2Fe-2S] cluster for incorporation into dethiobiotin to give biotin. The reaction leads to inactivation of the catalyst which must be repaired by iron-sulfur cluster synthesis proteins. In eukaryotes, the sulfur-incorporation step catalyzed by biotin synthase—and perhaps some other steps—occur in mitochondria.

Transport of Biotin Across Biological Membranes

Biotin uptake is required by non-producers, but in the presence of environmental biotin, even prototrophic prokaryotes shut down cost-intensive biotin synthesis and import the compound. Biotin transporters involved in uptake of the vitamin into cells were identified in mammals, fungi, and prokaryotes. In the intestine and several other tissues of mammals, dietary biotin and that produced from the intestinal microbiota is adsorbed by a sodium-dependent multivitamin transporter (SMVT).[4,8] This transporter is a membrane protein with 12 transmembrane segments and is responsible for uptake of the water-soluble vitamins biotin, pantothenate, and lipoate. The SMVT is also considered a potential target for delivery of drugs in cancer chemotherapy that are functionalized with a biotin moiety.[9] Monocarboxylate transporter 1 is considered to be another biotin-uptake system that may mediate biotin uptake into lymphoid cells and keratinocytes.[4] Proton-dependent biotin symporters act as uptake systems in the plasma membrane of the ascomycetous fungi *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.[10,11] Although the two proteins are unrelated on the sequence level, they share a 12-transmembrane helix architecture.

In prokaryotes, the molecular basis behind biotin uptake has long been unknown. A combined bioinformatic and biochemical approach led to the identification of energy-coupling factor (ECF) transporters as a widespread type of biotin uptake system.[12] ECF transporters constitute a special group of ATP-binding cassette-containing importers. They consist of a substrate-specific transmembrane protein (S unit, named BioY for biotin transporters), a moderately conserved but ubiquitous transmembrane protein (T unit, BioN), and pairs of ATP-binding cassette-containing ATPases (A units, two copies of BioM).[13] BioN plays a central role in stabilizing the BioMNY complexes, and the interaction sites between BioM and BioN have been unraveled on the molecular level.[14,15] Biotin transport activity was assigned to a lone BioY protein in the absence of its cognate BioMN module, but this conclusion was challenged by biochemical and structural studies of the purified component.[12,16] Recent in vitro and in vivo studies confirm the hypothesis, however, that (1) solitary BioY proteins indeed can transport biotin molecules across the cytoplasmic membrane in living bacteria, and (2) this activity may depend on oligomerization of these membrane proteins.[17-20]

A completely different type of biotin transporter (YigM) was identified in *E. coli*, and homologs thereof are present in other bacteria. YigM is a membrane protein with ten transmembrane helices and belongs to the carboxylate/amino acid/amine family of secondary active transporters.[21] Transport of biotin across organellar membranes in eukaryotes remains elusive. In animals, the vitamin must be exported from mitochondria into the cytosol for incorporation into cytosolic acetyl-CoA carboxylase. In plants, biotin protein ligase (also called holocarboxylase synthetase) activity that covalently attaches the vitamin to a lysine residue of the target apoenzymes, is found in the cytosol, but to a lesser extent also in mitochondria and chloroplasts.[22] This suggests that biotinylated enzymes located in mitochondria may receive their prosthetic group in that compartment. Biotin export from mitochondria into the cytosol and subsequent import into chloroplasts is a requirement for the biotin protein ligase reaction in the plastids. Plastids contain biotin-dependent acetyl-CoA carboxylase.[23]

Applications of a Biotin-Deficient *Escherichia coli* Strain

Recently, we constructed a biotin-auxotrophic and biotin transport-deficient *E. coli* reference strain to characterize activity of recombinant biotin transporters.[19] The Δ*bioH* deletion mutant contained in the Keio collection was used as the starting material.[24] This strain is unable to produce biotin since the last reaction of the upper biosynthetic pathway, the conversion of pimeloyl-ACP methyl ester to pimeloyl-ACP, is interrupted. It grows on trace levels of biotin due to uptake of the vitamin mediated by its endogenous high-affinity biotin transporter YigM. Prior to deletion of *yigM* in the Δ*bioH* background, we introduced a cloned pimeloyl:coenzyme A ligase gene (*pimA*) from a purple bacterium and introduced the corresponding plasmid into the strain. The PimA-containing Δ*bioH* cells grew in the absence of biotin on minerals salts medium supplemented with pimelate. This indicates that (1) the recombinants utilized exogenous pimelate as a biotin precursor and (2) pimeloyl-CoA can replace the natural intermediate pimeloyl-ACP. Then, we used a recombineering protocol to delete *yigM* in the Δ*bioH* ($PimA^+$) strain yielding the Δ*bioH* Δ*yigM*::Km double mutant in which *yigM* is deleted and replaced by a kanamycin resistance cassette.[19,25] For this purpose, the Δ*yigM*::Km genomic region of the corresponding strain from the Keio collection was amplified and the amplificate was electroporated into a variant of the

