Identification of the biotin transporter in *Escherichia coli*, biotinylation of histones in *Saccharomyces cerevisiae* and analysis of biotin sensing in *Saccharomyces cerevisiae*

Dissertation

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1 Introduction

1.1 History of vitamins

More than 100 years ago Sir Frederick Gowland Hopkins realized from the results of his experiments with young rats that the animals could not survive from being fed with only a mixture of pure protein, fat and carbohydrates [87]. He claimed there must be some other so called "minor" or "accessory factors" that are essential for normal growth and development. The term "vitamin" was invented in 1912 by Casimir Funk. He tried to isolate a substance to heal the Beriberi disease which is caused by a lack of thiamine. As Funk found out that the substance contained an amino group he called it vitamin (vita lat. = life, amin from amino-group). Several at that time uncharacterized growth factors, although not all of them contained an amino-group were then also designated as vitamins. Today vitamins are defined as organic substances that are essentiall in small amounts because they can not be synthesized at all or not in the required quantity. Vitamins are not needed as an energy-source but fulfill functions as cofactors, antioxidants or hormone-like substances. Although only very low doses in the mg or μg range of these substances are necessary, the symptoms of a lack of vitamins can lead to metabolic defects and severe illness. For humans 13 fat- and water-soluble vitamins are known today. An overview is shown in table 1.1.

Not all of the listed vitamins are essential for all organisms. Every organism has its own special set of vitamins. Several plants and microorganisms are still able to synthesize some or even all of these substances, but most higher organisms dependend on the uptake of a certain amount from food or in part from microbial synthesis in the intestine. The relative contribution of intestinal synthesis to vitamin supply may be different for individual vitamins and in different species and can in many cases not be precisely quantified.

Vitamin (class)	active substance	function in metabolsim
fat-soluble:		
vitamin A	retinol, retinal	light perception, antioxidants
vitamin D	calciferol	regulation of calcium- and phosphate-metabolism, hormon-like
vitamin E	tocopherol, tocotrienol	antioxidants for unsaturated membrane-lipids
vitamin K	phyllochinone, menachinone	γ-carboxylation of glutamate (blood clotting)
water-soluble:		
vitamin B_1	thiamine	aldehyde-transfer
vitamin B_2	riboflavin	oxidation and reduction
vitamin B_3 (x)(niacin)	nicotine acid, nicotinamide	oxidation and reduction
vitamin B_5 (x)	panthotenic acid	transfer of acyl-groups
vitamin B_6	pyridoxin, pyridoxal, pyridoxamin	decarboxylation and transamination of amino acids
vitamin B_7 (x)	biotin	carboxylation, decarboylation
vitamin B_9 (x)	folic acid	transfer of C_1-units
vitamin B_{12}	cobalamin	transfer of methyl-groups
vitamin C	ascorbic acid	antioxidant

Table 1.1: **Overview over the 13 vitamins.** (x) Historic names, not commonly used today

1.2 Biotin

E. Wildiers discovered in 1901 that yeasts need, beyond yeast-ashes, ammonium salts and a fermentable sugar [139], a substance called "bios" for growth [207]. Another result of a lack of biotin was discovered by W. G. Bateman who found out rats, rabbits, dogs and man suffer from so called egg-white-injury from consuming an adequate diet with additional raw egg withe [8]. This can be explained by the fact, that egg white contains a high amount of the biotin-binding protein avidin that prevented absorption of this vitamin both from dietary and intestinal sources. Biotin can be synthesized by bacteria, plants and lower fungi but has to be taken up by higher organisms. Rich sources of biotin are milk, boiled eggs (or egg-yolk), liver, kidney, several vegetables and cereals, but the bioavailability can vary greatly between 100 % and 5 %. Pure biotin was isolated for the first time from Kögl an Tönnis, who purified 1,1 mg of the substance from 250 kg dried egg-yolk in a 16-step-procedure [96].

1.2.1 Structure and chemistry

The IUPAC-name of biotin is cis-hexahydro-2-oxo-1H-thieno[3,4]imidazole-4-valeric-acid. With the three asymmetric centers of the molecule eight stereo-isomers are possible, but only one isomer with the configuration (3aS, 4S, 6aR), called d-(+)-biotin is biologically acitive and occurs in nature. With its sum formula $C_{10}H_{16}N_2O_3S$ biotin has a molecular weight of 244.31 kDa, is good soluble in hot water, dilute alkalies and 95 % ethanol, but only slightly soluble in cold water, dilute acids and almost insoluble in organic solvents. Crystalline biotin is stable in air and towards sunlight, thermally stable, but instable towards UV light, oxidizing agents, strong acids and strong bases. The structure was solved in 1942 by Kögl and coworkers as well as by the group of Vigneaud at the same time [53], [20]. The chemical synthesis of biotin was first established one year later by Harris [79]. Although great efforts have been made to produce biotin by genetically modified microorganisms, the major part of industrial production still comes from chemical synthesis based on a protocol of Gerecke [64].

1.2.2 Physiological role

Insufficient supplementation with biotin can result in hair-loss, brittle nails and skin rash. This is why biotin was originally called vitamin H (for "Haut" = skin).

Undersupplementation of biotin is rare and usually caused by genetic defects that are discussed in chapter 1.5.1. Negative effects of an oversupplementation by intake of high pharmalogical doses of biotin were not described.

Biotin plays an essential role as a cofactor in enzymes catalyzing carboxylation reactions, that means it is able to transfer 1-C-bodies. Between one and five different biotin-proteins can be found in one organism [41]. Biotin occurs covalently bound to the ϵ-amino-group of a lysine residue. These carboxylases are required for reactions in several branches in cellular metabolism e.g. lipogenesis, gluconeogenesis and amino acid degradation and can be classified into three groups [174], [100]. Transcarboxylase per definition transfers a carboxyl-group from a donor to an acceptor [215]. The enzyme mainly plays a role in propionibacteria and catalyzes the reversible transfer of a carboxyl-group from methyl-malonyl-CoA to pyruvate. Products of this reaction are propionyl-CoA and oxaloacetate. Transcarboxylases enable propionibacteria to metabolize distinct carbohydrates independently of ATP [214]. Decarboxylases appear in anaerobic procaryotes that are able to decarboxylate substrates like oxaloacetate, malonate, methyl-malonyl-CoA and glutaconyl-CoA. Decarboxylation is coupled to Na^+-transport out of the cell against a concentration gradient and helps the bacteria to accumulate energy by generating a Na^+-gradient [49]. Carboxylases are the most important and most widely distributed biotinylated enzymes. They transfer a carboxyl-group from bicarbonate as a donor to different substrates, mostly organic acids and occur in the three kingdoms of live. Acetyl-CoA-carboxylase is omnipresent and catalyzes the irreversible reaction from acetyl-CoA to malonyl-CoA, the first step in fatty acid biosynthesis. Further the enzyme plays an important regulatory role in fatty acid pathway. Transcription of yeast *ACC1* was reported to be repressed by the soluble lipid precursors inositol and choline and to be dependent of transcription factors Ino2p, Ino4p, and Opilp [81]. These results demonstrated that the rate-determining step of fatty acid synthesis catalyzed by Acc1p is regulated in conjunction with phospholipid biosynthesis in yeast and so is able to affect membrane properties and function. More examples for biotin dependent carboxylases from different organisms are pyruvate carboxylase, 3-methylcrotonyl-CoA-carboxylase and propionyl-CoA-carboxylase.

The reaction mechanism shared by carboxylases, decarboxylases and transcarboxylases is a two-step reaction. In the first step, the carboxyl-group is transferred

to the N_1-atom of enzyme-bound biotin. In carboxylases, the activation of HCO_3^- by ATP is necessary for this process, whereas it is not for de- and transcarboxylases as the carboxyl-group is cleaved from a substrate. In the second step the carboxyl-group is released as CO_2 (HCO_3^-) from decarboxylases or transferred to a specific substrate by carboxylases and transcarboxylases. Often metal ions such as Mg^{2+}, K^+ or Mn^{2+} are required for enzymatic activity.

The covalentely enzyme bound biotin results from a post-translational protein modification catalyzed by biotin protein ligase (BPL) or holocarboxylase-synthase (HCS). Each organism possesses a BPL that is able to modify different target-proteins. Catalytic sites in the BPLs are very well conserved throughout biology [30] (see fig. 1.1). The biotinylation itself is a two-step-process [108]. First BPL catalyzes an attack of the oxygen atom at the carboxyl group of biotin on a phosphate of ATP. Pyrophosphate is released and the intermediate biotinyl-5'-AMP is generated. In the second step biotin is covalently coupled to the target protein. This takes place by an attack of the nucleophilic ϵ-amino-group of a distinct lysine residue on the mixed anhydride of the biotin-adenylate, so that an amide bond is formed and AMP set free.

Similar to the BPLs, also the biotinylation-domains are strongly conserved from bacteria to men (see fig. 1.2). These domains mostly locate to the C-terminal end of the protein with the modified lysine residue about 35 amino-acids from the end [41], [174]. The primary structures of the biotinylation domains show strong similarity and all contain the sequence (A)MKM. The two methionine-residues flanking the lysine are absolutely essential for biotinylation and 35 - 40 further amino acids on both sides are required for efficient modification by BPL. Other studies showed the minimal sequence that is sufficient for biotinylation by BirA *in vitro* is 13 amino acids long when the peptide is fused to a protein [175]. Minimal sequence requirements for biotinylation are hard to determine, because the biotinylation domains require a certain lenght for proper folding. Another groups identified a minimal consensus sequence of 66 - 87 amino acids occuring in biotinylation domains of biotin proteins from procaryotes [186], [41] or eucaryotes [110], [195]. Efficiency of biotinylation requires besides these minimal consensus sequence further properties. Usually each BPL most efficiently modifies apo-biotin proteins of the own organism but sometimes cross reactivites allowing modification of biotinylation domains from other organisms are possible [118], [41], [30]. Nevertheless not all combinations between BPLs and biotinylation domains of different organisms allow biotinylation [118], [3].

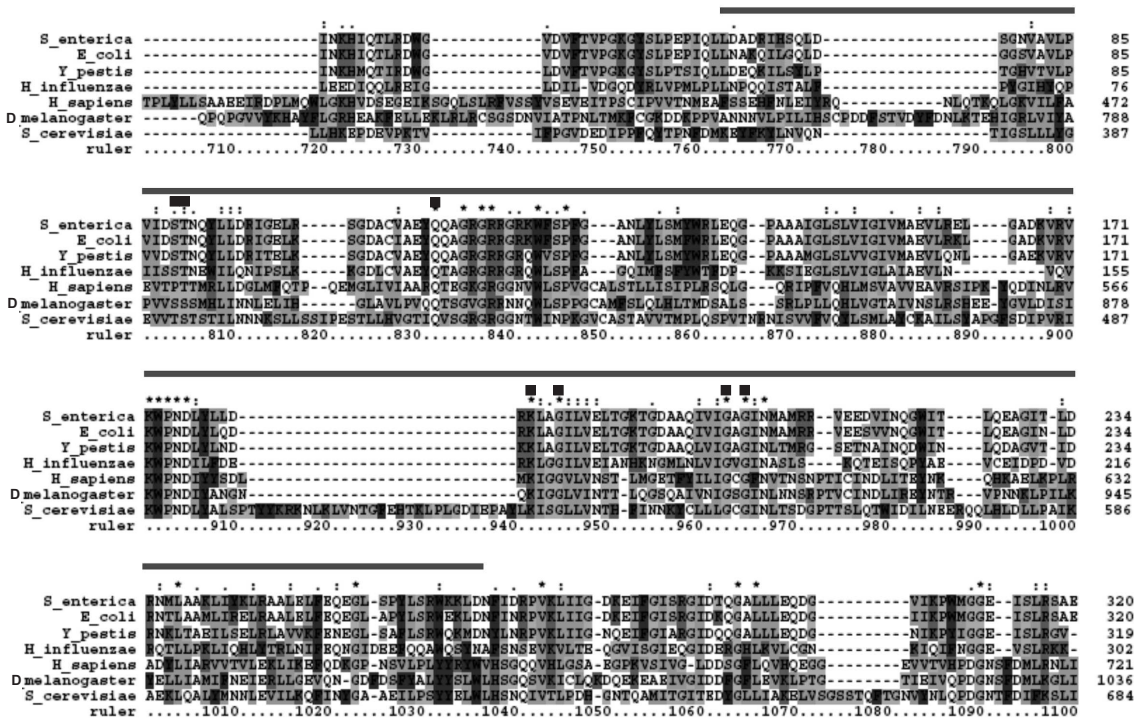

Figure 1.1: **Sequence alignment of different biotin protein ligases/holocarboxylase synthases.** The catalytic domains of the proteins are marked with a grey bar, the residues forming contacts with biotin (data taken from [208]) are marked with a black square.