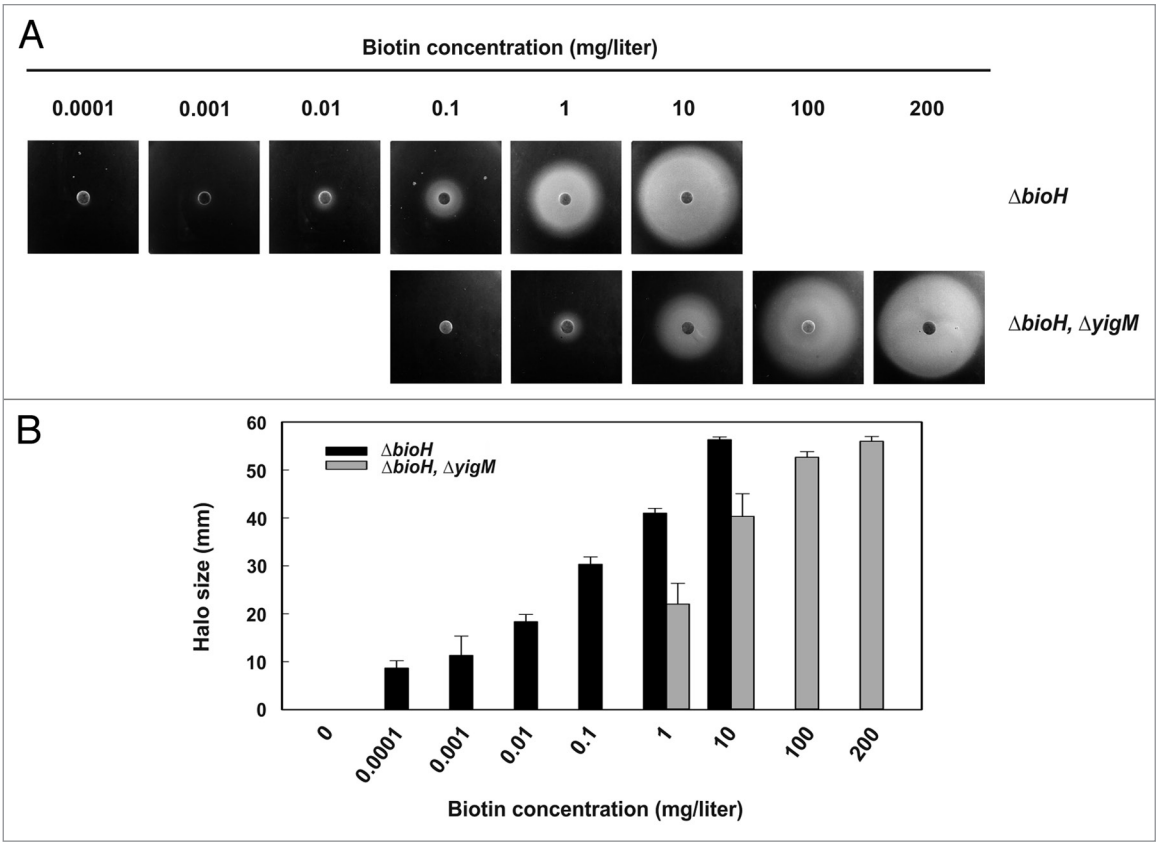

Figure 1. Bioassay for biotin quantification. (**A**) The *E. coli* Δ*bioH* Δ*yigM* strain and its parent (*E. coli* Δ*bioH*) were grown in mineral salts medium supplemented with biotin, harvested, washed and starved for biotin as described.[19] The starved cells (200 μl at OD_{600} = 1) were mixed with 5 ml of melted mineral salts soft agar (0.7% w/v agar), and the mixtures were poured onto mineral salts agar plates. Biotin solutions with the indicated concentrations were loaded on paper disks (10 μl for the Δ*bioH* Δ*yigM* strain, 5 μl for the Δ*bioH* strain), the disks were placed on the agar plates, and the plates were incubated for approx. twenty-four h at 37 °C. Diffusion of biotin into the agar allows growth of the biotin-deficient reporter strains around the disks resulting in a halo. (**B**) Correlation of halo sizes with biotin concentrations. Halo sizes of three independent assays were measured. The values represent the means of triplicate determinations ± the standard deviation. Sensitivity and detectable concentration are in a similar range as recently reported for a bioassay based on *Corynebacterium glutamicum* strains.[30]

Δ*bioH* strain that expressed the bacteriophage λ *exo bet gam* recombination genes. The Δ*bioH* Δ*yigM*::Km double mutants were selected on kanamycin-containing agar plates. The mutant cells were unable to grow on trace levels of biotin. They grew on media containing biotin at concentrations above 100 nM suggesting that nonspecific uptake at high external biotin concentrations is sufficient for growth.[19] We used the Δ*bioH* Δ*yigM* double mutant as the host for recombinant BioY proteins in order to the above-mentioned controversy whether or not the solitary S units represent functional biotin transporters. The analyses provided clear results. Eight out of 8 solitary BioYs allowed the recombinants to grow in mineral salts medium on traces of biotin (1 nM) confirming the hypothesis that BioY proteins can transport the vitamin across the membrane in the absence of a BioMN module.[19] This finding correlates with the fact that a number of BioY-containing prokaryotes lack recognizable BioMN modules or T- and A units in general.[12,13] Recent structural analyses of two ECF holotransporters uncovered that the S unit has a very unusual topology. Whereas the transmembrane helices of lone S units are oriented perpendicular to the membrane, they lie almost parallel to it when the S units are complexed with a cognate T-A module.[16,26,27] The reorientation within the membrane has been correlated with substrate translocation through the lipid bilayer. Whether or not solitary BioY proteins undergo a similar topological change even in the absence of T- and A units remains to be discovered.

Our recent work has demonstrated that the *E. coli* K-12-derived Δ*bioH* Δ*yigM* double mutant is a suitable tool to detect biotin uptake activity of recombinant transporters. For future screens for novel biotin transporters, the deletion mutations may be introduced into *E. coli* BL21 strains that are frequently used for recombinant protein production.

Bacterial and fungal strains have been used in the past as indicators in biotin quantification. Quantitative determination of biotin levels in food and food supplements, in pharmaceuticals as well as in fluids of humans suffering from biotin deficiency is crucial.[28] Bioassays represent a sensitive technique for biotin quantification.[29] Due to the limited requirements of the vitamin by microorganisms and the existence of high-affinity uptake systems, however, the upper detection limit is approximately 1 mg/ml. Biotin concentrations above this level cannot be discriminated. Recently,

the actinobacterium *Corynebacterium glutamicum* was engineered to yield an indicator strain for the determination of biotin levels up to 100 mg/l or slightly above.[30] *C. glutamicum* is a natural biotin auxotroph. Deletion of its *bioY* gene rendered the organism hyperauxotrophic resulting in an indicator strain with an increased biotin requirement. In a similar approach we used the *E. coli* Δ*bioH*/*E. coli* Δ*bioH* Δ*yigM* pair of strains in bioassays. As illustrated in **Figure 1**, the *E. coli* system is suitable for quantification of biotin levels ranging from 0.1 μg/l to approximately 200 mg/l. This range is very similar to that covered by the *C. glutamicum*-based bioassay. Our system may be expanded to allow discrimination of biotin from biotin precursors in sample fluids. In its current state, the system would respond to KAPA, DAPA, and dethiobiotin in addition to biotin which prevents selective determinations. Deletion of additional *bio* genes allows to differentiate between these compounds in the bioassay. Deletion of *bioB* (encoding biotin synthase) for instance, would result in a strain that responds to biotin selectively, because it cannot convert any precursor into the vitamin. Deletion of *bioD* (encoding dethiobiotin synthase) leads to a strain responding to both dethiobiotin and biotin and so on. In conclusion, the *E. coli* Δ*bioH* Δ*yigM* strain and variants thereof are versatile tools for biotin bioassays and searches for novel biotin transporters.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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