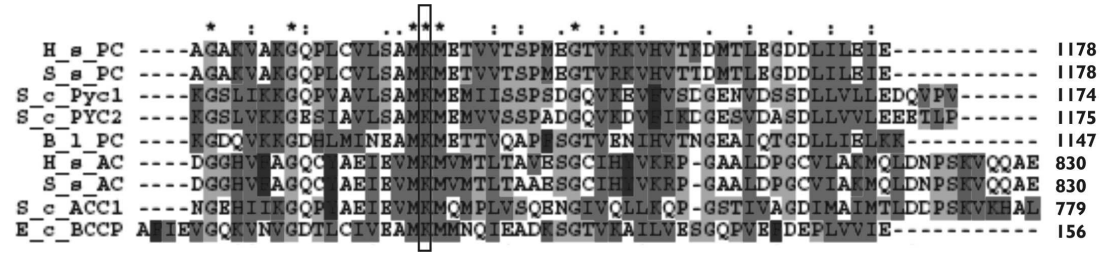

Figure 1.2: **Sequence alignment of biotinylation domains.** H_s_PC (*Homo sapiens* pyruvate carboxylase), S_ s_PC (*Sus scrofa* pyruvate carboxylase), S_c_PYC1/PYC2 (*Saccharomyces cerevisae* pyruvate carboxylase 1/2), B_l_PC(*Bacillus licheniformis* pyruvate carboxylase), H_s_AC (*Homo sapiens* acetly-CoA carboxylase), S_s_AC (*Sus scrofa* acetly-CoA carboxylase), S_c_ACC1 (*Saccharomyces cerevisae* acetyl-CoA carboxylase 1), E_c_BCCP (*Escherichia coli* biotin carboxyl carrier protein). The modified lysin-residue (K) is indicated by a black rectangle.

Beyond its function as a cofactor of carboxylases, new results suggest biotin plays also a role in gene regulation via different mechanisms that will be explained in detail

later in this work.

1.3 Biotin metabolism in *Escherichia coli*

Biotin in not essential for *E. coli* because the bacterium is capable of *de novo* synthesis. The only abundant protein covalently modified with biotin is the biotin carboxyl carrier protein (BCCP) that is part of the three-subunit enzyme acetyl-CoA-carboxylase (ACC) [71]. ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA and provides the first precursor required in fatty acid synthesis. The *E. coli* biotin protein ligase is called BirA (biotin retension) and is a 35.5 kDa protein with 321 amino acids encoded at 89.9 centisomes on the *E. coli* chromosome.

1.3.1 Biosynthesis in *E. coli*

Biotin prototrophy is wide spread among bacteria. The first common intermediate pimeloyl-CoA originates from different precursors, such as malonyl-CoA [90] or pimelic acid [16], depending on the organism. In contrast, the following four steps leading to biotin share common intermediates and enzymes and are well conserved [189] (see fig. 1.3). Five of the six involved genes (*bioA*, *B*, *C*, *D* and *F*) are organized in an operon at 17 min on the chromosome [72], [134]. Due to the fact that the operon is located in proximity of the λ-phage attachment site several mapping experiments have been possible and different biosynthesis mutants have been created [167], [36].

The sixth gene required for biotin-biosynthesis, *bioH*, can be found as a single gene at about 66 min [133], [189]. The first known precursor in the biosynthesis-pathway in *E. coli* is pimeloyl-CoA. Its synthesis depends on BioC and H, but the function of these proteins is not understood in detail yet. Pimeloyl-CoA and alanine are fused to α-keto-8-amino-pelargonic acid (KAPA) by KAPA-synthase BioF in presence of pyridoxalphosphate (PLP) as a cofactor. In a next step 7,8 di-amino-pelargonic acid (DAPA) is generated by DAPA-aminotransferase BioA. Here S-adenosyl-methionine (SAM) provides the N-atom for the reaction and again PLP serves as cofactors. *BioD* that codes for dethiobiotin-synthetase generates dethiobiotin (DTB) and CO_2 as well as ATP are required therefore. The last step to biotin is catalyzed by biotin-synthase encoded by *bioB*. Here the presence of NADPH, SAM and sulphur in form of an Fe-S-cluster is mandatory. The mechanism is a relatively uncommon radical reaction [117], [177], [189].

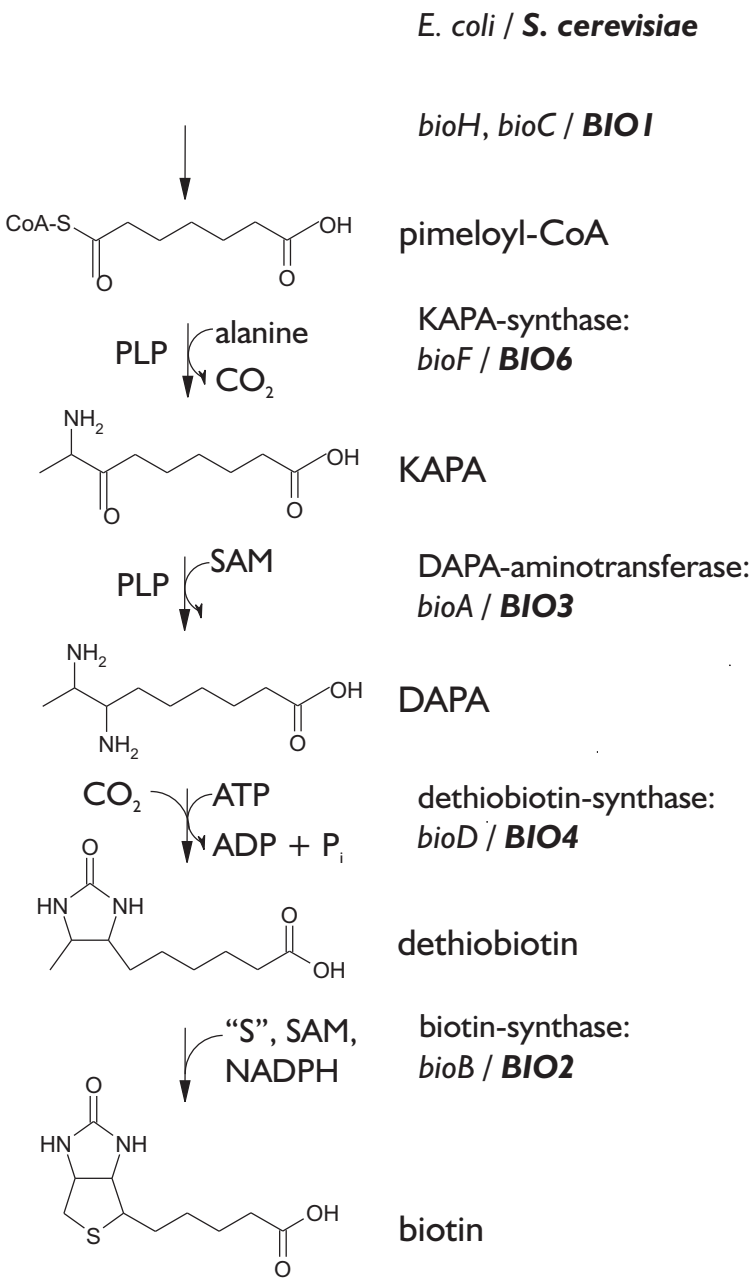

Figure 1.3: **Biotin biosynthesis in *E. coli* and *S. cerevisiae*.** Biosynthesis with the intermediates pimeloyl-CoA, KAPA (7-keto-8-amino pelargonic acid), DAPA (7,8-diamino pelargnoic acid) and dethiobiotin. Names of the enzymes and the corresponding genes are shown (bold genes for *S. cerevisiae*). The first two reactions catalyzed by *BIO1* and *BIO6* from the unknown precursor to pimeloyl-CoA and further to KAPA are only present in some prototrophic *S. cerevisiae* strains, but not in most of the laboratory strains.

1.3.2 Biotin transport in gram-positive bacteria and *E. coli*

Although a lot of bacteria are able to synthesize biotin, several of them are also able to take it up from the environment.

Biotin transport in gram-positive bacteria

The only experimentally confirmed biotin transport system in bacteria envolves the genes *bioY*, *M*, and *N* [84] (see fig. 1.4). Homologues of BioY with 95 % or more identity can be found in more than 120 genomes across firmicutes (34 species), α-proteobacteria (28 species), cyanobacteria (15 species), actinobacteria (15 species), and archaea (13 species) and form a unique protein family (pfam02632). Expression of BioY from *Rhodobacter capsulatum* in a biotin transport deficient *E. coli* mutant enables cells to take up the vitamin by facilitated diffusion with a K_M of 250 nM and a v_{max} of 60 pmol/(mg cells x min). The BioY protein has 6 predicted transmembrane domains with N- and C-terminus in the cytoplasm [84].

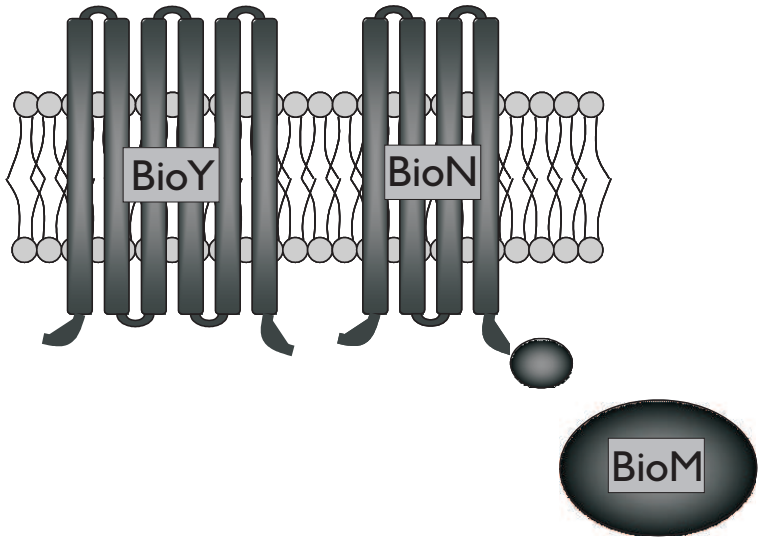

Figure 1.4: **Model of the BioMNY-system of gram-negative bacteria.** Topological model of the BioMNY-system with BioY (6 TMDs), BioN (4 TMDs and a cytoplasmic domain) and BioM (cytoplamic protein with ATPase motif). According to [84].

BioM and N can be found in about one third of the organisms that contain BioY. Coexpression of BioM and BioN with BioY change the properties of the biotin transport system: biotin uptake proceeds with a K_M of 5 nM and a v_{max} of 6 pmol/(mg cells and min), that means with higher affinity but lower capacity.

BioMNY-catalyzed uptake requires energy that is provided by ATP. BioM contains Walker A, Q loop, signature (LSGGQ), Walker B, and H motifs that are typical for ATPases. BioN contains 4 transmembrane domains and a cytoplasmic C-terminal extension with two amphiphatic helices that resemble to the EAA loop of classical ABC-transporters. Tripartite complexes of the three components could be experimentally shown, and ATPase-activity could be measured. Dimers of BioN and BioY as well as BioM and BioY were not found, whereas stable complexes of BioM and BioN could be purified, but this lacked ATPase-activity in contrast to the ternary complex [84].

Nevertheless the bioMNY system can be found in various species among bacteria and archea no homologues in the subdivisions of β-, γ-, δ- and ϵ-proteobacteria (including *E. coli*) could be found.

These modular transport systems called energy coupling factor (ECF)-systems could be identified in various organisms mainly among gram-positive bacteria and for a variety of substrates [157]. Comparative genome analysis revealed 21 families of substrate capture proteins that are responsible for substrate specificity of the systems, act as binding proteins and in some cases even allow facilitated diffusion of substrates. Additionally several energy-coupling modules composed of conserved transmembrane proteins and one or two nucleotide binding proteins similar to those of ATP binding cassette (ABC) transporters could be identified. Some substrate specific components interact only with one distinct energy-coupling module. Examples are systems for the uptake of biotin, cobalt, nickel, queosine, methionine, thiamine and methylthioadenosine. Some of them were experimentally proven [84], [156]. Other energy-coupling modules are shared by several substrate specific components. Among them systems for folate, thiamine, and riboflavin transporters were experimentally confirmed and others for pantothenate, niacin, tryptophane, cobalamin, lipoate, queosine, biotin, thiazole and pyridoxin proposed [84], [156].

Biotin transport in *E. coli*

Biotin transport in *E. coli* was first reported by C. H. Pai in 1972 and 1973 [137], [138]. He investigated the uptake of radioactively labeled biotin in wildtype cells and in a strain with a mutation in the operator of the biotin biosynthesis operon and found that biotin uptake is not affected by this mutation itself, but depends on how long cells have been starved for biotin before the uptake experiment. Pai proposed that intracellular content of biotin is the critical factor leading to stronger or weaker biotin uptake and found out, that growth in medium supplemented with 5 ng biotin per l almost abolished transport completely. In following years, the groups around Prakash and Eisenberg [151], [55], Cicmanec and Lichstein [35] and Piffeteau and Gaudry [143], [142] were successful in further characterizing biotin transport in kinetic parameters, pH- and temperature-optimum, substrate specificity and in parts energy-requirements. The mapping experiments of Eisenberg located the gene encoding the biotin transporter in the region between the *ilv*-operon for isoleucine biosynthesis and the *metE*-gene [55].

Another interesting finding was made by Walker and Altman in 2005 [199]. They reported that *E.coli* cells are able to take up biotinylated peptides up to a size of 31 amino acids in contrast to a maximum of five to six amino acids for unbiotinylated peptides. This uptake could be blocked by the extracellular addition of free biotin, avidin and the protonophore CCCP. In a transport mutant no uptake of biotinylated peptides could be observed.

Although this system has been exhaustively investigated by several groups the gene(s) coding for the *E. coli* biotin transport protein is/are still unknown.

1.3.3 Regulation

Cells have developed many mechanisms for regulation of gene expression in response to changing environmental conditions. One way of regulation is binding of a signal molecule to a kind of receptor that directly mediates the response by transcriptional regulation. The biotin regulatory system of *E. coli* that is a good example for this kind of regulation, was extensively examined and is well understood [22], [40], [12], [10], [11]. A key player in this process is the biotin-protein ligase BirA. The first mutants producing biotin at derepressed levels or requiring higher biotin concentrations have been isolated and described by Campbell [24]. BirA is not only the enzyme that is responsible for the biotinylation of BCCP, but also is

able to regulate transcription of biotin biosynthesis operon as a repressor. The N-terminal part of the protein contains a helix-loop-helix motif, that is able to bind to the operator sequence (bioO) of the bio-operon [106]. This takes place when the intracellular biotin concentration is high and most of the apo-BCCP-molecules are biotinylated. In this case biotinyl-AMP stays bound to BirA and leads to a conformational change in the molecule that favours homodimerization. The BirA homodimers are able to bind to bioO and block transcription. A maximal inhibitory effect could be observed at concentrations of 40 nm biotin [5], [56]. At lower biotin concentrations or when apo-BCCP accumulates, BirA-bound biotinyl-AMP is used for modifying apo-BCCP. This prevents dimerization and DNA-binding of BirA. Thus the transcriptional state of the bio-operon is regulated by intracellular free biotin, or accordingly, by the amount of unbiotinylated apo-BCCP in cells (see fig. 1.5).

These bifunctional properties of BirA have been conserved - with slight variation- in the *Bacillus*/*Clostridium* group, many proteobacteria, *Thermus thermophilus*, *Chlorobium tepidum* and in some archaea [158]. Apart from this mechanism Rodionov and Gelfand were able to eludicate by comparative genomic analysis that α-proteobacteria, *Rhizobiales* and *Rhodobacterales* use the GntR-family-protein BioR, that also contains a helix-loop-helix domain, but does not function in biotin transfer, for biotin-dependent gene regulation [156]. This protein binds to a conserved DNA-element designated the BIOR-box. The exact molecular mechanism and the ligand(s) binding to BioR still have to be investigated.

In addition to biotin biosynthesis, also the uptake of biotin was regulated by the biotin supply. It was maximally repressed at concentrations of 20 nM biotin in the medium resulting in a 50 % reduction of biotin-transport [142]. It is still unclear if biotin biosynthesis and biotin uptake are regulated by the same mechanism.

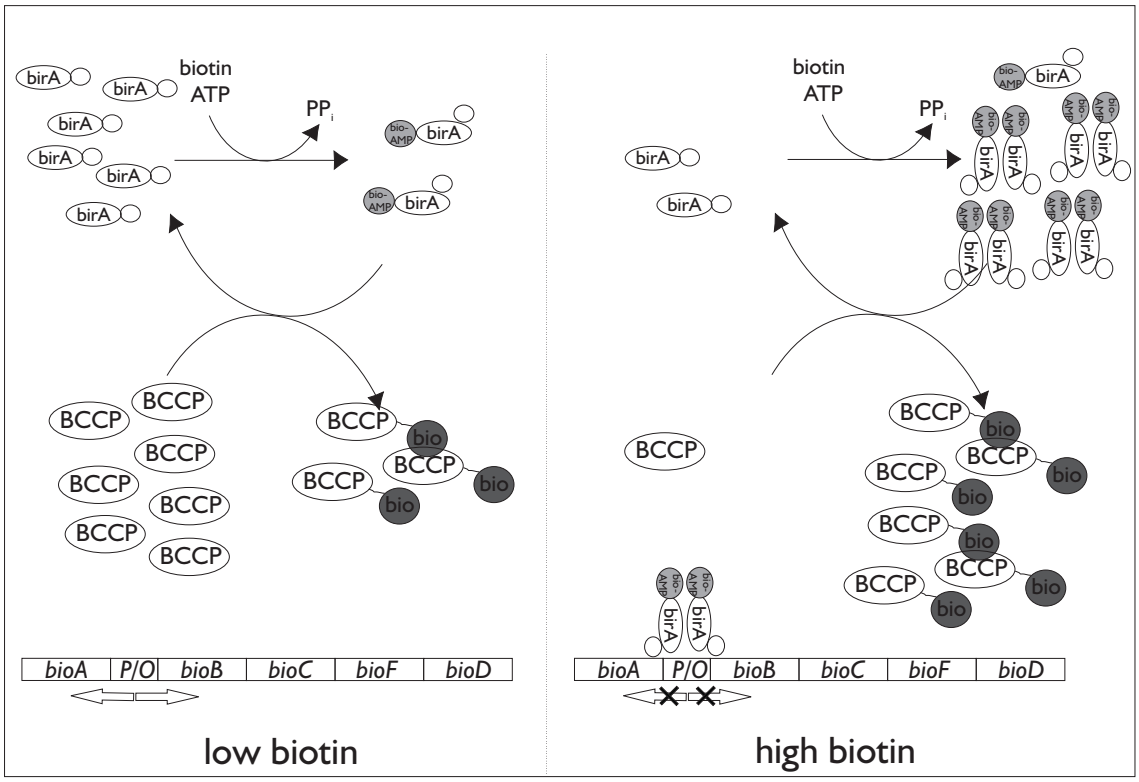

Figure 1.5: **Model of biotin dependent regulation by BirA in *E. coli*.** At low biotin concentrations the majority of BCCP is unbiotinylated and appears in its apo-form. BirA as well does not carry biotinyl-AMP and is available in monomers. It is unable to bind to DNA and transcription from the biotin biosynthesis operon can take place. At high biotin concentrations, when most of BCCP is modified biotinyl-AMP stays bound at BirA and leads to a conformational change in the protein. In this conformation BirA favours homodimerization, is able to bind to the promoter/operator region of the operon (P/O) and suppresses transcription. According to [11].

1.4 Biotin metabolism in *Saccharomyces cerevisiae*

A quite uncommon situation concerning biotin can be found in yeasts. Most yeast strains are not able to synthesise biotin *de novo*, and need to be supplemented with it. Nevertheless in most cases the cells retained the genes that are required for the last three steps of biotin biosynthesis. The biotin prototrophic yeasts used in sake brewing are exceptional as they contain two additional genes that are sufficient for *de novo* biosynthesis. These could be identified recently [216], [75]. *Saccharomyces cerevisiae* carries six different biotinylated proteins that are except of one all located in the cytoplasm. The only essential biotin enzyme is acetyl-CoA-carboxylase (Acc1p) that catalyses the reaction of acetyl-CoA to malonyl-CoA which is the first comitted step in fatty-acid elongation [81]. Pyc1p and Pyc2p are two isoforms of pyruvate carboxylase [190]. They catalyse the reaction from pyruvate to oxaloacetate, the first step in gluconeogenesis. Oxaloacetate is also required for being funnelled into the citrate-cycle and therefore has anaplerotic function. Dur1,2p is the urea-carboxylase and allows yeasts to grow on urea as sole nitrogen source. It has two catalytic activities and is able to carboxylate urea to allantoate and further hydrolases allantoate to CO_2 and NH_3 [39]. A special biotinylated protein and unique to *S. cerevisiae* is Arc1p [97]. It does not require biotin as a cofactor for an enzymatic activity, and the reason for biotinylation is still unclear. Arc1p associates with acyl-tRNA-synthetases metRS and gluRS and the corresponding tRNAs and is important for cytoplasmic localization of the complex and so enhances efficiency of the acylation-reaction [46]. The only mitochondrial biotin-protein Hfa1p is an isoform of the acetyl-CoA-carboxylase. In mitochondria, the produced malonyl-CoA is predominantly needed for lipoate-biosynthesis [86].

The biotinylation of the different proteins is carried out by biotin-protein-ligase (BPL) encoded by the gene *BPL1* [42]. Concerning the catalytic center Bpl1p shows homology to bacterial biotin protein ligases like *E. coli* BirA. The biological and kinetic properties of the enzyme are well understood [146]. Polyak *et al.* also investigated the domain strucutre of the enzyme by limited proteolysis and found two cleavage sites, one in an interdomain region and one within the catalytic site. Interestingly they found that the protein was less sensitive to proteolytic cleavage in presence of the ATP and biotin, indicating a conformational change after substrate binding. Another result was, that the N-terminal domain has no DNA-binding ability, but is essential for catalytical activity of the enzyme [146].

1.4.1 Biosynthesis

In contrast to *E. coli* most of the commonly used laboratory-yeast-strains are not capable of biotin *de novo* biosynthesis. Some fungal species completely lack biosynthesis like *Ashbia gossypii* [48], but many *S. cerevisae*-strains retained the last three steps of the pathway and are able to use KAPA, DAPA and DTB as precursors (see fig. 1.3). The first two reaction-steps from KAPA to DTB are identical to the *E. coli* pathway and catalyzed by the proteins Bio3p and Bio4p [141], respectively (see. fig 1.3). In the third and final step DTB is converted into biotin by Bio2p. This reaction takes place in mitochondria. *BIO2* was the first biosynthesis gene that was discovered in yeast in 1994 by Zhang and coworkers [221]. It shows homology to *bioB* of *E. coli* and *Bacillus*-strains. *BIO3* and *BIO4* are encoded an chromosome XIV in a gene cluster with *BIO5*. Recently two genes were reported, that promote growth of certain *S. cerevisiae*-strains on biotin-free medium [216], [75]. Wu *et al.* have found a gene with about 50 % identity to *BIO3* called *BIO6* in strains used for sake brewing. *BIO6* occurs in different copy numbers in the genome of certain strains and is involved in prototrophy for biotin. They showed that prototrophic strains became auxotrophic after a knockout of *BIO6*, indicating the gene is essential for biotin biosynthesis. But the authors could only speculate about the function of Bio6p and proposed it to act somewhere upstream of KAPA in the biosynthesis pathway, maybe as KAPA synthase, because it contains putative binding sites for SAM and PLP that are also present in the KAPA synthase *bioA* of *E. coli*. In 2007 Hall and Dietrich showed that *BIO6* is essential but not sufficient for biotin prototrophy [75]. They found an additional gene called *BIO1* that when coexpressed with *BIO6* enables an auxotrophic laboratory strains to grow in biotin free medium. Bio1p fullfills the function of pimeloyl-CoA synthase, like the *BioC-H* complex in *E. coli*. However de novo synthesis with *BIO1* and *BIO6* seems to be very slow and only promotes slow growth of cells [75].

1.4.2 Transport

Biotin transport in yeast was already described in 1969 by Rogers and Lichstein [165]. They reported an energy dependent uptake-system with a K_M of 323 nM, a temperature optimum of 30 °C and a pH optimum of 4.0 in *S. cerevisiae*. Biotin transport was stimulted by glucose, inhibited by structural analogues of biotin and was sensitve to iodoacetate and sodium azide. Yeast cells were able to accumulate

the vitamin until the intracellular concentrations exceeded the concentration in the medium by a factor of 1000.

S. cerevisae VHT1

Biotin can be taken up by Vht1p (vitamin H transporter 1), that was found by Stolz *et al.* [185]. The protein consists of 593 amino acids, has 12 putative transmembrane domains, with N- and C-terminus directed towards the cytoplasm. *VHT1* shows similarity to plasmamembrane transporter *DAL5* for allantoate, that is structurally related to biotin, and to *FEN2*, a transport protein for pantothenate. The *VHT1*-gene is located on chromosome VII and the open reading frame implements 1782 basepairs. Transport by Vht1p was shown to be specific for biotin, as structural similar substances like allantoin, allantoate, xanthine, uric acid, and urea were not able to inhibit uptake. Additionally a proton symport mechanism could be proposed, as uptake was inhibited by the protonophores CCCP and 2,4-dinitrophenol.

Sz. pombe vht1+

Beyond *S. cerevisae VHT1*, also *Sz. pombe* biotin transporter encoded by *vht1+* could be identified [184]. The protein shows only little sequence homology to *S. cerevisae* Vht1p but is able to complement a *S. cerevisae vht1*-mutant and has similar biological properties. *vht1+* encodes a protein that is predicted to contain 12 transmembrane domains. Like *S. cerevisae VHT1 S. pombe vht1+* also belongs to the familiy of allantoate transporters. Vht1+p as well enables active transport and biotin accumulation in the cell in a H^+-dependent manner and is sensitive to protonophores. The pH optimum of 4.0 is identical to that of *S. cerevisae VHT1* and the K_M of 230 nM is very similar, too. Even the substrate specificities of the two transport systems are comparable [184].

S. cerevisiae BIO5

S. cerevisiae can also utilize KAPA and DAPA in the growth medium as biotin precursors. The protein responsible for KAPA and DAPA transport across the plasmamembrane was shown to be Bio5p [141]. Bio5p contains 12 transmembrane domains and enables energy dependent uptake of the two substrates, demonstrated by glucose stimulation [141]. The K_M for KAPA is 0.8 μM. An interesting feature of *BIO5* is that it lies in a gene cluster with *BIO3* and *BIO4* on chromosome XIV. Re-

calling the biological meaning this clustering makes sence, because Bio5p enables the uptake of KAPA and DAPA that can be converted into DTB by Bio3p and Bio4p. Another reason for clustering might be that both genes were probably aquired by horizontal gene transfer [74]. So *S. cerevisiae* has, beyond taking up biotin, another alternative to obtain the vitamin, although it is not capable of *de novo* biosynthesis.

One transport component of biotin metabolism in yeast is still missing for complete understanding. Because the last step of biosynthesis from DTB to biotin, catalyzed by Bio2p is taking place in mitochondria, there has to be one or more transport protein(s) that enable(s) the cells to transport DTB into the mitochondria and biotin out of them, as it seems unlikely this exchange occurs via passive diffusion. So efforts to find the responsible gene and to characterize it still have to be made.

1.4.3 Regulation

Like in *E. coli* also in *S. cerevisiae* several genes that are associated with transport and biosynthesis of biotin are regulated by the vitamin. First evidence was shown by Rogers and Lichstein who reported, that biotin uptake was low in cells grown in medium with excess of biotin (25 μg/l), whereas high transport rates were measured in cells from medium with low biotin (0,25 μg/l) [166]. The results could be confirmed by Stolz *et al.* who showed a biotin-dependent regulation of the *S. cerevisiae* biotin transporter *VHT1* [185] and biotin transporter *vht1+* from *Sz. pombe* [184]. Hints for a regulation of the biosynthesis genes *BIO3*, *BIO4*, *BIO5* and again *VHT1* were found with a genome wide expression study by Wodicka *et al.* [210]. They showed higher abundance of mRNAs of the four genes in cells from minimal medium in contrast to a rich medium. This result was confirmed by Pirner and Stolz [144]. The same authors, as well as Weider *et al.* [201] additionally demonstrated the existence of a promoter element called BRE or VHRE (biotin response element or vitamin H response element) that mediates biotin dependent transcription of *BIO3*, *4* and *5*, as well as *VHT1* in a biotin dependent manner. The BRE is a conserved 20 bp [144], VHRE a 18 bps [201] palindromic upstream activating sequence (UAS) located between 250 and 370 bps upstream of the start codons of *BIO2*, *BPL1* and *VHT1* in *S. cerevisiae*, but also in related species like *Saccharomyces paradoxus*, *Saccharomyces mikatae*, *Saccharomyces kudriavzevii*, *Saccharomyces bayanus*, and *Saccharomyces castellii*.

Another factor required for biotin dependent regulation is VHR1 (VHT1 regulator 1) [201]. Beyond *VHR1* another necessary protein for biotin sensing is Bpl1p. Miss-

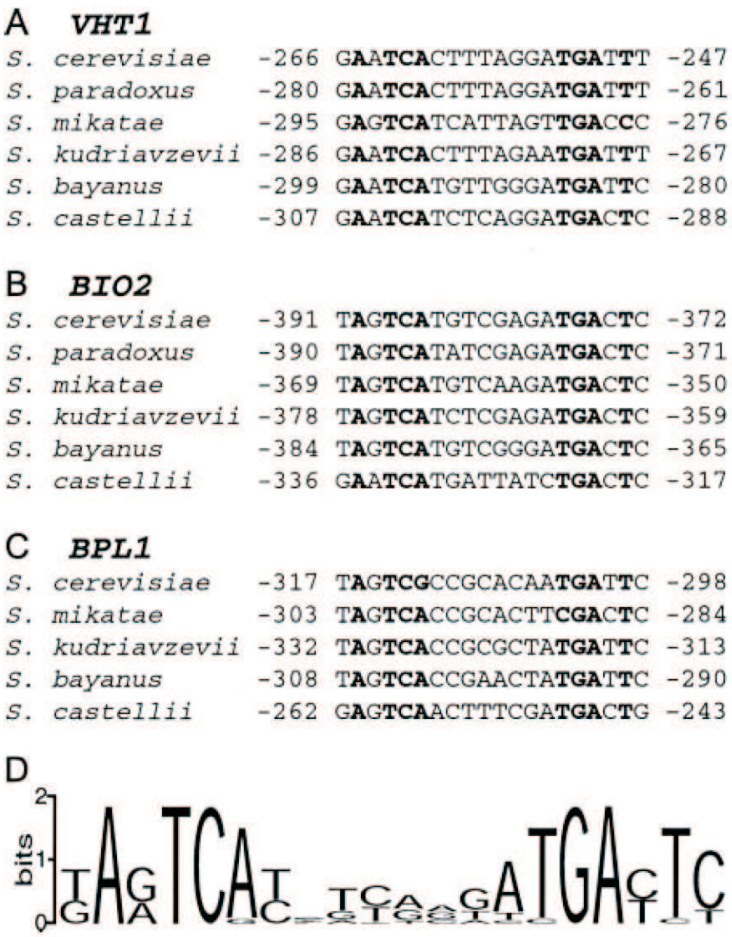

Figure 1.6: **Biotin response element (BRE) (from Pirner and Stolz [144]).** The BRE was identified in the genes of biotin permease VHT1 (A), biotin synthase BIO2 (B), and biotin-protein ligase BPL1 (C) of the related yeast species *S. cerevisiae*, *Saccharomyces paradoxus*, *Saccharomyces mikatae*, *Saccharomyces kudriavzevii*, *Saccharomyces bayanus*, and *Saccharomyces castellii*. The numbering indicates the position relative to the start ATG (where A is +1) of the gene. Positions that are identical in at least 16 of the 17 sequences are printed in bold. D: Weblogo (available online) was used with default settings to create a graphic representation of the sequences displayed in A, B and C.

ing Bpl1p-activity leads to a low-biotin signal even if high concentrations of biotin are available. If biotinyl-5'-AMP that is built by Bpl1p, is the crucial metabolite for biotin sensing, like in *E. coli* is still unknown. Another important point is that biotin sensing is independent of extracellular biotin concentration and of *VHT1*. This was experimentally proven by Pirner and Stolz with a *vht1*-mutant lacking biotin transport [144]. The mutant requires very high levels of biotin in the medium to grow,

whereas is has very a low content biotin inside the cell. Reporter assays showed that these cells behaved like in low-biotin medium, demonstrating that the intracellular concentration is crucial for low biotin sensing. Adding biotin precursors like KAPA or DAPA to the medium that and can be converted into biotin abolished the low-biotin signal. Vht1p takes no influence on the sensing [144].

In summary, yeast cells are able to sense the intracellular biotin concentration and are able to react with increased transcription of transporter genes *VHT1* and *BIO5*, as well as biosynthesis genes *BIO2*, *3*and *4* on low intracellular biotin concentration. The mentioned genes and *BPL1* contain the UAS element BRE in their promoters. Proteins essential for the biotin sensing process are the biotin protein ligase Bpl1p [144], [183] and Vhr1p [201]. Knockout mutants of *VHR1* were shown to be unable to activate expression from promoters containing the BRE element in low biotin medium. Further experiments with yeast one hybrid assays using the transcription factor Gal4p were performed by Weider. Gal4p contains a DNA binding domain (DBD) and an activating domain (AD) that are both required to drive expression from Gal4p-dependent promoter [50]. A fusion protein of the N-terminal part of Vhr1p that contains a predicted helix-turn-helix motive and the AD of Gal4p was shown to be able to activate transcription from a promoter containing the BRE. Thus Weider suggested the N-terminus of Vhr1p to be able to bind to a BRE. Activation also seemed to be biotin dependent on biotin concentrations, with weaker expression at high biotin concentrations and stronger expression at low biotin levels [202]. Fusing the DBD of Gal4p to the C-terminal part of Vhr1p resulted in expression from the *GAL1*-promoter containing four Gal4p binding sites, demonstrating the function of Vhr1p C-terminus as an AD [202]. Different biotin requirements for wt yeast cells and *vhr1*Δ cells could not be observed [202]. However, it is unclear which information is recieved by Vhr1p and this is missing for a complete understanding of the *S. cerevisiae* biotin sensing pathway.

1.5 Biotin in mammals

Biotin is, in contrast to bacteria essential for mammals and man. Although the metabolic function and metabolism is well understood, not much is known about nutritional requirements. Studying requirements is difficult, as resorption of biotin from dietary intake and from intestinal synthesis by the microbial gut flora can not be quantified or discriminated. A new field besides the functions of biotin as a cofactor is

coming up recently and there are several indications for a participation of biotin and its metabolic degradation products in different regulatory processes. Biotinylation of histones as a epigenetic modification was not established since about ten years ago. Meanwhile several biotinylation sites have been identified, but understanding of the function of this modifications is still quite scarce.

1.5.1 Biotin as a vitamin

Adults should obtain 30-100 μg biotin per day. The intake of young Brithish adults was estimated at about 35 μg per day [21], whereas intake in Switzerland was suggested to be about twice as high [78]. The vitamin can not only originate from food, but also from bacterial synthesis in the intestine [38], but how much of this biotin can be absorbed is still discussed [18], [170].

Mammalian cells contain five proteins that carry biotin as a cofactor [100], [214]. One of them is like in bacteria and yeasts acetyl-CoA-carboxylase. Cells possess one mitochondrial (ACC-1) and one cytoplasmic isoform (ACC-2). Biotin-enzymes except the ACC2-protein are located in mitochondria, which is in contrast to *S. cerevisiae*, where all biotin proteins except Hfa1p (mitochandria) are located in cytoplasm. For replenishing the citric acid cycle pyruvate-carboxylase (PC) generates oxaloacetate from pyruvate. Methylcrotonyl-CoA-carboxylase (MCC) is involved in degradation of the branched-chain amino acid leucin to acetoacetate. Methylmalonyl-CoA that can enter the citric acid cycle after being converted to succinyl-CoA results from a carboxylation of propionyl-CoA that is catalysed by propionyl-CoA-carboxylase (PCC).

In mammals the enzyme responsible for post-translational modification with biotin is called holocarboxylase synthetase (HCS) [217] and shows homology to biotin transfer-domains of *E. coli* BirA and yeast Bpl1p [191], [111]. An enzyme that is not present in *E. coli* and yeast, but in man and mammals is biotinidase. It is able to cleave biotin from the lysin residue of biotinylated proteins, peptides and biotinyl-lysin (biocytin) and so to set free covalentely bound biotin from dietary intake, as well as to recycle biotin in metabolism [37], [99]. The protein contains a signal sequence and six sequons for N-glycosylation and enables secretion of the protein to serum and pancreas where it can fulfill its function [128]. Apart from releasing biotin, biotinidase is also speculated to serve as a biotin binding an transport protein in serum [31].

Mutations in HCS or biotinidase can lead to multiple carboxylase deficiency (MCD)

which results from reduced biotinylation of the biotin-dependent carboxylases leading to reduced enzyme activities. The consequence is metabolic ketoacidosis with lactic acidosis that manifests in neurological deficiencies, skin rash and hair loss. MCD can also result from mutations in biotinidase presumably because of reduced absorption, recycling and reutilization of biotin [9], [193], [212]. Several mutations in HCS and biotinidase have been described in the literature [15], [131], [130], [129], [149], [150], [148]. HCS deficiency can be life threatening in infancy, whereas the onset of MCD with biotinidase defects tends to occur later and with milder symptoms than a loss of function of HCS [211]. MCD may also result from protein malnutrition [198], [197]. After timely diagnosis by presymptomatic screening a therapy of MCD by pharmacological doses of biotin is possible and effective.

1.5.2 Transport

Biotin can only be taken up efficiently in its free form. Biotinyl-lysine, resulting from breakdown of biotinylated proteins can not be transported, so biotinidase activity is required to release free biotin from the diet. Biotin was shown to be co-transported with sodium in a 1:1 stochiometry and transport is driven by a sodium-gradient. Competition was observed with structural analogs of biotin, such as dethiobiotin [169]. Maximal biotin absorption takes place in the jejunum, lower affinity is found in ileum and less in proximal colon [170]. The gene responsible for biotin uptake was found in 1998 by Prasad and coworkers [152]. They identified a cDNA from rat placenta termed sodium dependent multi vitamin transporter 1 (SMVT1) that is predicted to encode a 634 amino acid protein with about 69 kDa and 12 putative transmembrane domains and N-, as well a C-terminus facing the cytosol. Apart from biotin transport the protein is also capable to translocate pantothenic acid when overexpressed in Caco-2 cells [168], whereas the affinity is highest for pantothenate followed by lipoate and lowest for biotin after overexpression in HeLa cells [152]. The K_M-values for biotin and pantothenic acid are 15.1 and 4.9 μM, respectively. SMVT1 belongs to the familiy of sodium dependent glucose transporters and shows significant homology to other vitamin transport proteins, like the sodium-dependent pantothenate-transporter of *E. coli*, PanF [92] and of *Hemophilus influencae* [61]. Highest similarity is found to the mammalian iodide, glucose and myo-inositol transporters. In rat SMVT is distributed in several tissues with highest abundance of the mRNA in absorptive tissues like intestinal mucosa, kidney and placenta but also easily detectable in liver, brain, heart, lung and skele-

tal muscle [152]. An additional biotin transporter to SMVT in human peripheral blood mononuclear cells (PBMCs) has been proposed by Mardach *et al.* [115]. Possibly also monocarboxylate transporter 1 (MCT1) plays a role in biotin uptake in lymphoid cells [43], but this has not been shown for other tissues. One improtant function of MCT1 may be the transport of biotin into mitochondria as the protein was detected in mitochondrial membranes [23], [73].

1.5.3 Regulation

Regulation of gene expression by biotin is not unique to bacteria and yeasts. One of the first hints in this field came from Dakshinamurti and co-workers 40 years ago, who showed biotin deficiency caused a 40 % decrease of liver glucokinase activity in rats [44]. As expected, biotin-regulated genes include biotin transporters and biotin-dependent carboxylases [164]. Up to now the data of DNA microarrays with mostly HepG2 cells and PBMCs showed that expression of more than 2000 human genes is dependent on biotin [160], [205], [206]. It was observed that the distribution and function of the biotin-dependent genes is not at random, but genes are often clustered and e.g. genes playing a role in signal transduction and gene products locating in the nucleus are overrepresented among regulated genes. The most prominent transcription factors mediating biotin effects are nuclear factor (NF) -κB [163], the Sp/Krüppel-like factor (KLF) familiy proteins SP1, SP3 [70] and receptor tyrosine kinases [159]. The exact mechanisms and target genes following biotin signals on these transcription factors are quite complex, miscellaneous and only partially explored. Metabolites that direct a biotin signal downstream the the above mentioned mechanisms are biotinyl-AMP and cGMP [180]. According to Solorzano-Vargas biotinyl-AMP that is provided by HCS activates guanylate cyclase and so increases cGMP-levels by a yet unknown mechanism. This leads to subsequent stimulation of protein kinase G and activation of downstream targets by phosphorylation. Coincident with this model Singh and Dakshinamurti reported increased activity of guanylate cyclase in HeLa cells and fibroblast upon biotin addition to cells grown in biotin-deficient medium [179]. Apart from effects of biotin and biotinyl-AMP on gene expression there is evidence that biotin catabolites also might play a role in cell signalling [161]. The most prominent degradation products of biotin are bisnorbiotin, tetranorbiotin, biotin-d,l-sulfoxides and biotin-sulfone [119]. These can occur in similar concentration like biotin in body fluids and cells, but the mechanisms by which they can affect cell signaling are unknown. What can be excluded is competi-

tion with biotin for binding to HCS or a conversion into AMP-esters by this enzyme [194].

1.5.4 Biotinylation of histones

A quite new and interesting aspect is modification of histones with biotin. Other histone-modifications like lysine and arginine methylation, lysine acetylation, serine and threonine phosphorylation, lysine ubiquitination and poly-ADP-ribosylation are well known and their functions are at least in part understood [91], [14], [222], [32]. These modifications that can be found in various eucaryotic cells [62] occur predominantely at the N-terminal ends of the histone proteins, the so called "tails" that protrude in chromatin and are quite well accessible in contrast to the globular C-termini of the proteins that make up the protein core of the nucleosome structure.

Using synthetic peptides with the amino acid sequence of histone tails [25] and incubation with biotinidase or HCS meanwhile following distinct biotinylation sites could be identified (see tab. 1.2).

Histone	Biotinylated amino acid	Reference
H1		
H2A	K9, K13, K125, K127, K129	[33]
H2B		
H3	K4, K9, K18, (K23)	[101], [102]
H4	K8, K12	[28]

Table 1.2: **Overview over biotinylated amino acids in histones**

Insight into biological functions of biotinylated histones is still quite scarce, although some functions like regulating the expression of human SMVT [220], marking heterochromation [27] and others have been reported [34], [103],[220].

1.6 Aims of this work

The ability of *E. coli* cells to take up biotin is known for more than 35 years. Several groups collected lots of data about substrate specificity, pH- and temperature-optimum, kinetic parameters and the mode of transport but not all of these results are consistent. Although *E. coli* is one of the best understood model organisms, nevertheless until today the gene(s) for the *E. coli* biotin transporter(s) still is/are unknown.

One goal of this work was to identify the gene encoding the biotin transport protein and to further characterize it by cloning and overexpression. Therefore knockout mutants and cells overexpressing the transport gene have been used and been tested in uptake experiments with radiolabelled substrate in whole cells and membrane vesicles. Additionally the regulation of the transporter gene and biotin transport in general was studied. These assays used luciferase reporter constructs for biosynthesis genes as a reference for the transporter gene. As biotin transport genes from mammalian cells, eucaryotic unicellular organisms (*S. cerevisiae*, *Sz. pombe*) and from gram-positive bacteria and archaea are already known, the *E. coli* biotin transporter will add to the set of biotin-transporters.

In the last years new insights into biotin dependent gene regulation in yeast have been gained but some parts of the sensing pathway are still missing. Thus another aim of the present work was to fill the existing gaps of this pathway in *S. cerevisiae*. Special attention was dedicated to biotin-dependent pyruvate carboxylases 1 and 2 by investigating knockout and overexpression mutants and examining the full length and truncated versions of the protein. Monitoring expression, biotinylation and enzyme activities was part of the present work.

A third approach was to investigate protein biotinylation in *S. cerevisae*, as biotinylated histones have been found in several mammalian cells during the last decade.

2 Material and methods

2.1 Material

2.1.1 Instruments

Manufacturer	Device
Amersham	Photometer Ultrospec 3100 pro
Beckman Coulter	Allegra 64R Centrifuge
Bio101	Thermo Savant Fast Prep FP 120 (Ribolyser)
Bioblock Scientific	UV-handlamp
Biometra	T3-Thermocycler, gel documentation BiodocAnalyze
BioRad	power supplies, mini protean II gel chambers
Christ	SpeedVac RVC 2-18
Clean Air	Sterile hood CA/RE 4
Edmund Bühler	TH30, SM30-Control (Thermo Shaker)
Eppendorf	Centrifuge 5417R, Centrifuge Minispin plus, Thermomixer 5436, Thermostat 5320
Epson	Epson Scanner Epson Perfection 3200
Heidolph	Magnet Stirrer MR3000, MR3001
Hettich	Centrifuge Rotina 35R
Hielscher GmbH	(Ultrasound processor)
ICN Biomedicals	sterile hood BSB 4A
ISA Instruments	Fluoromax-2
Jouan	Speedvac RC 10.10, EB 18
Leica	Microscope DMLS
LTF Labortechnik	PP-IP-008SD gel documentation
Mettler	Balance PJ3000
Millipore	Milli Q biocel A10
Perkin Elmer	TriCarb counter
Pharmacia	Gene Power Supply GPS 200/400
Protec	Optimax 2010
Sartorius	Accuracy Balance R160P
Schott	Glass wares
Scientific Industries	Vortex Genie 2

Manufacturer	Device
Singer	System 300 Tetrad Dissection Mikroscope
Tecan	Multimode Microplate Reader Infinite 200
Thermo	Varioscan
Wolf	SANOclav table autoclav
WTB Binder	Thermo Cabinets
WTW	Digital-pH-meter pH525, Digital pH-Meter pH540 GLP

Table 2.1: **Instruments**

2.1.2 Databases, websites and software

Chemical structure drawing	ISIS Draw 2.3
Diagramms and tables	Micorosoft Excel
DNA melting point calculations	http://nature.berkeley.edu/žimmer/oligoTMcalc.html
E. coli Genetic Stock Center	http://cgsc.biology.yale.edu/
ExPASy / Swiss-Prot	http://kr.expasy.org/
Graphics	Adobe Photoshop
Hydropathy-analysis TOPCONS	http://topcons.net/
Literature search	http://www.pubmed.com
NCBI Blast	http://www.ncbi.nlm.nih.gov/BLAST/
Saccharomyces genome database SGD	http://www.yeastgenome.org/
Statistics	GraphPad Prism
Text	LaTeX
Working with DNA-sequences	DNA Strider, ApE-Plasmideditor v1.09.1

Table 2.2: **Databases, websites and software**

2.1.3 Chemicals and enzymes

If not otherwise stated chemicals were purchased from Merck (Darmstadt) in analytical grade. Cosumables were purchased from Greiner, Roth, Sarstedt and Schubert & Weiss.

Manufacturer	Chemicals
Applichem (Darmstadt)	IPTG, glycerol (87%)
Amersham Bioscience (Braunschweig)	D-(carbonyl)-^{14}C biotin
Becton Dickinson (LePont de Claix, Frankreich)	Yeast Extract, Trypton, Bacto Pepton, Bacto Casaminoacids, BactoAgar (Difco)
Biomol (Hamburg)	d-biotin
Calbiochem (San Diego, USA)	G418
Fluka (Buchs)	myo-inositol, tryptophane (nr. 93659), methionine
Fujifilm Europe GmbH (Düsseldorf)	Fuji medical X-ray film (100 NIF) Super RX
MBI Fermentas (St. Leon-Rot)	dNTPs, restriction enymes and buffers, T4-DNA ligase and buffer, Taq DNA polymerase and buffer, GeneRuler 1 kb
Millipore (Molsheim, Frankreich)	Steritop-sterile filter (500 ml), Steriflip (50 ml vacuum sterile filtration)
MP Biomedicals/Qbiogene (Heidelberg)	YNB without amino acids and vitamins
neoLab (Heidelberg)	plastic picks, sterile
New England Biolabs (Schwalbach)	alkaline Phosphatase (CIP), restriktion enzymes and buffer, Phusion High-Fidelity DNA polymerase, protein marker broad range, T4 DNA polymerase
Operon Biotechnologies GmbH (Koln)	oligonucleotides
Pall (Dreieich)	GN-6 Metricel Membran (0,45 μm pores)
Peqlab (Erlangen)	Universal agarose
Perbio Science Deutschland GmbH (Bonn)	Streptavidin Horseradish Peroxidase conjugated (Pierce), SuperSignal West Pico Chemiluminescent substrate (Pierce), SuperSignal West Dura Extended Duration Substrate (Pierce)
Qiagen (Hilden)	QIAprep Spin Miniprep Kit (50), QIAquick Gel Extraction Kit (50), MinElute Gel Extraktion Kit (50), Ni-NTA Agarose, Penta-His antibody (nr. 34660)

Manufacturer	Chemical
Roth (Karlsruhe)	acrylamide, ampicillin (Na-salt), Agar-Agar, BSA (Albumin Fraktion V), 2-ml cryotubes, ethidiumbromide, glass beads (0,25-0,5 mm), microtiterplates rotilabo 96U, 0,2-ml PCR-MikroUltraTubes, RotiR-Aqua-Phenol, RotiR-Phenol, RotiszintR eco plus, sterile filters (Rotilab 0,22 μm), tryptophane, X-Gal
Santa Cruz Biotechnology Inc. (Santa Cruz, USA)	polyclonal HA-probe (Y-11) antibody (sc-805), monoclonal HA-probe (F-7) antibody (sc-7392)
Sarstedt (Numbrecht)	3-ml fluorescence-cuvettes
Schleicher and Schuell (Dassel)	nitrocellulose-transfer-membran "Protran"
Serva (Heidelberg)	APS
Sigma-Aldrich (Steinheim)	amino acids, Anti-Mouse IgG-Peroxidase antibody (A9044), anti-Rabbit Horseradish Peroxidase conjugated (A6154), lysozyme (L2879), Triton X-100, TEMED, Ponceau S, RNAse A
Thermo Electron GmbH (Ulm)	oligonucleotides
USB (Bad Homburg)	Tris Base

Table 2.3: **Chemicals**

2.1.4 Buffers and solutions

Acrylamide (for gels)	30% (w/v)	acrylamide
	0.8% (w/v)	bisacrylamide
Bradford (5x)	0.05% (w/v)	Coomassie brilliant blue G-250
	24% (v/v)	ethanol
	50% (v/v)	H_3PO_4
Buffer A	15 mM	Tris-HCl (pH 7,4)
	80 mM	KCl
	2 mM	EDTA
	10 mM	PMSF (in DMSO) added before use

Chemiluminescence solutions:		
ECL1	2,5 mM	luminol (in DMSO)
	0,4 mM	p-coumaric acid (in DMSO)
	0,1 M	Tris/HCl pH 8,5
ECL2	0,019 %	hydrogenperoxide
	0,1 M	Tris/HCl pH 8,5)
Cell breaking buffer	2 % (w/v)	Triton X-100
	1 g (w/v)	SDS
	100 mM	NaCl
	10 mM	Tris/HCl, pH 8,0
	1 mM	EDTA
Coomassie staining solution	0.05% (w/v)	Coomassie blue R
	10% (v/v)	acetic acid
	25% (v/v)	isopropanol
Loading dye (agarose gels)(10x)	100 mM	EDTA
	60% (w/v)	glycerol
	0,25% (w/v)	bromphenolblue
	0,25% (w/v)	xylencyanol
PBS(T) (10x)	8% (w/v)	NaCl
	0,2% (w/v)	KCl
	1,44% (w/v)	Na_2PO_4
	0,24% (w/v)	KH_2PO_4 pH 7.4
	(0,1% (v/v)	Tween 20)
RNase A	10 mg/ml	Ribonuclease A
	0,01 M	NaAc, pH 5.2
		15 min at 100 °C, cool to RT,
		adjust pH with 0.1 volumes
		1M Tris/HCl pH 7.4
		store at -20 °C

Stacking gel buffer	0,139 M	Tris/HCl, pH 6.8
	0,11% (w/v)	SDS
SDS-running buffer	25 mM	Tris/HCl
	192 mM	glycerol
	0,1% (w/v)	SDS
SDS-sample buffer (4x)	250 mM	Tris/HCl, pH 6.8
	8% (w/v)	SDS
	20% (w/v)	glycerol
	20% (w/v)	β-mercaptoethanol
	0,4% (w/v)	bromphenolblue
Separating-gel-buffer(3x)	1,126 M	Tris/HCl, pH 8,8
	0,3 % (w/v)	SDS
STET-buffer	8% (w/v)	sucrose
	5% (v/v)	Triton X-100
	50 mM	EDTA
	50 mM	Tris/HCl, pH 8.0
TB-buffer	10 mM	Pipes
	15 mM	$CaCl_2$
	250 mM	KCl, adjust pH to 7.6
	55 mM	$MnCl_2$, steril filtered
TBE-buffer(5x)	445 mM	Tris/HCl, pH 8,0
	445 mM	boric acid
	5 mM	EDTA
Transfer-buffer(2x)	0,48 % (w/v)	Tris
	2,24 % (w/v)	glycin
	40 % (v/v)	methanol
	0,04 % (w/v)	SDS

Uptake-buffer	50 mM	KPO_4 pH 6.0
	10 mM	$MgSO_4$
Z-buffer	40 mM	NaH_2PO_4
	60 mM	Na_2HPO_4
	10 mM	KCl
	1 mM	$MgSO_4$ pH 7,0 (adjusted with NaOH or HCl)
	40 mM	β-mercaptoethanol (added before use)

2.1.5 Culture media

Media for *E. coli*

2TY (rich medium)	16 g/l	bacto trypton
	10g/l	yeast extract
	5 g/l	NaCl
M9 (minimal medium)	6 g/l	Na_2PO_4
	3 g/l	KH_2PO_4
	1 g/l	NH_4Cl
	0.5 g/l	NaCl
		pH 7.4
	11 mg/l	$CaCl_2$
	0.25 g/l	$MgSO_4$ x 7 H_2O
	0.34 g/l	thiamine HCl
	20 mg/l	proline
	2 g/l	glucose
SOB	20 g/l	tryptone
	5 g/l	yeast extract
	0.584 g/l	NaCl
	0.186 g/l	KCl
SOC (50x)	180 g/l	glucose
	47.6 g/l	$MgCl_2$
	60 g/l	$MgSO_4$

2TY plates were prepared with 2 % agar and M9 plates with 2 % bacto agar. For selection purposes the media were supplied with ampicillin (100 mg/l), chloramphenicol (33 mg/l) or kanamycin (10 mg/l). In case of blue/ white screening on 2TY plates, 10 mg/l IPTG and 0.004 % X-gal were used.

Media for *S. cerevisiae*

YPD (rich medium)	1 % (w/v)	yeast extract
	2 % (w/v)	Bacto peptone
	2 % (w/v)	glucose
MMA (synthetic medium)	0,67 % (w/v)	YNB w/o amino acids
	2 % (w/v)	glucose

Plates were prepared with 2 % agar. For selection purposes YPD was supplied with 100 mg/l G418. For cultivation with galactose medium, glucose in media has been replaced by the same ammounts of galactose.

Amino acids and nucleobases:

For synthetic media required amino acids and nucleobases were added after autoclaving the media. Therefore 100-fold concentrated stock solutions were prepared and autoclaved seperately. Stock solutions for tryptophane were sterile filtered and stored dark. Final concentrations of the components were: adenin, uracil, histidin, methionine und tryptophane: 20 mg/l; leucin und lysin: 30 mg/l.

Vitamins:

In synthetic media YNB without vitamins has been used and several vitamins added in standard concentrations [203], [204]. These are calcium-panthothenate (400 μg/l), folic acid (2 μg/l), inositol (2 mg/l), niacin (400 μg/l), p-aminobenzoic acid (200 μg/l), pyridoxin/HCl (400 μg/l), riboflavin (200 μg/l) and thiamin/HCl (400 μg/l). Biotin has been added as indicated. For plates with defined vitamin concentrations Difco Bacto agar was used.

Aspartate (+Asp):

Pyruvate carboxylase mutants required L-aspartate for growth that was added 40 mM with additionally 15 mM potassium hydrogenphtalate. pH was adjusted to 5.0 with KOH.

2.1.6 Organisms

E. coli strains

Strain	Genotype	Source
BL21(DE3)	F *ompT gal dcm lon hsdS*$_B$(rB mB) λ(DE3)	Invitrogen
DH5α	F *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* ϕ80d*lacZ*CΔ*M15* Δ(*lacZYA-argF*) *U169 hsdR17*(rK mK$^+$) λ	[76]
XL-1 blue	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 lac	Stratagene
BW25113 Keio-wt	F$^-$, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ^-, rph-1, Δ(rhaDrhaB)568, hsdR514	EGSC No. 10756; The Keio Collection [2]
*yigM*772Δ::*kan*	F$^-$, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ^-, rph-1, Δ*yigM*772::*kan*, Δ(rhaDrhaB)568, hsdR514	EGSC No. 10756; The Keio Collection [2]
*bioA*746Δ::*kan*	F$^-$, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ^-, rph-1, Δ*bioA*746::kan, Δ(rhaDrhaB)568, hsdR514	EGSC No. 10756; The Keio Collection [2]
*bioB*747Δ::*kan*	F$^-$, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ^-, rph-1, Δ*bioB*B747::kan, Δ(rhaDrhaB)568, hsdR514	EGSC No. 10756; The Keio Collection [2]
*yifK*746Δ::*kan*	F$^-$, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ^-, rph-1, Δ*yifK*746::kan, Δ(rhaDrhaB)568, hsdR514	EGSC No. 10756; The Keio Collection [2]
*rar*D764Δ::*kan*	F$^-$, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ^-, rph-1, Δ*rar*D764::kan, Δ(rhaDrhaB)568, hsdR514	EGSC No. 10756; The Keio Collection [2]
*bir*A1	F$^-$, [*araD139*]$_{B/r}$, Δ*(argF-lac)169*, λ^-, *TP(bioF-lacZ)501*, *flhD5301*, Δ*(fruK-yeiR)725(fruA25)*, *relA1*, *rpsL150*(strR), *rbsR22*, *birA1*, Δ*(fimB-fimE)632(::IS1)*, *deoC1*	[7]
*bir*A85	F$^-$, [*araD139*]$_{B/r}$, Δ*(argF-lac)169*, λ^-, *TP(bioF-lacZ)501*, *flhD5301*, Δ*(fruK-yeiR)725(fruA25)*, *relA1*, *rpsL150*(strR), *rbsR22*, *birA1*, Δ*(fimB-fimE)632(::IS1)*, *deoC1*	[7]

Table 2.5: ***E. coli* strains**

S. cerevisiae strains

Strains *VHR1*-3HA, -ZZ and -GFP were generated by transformation of PCR-products 3HA-*kanMX4*, ZZ-*kanMX4*, GFP-*kanMX4*, respectively and homologous recombination of the pcr-products into yeast genomes. Colonies were selected for resistance to G418 on YPD_{G418}-plates. Correct integration of constructs was confirmed by PCR with specific primers.

Strain	Genotype	Source
W303-1A	*MATa*, *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*	[192]
VHR1-3HA	*MATa*, *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*, *VHR1*-3HA-*kanMX4*	this work
VHR1-ZZ	*MATa*, *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*, *VHR1*-ZZ-*kanMX4*	this work
VHR1-GFP	*MATa*, *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*, *VHR1*-GFP-*kanMX4*	this work
YGALSNU13	*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *LYS2*, *ura3Δ0*, *kanMX6*:P_{GAL1}-*SNU13*	[51]
arc1Δ	*MATa*, *ura3Δ0*, *leu2Δ0*, *his3Δ1*, *lys2Δ0*, *arc1Δ::kanMX4*	EUROSCARF
hfa1Δ	*MATa*, *ura3Δ0*, *leu2Δ0*, *his3Δ1*, *lys2Δ0*, *hfa1Δ::kanMX4*	EUROSCARF
dur1,2Δ	*MATa*, *ura3Δ0*, *leu2Δ0*, *his3Δ1*, *lys2Δ0*, *dur1,2Δ::kanMX4*	EUROSCARF
pyc1Δ	*MATa*, *ura3Δ0*, *leu2Δ0*, *his3Δ1*, *lys2Δ0*, *pyc1Δ::kanMX4*	EUROSCARF
pyc2Δ	*MATa*, *ura3Δ0*, *leu2Δ0*, *his3Δ1*, *lys2Δ0*, *pyc2Δ::kanMX4*	EUROSCARF
vhr1Δ	*MATa*, *ura3Δ0*, *leu2Δ0*, *his3Δ1*, *lys2Δ0*, *vhr1Δ::kanMX4*	EUROSCARF
pyc1Δpyc2Δ	*MATα*, *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*, *pyc1Δ::kanMX4*, *pyc2Δ::kanMX4*	Jürgen Stolz
pyc1Δacr1Δ	*MATα*, *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*, *pyc1Δ::kanMX4*, *arc1Δ::kanMX4*	Jürgen Stolz
pyc2Δarc1Δ	*MATα*, *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*, *pyc2Δ::kanMX4*, *arc1Δ::kanMX4*	Jürgen Stolz

Strain	Genotype	Source
H2B-3HA	*ade2-1*, *ura3-1*, *leu2-3,112*, *trp1-1*, *his3-11*, *can1-100*, *HTB2*-MNase-*kanMX6*-3HA	YKM01 [122]
H3-3HA	*ade2-1*, *ura3-1*, *leu2-3,112*, *trp1-1*, *his3-11*, *can1-100*, *HHT1*-MNase-*kanMX6*-3HA	YKM04 [122]
H4-3HA	*ade2-1*, *ura3-1*, *leu2-3,112*, *trp1-1*, *his3-11*, *can1-100*, *HHF2*-MNase-*kanMX6*-3HA	YKM25 [122]
c13-ABYS-86	*MAT*α, *pra1-1*, *prb1-1*, *prc1-1*, *cps1-3*, *ura3*Δ*5*, *leu2-3,112*, *his*$^-$	[85]

Table 2.6: ***S. cerevisiae* strains**

2.1.7 Plasmids

E. coli plasmids

Name	Description	Source
pUC19	pMB1 replicon rep, *ori*, *bla*, *lacZ*α; used for subcloning of PCR-products	[218]
pPCR-Script Amp Sk(+)	pUC *ori*, *f1(+) ori*, *Amp*^{R}, *P-lac*, *lacZ*; used for subcloning of blunt end PCR-products in EcoRV-site	Stratagene
pBluescript II SK(-)	*ori*, *bla*, *lacZ*α	Stratagene
pBS-P$_R$	P$_R$ promotor of the λ-phage (PCR-product Daniela) ligated over *SalI-HindIII*-sites into pBluescript II SK(-)	Daniela Kolmeder, TUM
pBS-P$_R$-*yigM*	Ligation of *yigM*-ORF (PCR-product SR62-SR64, *HindIII-NcoI*)and T7-terminator (PCR-product SR67-SR69 *NcoI-EagI*) into *HindIII*- and *EagI*-sites of pBS-P$_R$	this work
pBS-P$_R$-*yigM*-HA	Ligation of *yigM*-HA (PCR-product SR62-SR63, *HindIII-NcoI*)and T7-terminator (PCR-product SR67-SR69 *NcoI-EagI*) into *HindIII*- and *EagI*-sites of pBS-P$_R$	this work
pBS-P$_R$-HA-*yigM*	Ligation of HA-*yigM*-T7-terminator (PCR-product SR69-SR64, *HindIII-EagI*) into same sites of pBS-P$_R$	this work
pBS-P$_R$-*yigM*$_{CO}$	Codon-optimized version of *yigM* cloned as *HindIII-NcoI*-fragment into pBS-P$_R$ *HindIII-NcoI*-fragment after restriction of pBS-P$_R$-*yigM*	this work

Name	Description	Source
pBS-P_R-*yigM*-S28R	PCR-products S1039-*yigM* SR70-SR85 (*HindIII-PstI*) and pcr wt-*yigM* SR84-SR64 (*PstI-NcoI*) ligated into same sites of pBS-P_R	this work
pBS-P_R-*yigM*-P243S	PCR-products wt-*yigM* SR70-SR85 (*HindIII-PstI*) and pcr S1039-*yigM* SR84-SR64 (*PstI-NcoI*) ligated into same sites of pBS-P_R	this work
pBS-P_R-*yigM*-S28R P243S	PCR-product S1039-*yigM* SR70-SR64 (*HindIII-NcoI*) ligated into same sites of pBS-P_R	this work
pET24a(+)	expression vector, pBR322 origin, f1 origin, kan^R, *lacI*, T7 promoter, T7-epitope (N-terminal), His-epitope (C-terminal), T7 terminator	Novagen
pET24-$yigM_{CO}$	*XbaI-SacI* fragment of $yigM_{CO}$ ligated into same sites of pET24a(+)	this work
pET24-$yigM_{CO}$w/o C	*NdeI-NcoI* fragment from PCR-product T7-SR97 ligated into same sites of pET24a(+)	this work
pQE60	Expression vector, T5 promoter, lac operator element, 6His-taq coding sequence, mcs, λt_0 terminator, *rrnB* T1 terminator, ColE1 *ori*, Amp^R	Quiagen
pQE60-birA-6His	*birA*-PCR-product (SR90-SR92) cloned in pQUE60 over *NcoI*- and *BglII*-site; fusion of BirA with 6His	this work
pDEW20	Reporter construct with promoterless *luxCDABE* gene-cluster from *Photorhabdus luminescens* for bioluminescence emission, *ori* from pBR322, *bla*, T14 terminator	[196]
pDEW201-P*bioB*	PCR product of the *bioB*-promoter (SR77-SR74) inserted into pDEW201 as *BamHI-EcoRI* fragment	Carina Mann, TUM
pDEW201-P*yigM*	PCR product of the *yigM*-promoter (SR75-SR76) inserted into pDEW201 as *BamHI-EcoRI* fragment	this work

Table 2.7: Plasmids used in *E. coli*

S. cerevisiae plasmids

Name	Description	Source
YCplac33	*ori*, *CEN*, Amp^R, *URA3*	[67]
YEplac195	*ori*, *2µ*, Amp^R, *URA3*	[67]
YEplac195-GFP	plasmid for C-terminal GFP fusions	Jürgen Stolz
p416-Met25	*CEN*, *URA3*, Amp^R, *MET25*-promoter, *CYC1*-terminator	ATCC 87324 [124]
pUG36	*ori*, *CEN*, Amp^R, *URA3*	[127]
p3HAT-kanMX5	cloning vector with 3HA epitope and *kanMX*-cassette	Jürgen Stolz
pZZT-kanMX5	cloning vector with ZZ epitope and *kanMX*-cassette	Jürgen Stolz
pGFPT-kanMX5	cloning vector with GFP and *kanMX*-cassette	Jürgen Stolz
pMELβ2-fragment D	VHT1-promotor PCR-fragment (bp -286 to -223) in pMELβ2	Heike Stüer
YEplac112-pMEL-VHT1-lacZ	pMEL-VHT1 promotor (*HindIII-SphI*)-fragment from PCR-product HP15-HP16 of pMELβ2-fragment D fused to *lacZ* (*SphI-XbaI*) in YEplac112-lacZ	Heike Stüer
YEplac195-pMEL-VHT1-GFP	*HindIII-BamHI*-fragment (pMEL-VHT1) of PCR-product JST83-SR19 integrated into same sites of YEplac195-GFP	this work
p416-Met25P-*pyr1+*	Ligation of *AvrII-XhoI*-fragment for PCR-product JST361-JST362 into *XbaI*- and *XhoI*-sites of p416-MET25.	this work
YCplac33-3HA	YCplac33 with 3HA inserted as *HindIII-MfeI* fragment into *HindIII-EcoRI* of YCplac33	Jürgen Stolz
YCplac33-*PYC1*-3HA	Ligation of *PYC1 SalI-NcoI*-fragment from pRS316-*PYC1*, *PYC1 NcoI-XbaI*-fragment from PCR-product SR27-SR28 and YCplac33-3HA *XhoI-XbaI*-fragment	this work
YCplac33-*PYC2*-3HA	Ligation of *PYC2*(*HindIII-Mph1103I*)-fragment from pRS316-*PYC2* and (*Mph1103I-XbaI*)-fragment from PCR-product SR25-SR26 in YCplac33-3HA(*HindIII-XbaI*)-fragment	this work

Name	Description	Source
pUG36-Met25P-3HA	N-terminal 3HA tagging plasmid. *CEN URA3*. Carries the *MET25* promotor. Constructed by ligation of *XbaI-BamHI* fragment from PCR product JST363-JST364 into the same sites of pUG36	Jürgen Stolz
pUG36-Met25P-3HA-*VHR1*	*VHR1* cloned as *NotI-XhoI* fragment from PCR-product JST366-JST367 into same sites of pUG36-3HA-MET25P	this work
pUG36-Met25P-3HA-*PYC2*	*PYC2* cloned as *NotI-XhoI* fragment from PCR-product SR34-SR35 into same sites of pUG36-3HA-MET25P	this work
pUG36-Met25P-3HA-*PYC2*-(-2)	*BamHI-XhoI*-fragment from PCR-product SR42-SR47 ligated into same sites of pUG36-Met25P-3HA-*PYC2*	this work
pUG36-Met25P-3HA-*PYC2*-(-6)	*BamHI-XhoI*-fragment from PCR-product SR44-SR47 ligated into same sites of pUG36-Met25P-3HA-*PYC2*	this work
pUG36-Met25P-3HA-*PYC2*-(-10)	*BamHI-XhoI*-fragment from PCR-product SR46-SR47 ligated into same sites of pUG36-Met25P-3HA-*PYC2*	this work

Table 2.8: Plasmids used in *S. cerevisiae*

2.1.8 Oligonucleotides

Name	Sequence (5'→ 3')	Description
HP15	ATATGCATGCCGTCGTTGC TTTTATTACCG	antisense primer before lacZ-Startcodon in pMELβ2 with *SphI*-site
HP16	ATTCCCGAATTGGGAAGC	sense Primer at HindIII-site in pMELβ2
JST83	GCGGATAACAATTTCACACAGG	forward primer binds 25 bp away from *HindIII*-site in pUC19-polylinker
JST361	ATATAGCGGCCGCCTAGGATGA CTTCTAAATATGATGCGTTGTTGC	for cloning *S. pombe pyr1+* with *NotI*- and *AvrII*-sites for cloning into *XbaI*- and *XhoI*-sites of p416-MET25; used with JST362
JST362	ATATGCGGCCGCTCGAGTTACTC GTGTTCAAGAACAGCACAC	for cloning *S. pombe pyr1+* with *NotI*- and *XhoI*-ends; used with JST361
JST363	ATTCTAGAAAAAATGGCATACCC GTACGACGTCC	upstream oligo for amplification of 3HA tag for making N-terminal fusions in pUG36
JST364	ATGGATCCTGCGGCCGCGCCGG CGTAATCCGG	downstream oligo for amplification of 3HA tag for making N-terminal fusions in pUG36
JST366	ATATGCGGCCGCAAACGGTCCT CCAACATTCACTC	used with JST367 to amplify *VHR1* (NotI/XhoI) for cloning into pUG36-3HA
JST367	ATATCTCGAGTTATCAAAGTAAA GGCTGGAAAACCG	used with JST366 to amplify *VHR1* (NotI/XhoI) for cloning into pUG36-3HA
SR19	ATATGGATCCCATTTTCGTCG TTGCTTTTATTACCG	reverse primer for amplifying *VHT1*-promoter to fuse with GFP in frame over *BamHI*-site (contains AAA-ATG directly upstream of *BamHI*-site)
SR25	GATACTGGGATTAACGTTGAGC	forward primer for amplification of C-terminal *PYC2*-fragment; used with SR26
SR26	TATATCTAGACTTTTTTTGGGA TGGGGGTAGGG	reverse primer for C-terminal 3HA-tagging of *PYC2*, for ligation in YCplac33-3HA over *XbaI*-site

Name	Sequence (5'→ 3')	Description
SR27	GGAAAGATTCTTGGACAAGCC	forward primer for use with SR28 to amplify C-terminal end of *PYC1* with natural *NcoI*- and introduced *XbaI*-site for fusion with 3HA, used with SR28
SR28	TATATCTAGATGCCTTAGTTTCA ACAGGAACTTG	reverse primer for C-terminal 3HA-tagging of *PYC1*, for ligation in YCplac33-3HA over *XbaI*-site; used with SR27
SR34	ATATATGCGGCCGCCAGTAGCAA GAAATTGGCCGG	forward primer for ampfication of *PYC2* from YCplac33-*PYC2* for N-terminal tagging in pUG36-Met25p-3HA; contains *NotI*-site, starts after ATG of *PYC2*
SR35	ATATCTCGAGTTATTACTTTTT TTGGGATGGGGG	reverse primer for ampfication of *PYC2* from YCplac33-PYC2 for N-terminal tagging in pUG36-Met25p-3HA; with natural plus additional stop-codon just before *XhoI*-site
SR42	ATATCTCGAGTTATTATTGGGAT GGGGGTAGGGTTTC	reverse primer for deletion of last two amino acids of PYC2p, used with SR47
SR44	ATATCTCGAGTTATTATAGGGTT TCTTCTTCTAGGACAACCAAC	reverse primer for deletion of last six amino acids of PYC2p, used with SR47
SR46	ATATCTCGAGTTATTATTCTAGG ACAACCAACAAATCTGATGC	reverse primer for deletion of last ten amino acids of PYC2p, used with SR47
SR47	CGCTTTAGAATGTTGGGGTGG	forward primer for C-terminal truncated versions of *PYC2* in pUG36-3HA-*PYC2*, used with SR42-46
SR62	ATATAAGCTTGTGGCGCTACTTA TCATCACC	forward primer for amplification of *E. coli yigM*, contains *HindIII*-site, used with SR63
SR63	ATATCCATGGTTACGCGTAGTCA GGCACGTCGCCTTCGCTCAGC GCGC	reverse primer for amplification of *E. coli yigM* with HA-tag, contains *NcoI*-site, used with SR 62
SR64	ATATCCATGGTTATTCGCTCAGC GCGC	reverse primer for amplification of *E. coli yigM*, contains *NcoI*-site, used with SR 62
SR67	ATATCCATGGAATAACTAGCATA ACCCCTTGGG	forward for amplification of T7-terminator, contains *NcoI*-site, used with SR68

Name	Sequence (5'→ 3')	Description
SR68	GCCACGCTTCCCG	reverse for amplification of T7-terminator, binds at 3770-3784 in P_R-YjdL behind *SapI*- and *EagI*-sites, used with SR67
SR69	ATATAAGCTTATGGGCGACGTGC TTGACTACGCGGGCGCGGGCGC GGCGCTACTTATCATCACCACG	forward primer for amplification of HA-*yigM*, ATG-start-tag(MGDVLDYA)-linker(GAGA)-*yigM*, natural start-GTG of *yigM* deleted, contains *HindIII*-site, used with SR64
SR70	ATATAAGCTTTCTAGAATGGCG CTACTTATCATCACC	forward primer for amplification of *yigM*, with natural start-GTG replaced by ATG-start-codon, contains *HindIII*-site and *XbaI*-site, used with SR64
SR74	ATATGGATCCGGGGCTTCTCC AAAACG	reverse primer for amplification of *bioB*-promoter, contains *BamHI*-site, used with SR73
SR75	ATATGAATTCTCTAGATCGTTC TTTATTTGGTCAGTTGT	forward primer for amplification of *yigM*-promoter, contains *EcoRI*-site, used with SR76
SR76	ATATGGATCCAATGGAAAACTCT TTGATTAACGG	reverse primer for amplification of *yigM*-promoter, contains BamHI-site, use with SR75
SR84	GCAAACCACGCTGCAGTGGGG CATTCTGGTGTT	mutation primer for cloning *yigM* from S1039, introduces *PstI*-site in *yigM* ORF over silent mutation, used with SR64
SR85	AACACCAGAATGCCCCACTGCA GCGTGGTTTGC	mutation primer for cloning *yigM* from S1039, introduces *PstI*-site in *yigM* ORF over silent mutation, used with SR70
SR90	TATACCATGGATGAAGGATAAC ACCGTGCC	forward primer for amplification of *birA*, used with SR92
SR92	ATATAGATCTTTTTTCTGCACTA CGCAGG	reverse primer for amplification of *birA*, used with SR90
SR97	TATACCATGGTTATTATGCGGTC TGGCTGCTAC	reverse primer for C-terminal deletion of $yigM_{CO}$ (last 11 aa DDRRRDCALSE missing), used with T7 primer on pET24-$yigM_{CO}$
T7	GCGTAATACGACTCACTATAGGGC	forward primer for amplification of $yigM_{CO}$ from pET24-$yigM_{CO}$, used with SR97

Table 2.9: Oligonucleotides

2.2 Methods

2.2.1 Cell maintainence

Culturing cells

Liquid cultures for *E. coli* were grown aerobically in shakers at 37 °C, plates aerobically in incubators at 37 °C over night on 2TY rich, or for 24 h on M9 minimal media. Cultures for *S. cerevisiae* were grown aerobically on plates incubated at 30 °C for several days, and liquid cultures aerobically under shaking at 30 °C. Cells from glycerol stocks were thawed and grown on YPD rich medium before cultivating them on MMA minimal media. Cell concentrations were measured over optical density at 600 nm (OD_{600}). For *S. cerevisiae* cells 1 ml of an OD_{600} corresponds to about 10^7 cells.

Glycerol stocks of *E. coli*- and *S. cerevisiae*- strains

Freshly grown cells from 2TY- or YPD-plates for *E. coli* and *S. cerevisiae*, respectively, were resuspended in the same medium with 15 % glycerol, frozen in liquid nitrogen and stored at -80 °C.

2.2.2 Molecular biology methods

Standard methods

Molecular biology standard methods (agarose-gel-electrophoresis, digestion with restriction enzymes, ligation) were performed as described in [173]. DNA was extracted from agarose gels QIAGEN gel exctraktion kits as indicated by the manufacturer.

Chemical preparation of competent *E. coli* cells

3 ml 2TY medium were inoculated with the strain and incubated under shaking overnight at 37 °C. From each strain 50 ml SOC medium were inoculated with 0.5 ml overnight culture and incubated till an OD_{600} of 0.2 - 0.3. 35 ml of these cultures were transfered into a sterile 50 ml tube and kept on ice for 15 min. Afterwards cells were centrifuged for 5 min at 4 °C and 3500 g. The supernatant was discarded and cells were carefully resuspended in 35 ml ice cold 100 mM $CaCl_2$ solution. After an 20 min incubation on ice the cells were centrifuged for 5 min at 4 °C and 3500

g. Supernatant was again discarded and cells were resuspended in 3 ml ice cold 100 mM $CaCl_2$ solution. Cells are then ready for transformation. In case the competent cells were stored at -80 °C, 105 μl DMSO were added, cells kept on ice for 15 min, then aliquoted in 100 μl and finally frozen in liquid nitrogen.

Preparation of highly competent *E. coli* cells

10 ml 2TY were inoculated with a single colony of cells and incubated in a shaker at 37 °C over night. 250 ml SOB-medium with SOC was inoculated to an OD_{600} from this overnight culture and incubated in a shaker at 18 °C until an OD_{600} was reached (12-18 h). Cultures were cooled on ice/water under slow shaking for 10 min and harvested (10 min, 2500 g, 4 °C). Cells were resuspended in sterile TB-buffer, incubated on ice for 10 min and centrifuged again (10 min, 2500g, 4 °C). Cell pellets were resuspenden in 20 ml of cold, sterile TB-buffer, 20 % glycerol were added, cells frozen in liquid nitrogen in 400 μl aliquots and stored at -80 °C.

Transformation of *E. coli* cells

Competent cells were thawed on ice and stored for 5-10 min on ice after addition of DNA. After at 30 sec heat pulse at 42 °C cells were cooled for 1 min on ice and subsequently mixed with 1 ml 2TY. Incubation at 37 °C for was performed, cells centrifuged 1 min at 13,200 g, resuspended in 100 μl 2TY and plated on selective plates.

Integration of T7 polymerase into the *E. coli* chromosome

For expression of *yigM* from the T7-based expression system of pET24 λDE3 prophages were introduced into the *E. coli* chromosome using a λDE3 Lysogenization Kit (Novagen) according to manufacturers instructions.

Transformation of *S. cerevisiae* cells

Transformation of yeast cells was performed as described previously [66], [68].

High efficiency transformation: 50 ml YPD were inoculated to an OD_{600} of 0.5 from an overnight culture and incubated in a shaker at 30 °C until OD_{600} of 2 was reached. Cells were harvested (RT, 4000 g, 3 min) washed with water, resuspended in 1 ml 100 mM LiAc and transfered to a 1.5 ml Eppendorf tube. After pelleting cells (RT, 13,200 g, 10 sec) supernatant was discarded and pellets resuspendend in 400 μl

100 mM LiAc. For each transformation 50 μl of these cells were used. 240 μl of 50 % PEG, 36 μl 1 M LiAc, 50 μl carrier DNA (2 mg/l in TE, freshly boiled) 34 - x μl H_2O and x μl DNA (usually 2 μl plasmid DNA or 20 μl PCR-product) were added and mixed thoroughly by vortexing. Tubes were incubated for 30-60 min at 30 $^\circ$C, 20 min at 42 $^\circ$C and cooled on ice. Cells were pelleted by centrifugation (RT, 5,000 g, 2 min), supernatant discarded, pellets carefully resuspended in 100 μl sterile H_2O and plated on selective plates. If cells were selected in G418 transformation assays were mixed with 1 ml YPD and incubated for 4 h at 30 $^\circ$C before plating.

Quick transformation: For quick transformation of plasmid-DNA the protocol given before was modified. Freshly grown yeast cells from a plate were resuspended in 1 ml H_2O, pelleted and resuspended in 1 ml 100 mM LiAc. After a 5 min incubation at 30 $^\circ$C, cells were distributed to 1.5 ml tubes, mixed with PEG, LiAc, carrier-DNA and DNA as describes above and carefully resuspended. Heat shock and plating was again performed as described before.

2.2.3 Methods with DNA

Plasmid isolation from *E. coli*

1 ml of an overnight culture with cells containing the plasmid was transfered to an Eppendorf tube, centrifuged 1 min at 13,200 g and supernatant discarded. 500 μl STET buffer were added to the cells and vortexed until the pellet was completely resuspended. For cell lysis 100 μl lysozyme (10 mg/ ml in STET buffer) were added, the cells were resuspended and suspension was incubated at 95 $^\circ$C for 2 min. Tubes were then centrifuged for 5 min at 13,200 g and afterwards pellets were removed with a sterile toothpick and discarded. 400 μl isopropanol were added to the supernatant containing the DNA and the latter precipitated. Samples were vortexed and centrifuged for 5 min at 13,200 g. Supernatant was discarded and the pellet was washed with 200 μl 70 % ethanol and subsequently centrifuged for 5 min at 13,200 g. The pellet containing DNA was then dried in the Speedvac. 100 μl ribonuclease A (100 μg/ ml) in TE-buffer were added and incubated for 5 min at 65 $^\circ$C shaking. Tubes were centrifuged for 3 min at 13,200 g and supernatants containing plasmid DNA used for further analysis. For more pure plasmid purifications (e.g. for sequencing) QIAprep Spin Miniprep Kits were used according to manufactureres instructions.

Isolation of genomic DNA

5-10 ml culture in 2TY medium was grown overnight at 37 °C to saturation. Cells were harvested, resuspended in 0.5 ml H_2O, transferred into an Eppendorf tube and centrifuged for 30 s at 13,200 g. Supernatant was discarded and the pellet resuspended in the remaining liquid. 200 μl lysis buffer, 200 μl phenol : chloroform : isoamylalcohol (25 : 24 : 1) and 0.3 g glass beads with 0.5 diameter were added before the sample was placed in the ribolyser for 30s at level 5. Subsequently 200 μl TE buffer (10 mM Tris pH 7,0; 1 mM EDTA) were added and the cell lysate mixed carefully. Afterwards tubes were centrifuged for 5 min at 13,200 g. 200 μl from the aqueous supernatant were mixed with 1 ml 100 % ethanol by inverting the tube. Precipitated DNA was centrifuged for 2 min at 13,200 g and supernatant discarded. The humid pellet was resuspended in 400 μl TE buffer with 4 μl RNAse A (10 mg/ml) and incubated at 65 °C for 5 min. After incubation 1 ml 100 % ethanol and 10 μl 4M NH_4Ac were added and mixed by inverting the tube. Pellet were collected by centrifugation for 2 min at 13,200 g and dried in the speedvac. Dried genomic DNA was resuspended in 50 μl water.

Polymerase Chain Reaction (PCR)

PCR-assays with Taq-Polymerase contained 50-100 ng DNA, 1x ThermoPol-buffer (New England Biolabs), dNTPS (0.25 mM each), primer (100 pmol, each) and 0.5 μl polymerase in a 25 μl-assay. Reactions with Phusion High-Fidelity PCR Kit (Finnzymes) were performed according to manufacturers instructions.

Colony-PCR

Colony-PCR was used for fast control of *E. coli* transformants. Single colonies were collected from plates with sterile toothpicks and transfered to PCR-tubes. With the same toothpick a 2 ml 2TY overnight cultures with corresponding antibiotic was inoculated for plasmid isolation. PCR assays for cells were completed with 1x ThermoPol buffer, 0.25 mM dNTPs (each), 30 pmol primer (each) and 1U Taq polymerase in a final volume of 15 μl. Subsequently a PCR with 20 cycles was run.

Measurement of DNA concentration

DNA was diluted in water (1:20) and concentration was measured in a quartz cuvette with a UV-photometer from 240 nm to 290 nm. The quotient A260(DNA)

/ A280(protein) informs about the purity of the DNA sample. Ratio should ideally be 1.8.

DNA ethanol precipitation

PCR products for EMSAs were purified by ethanol precipitation. 1/10 volume 3 M NaAc pH 5.0 and 3 volumes 100 % ethanol were added to the PCR samples. DNA was precipitated by storage for 2 h at -80 °C or overnight at -20 °C. The samples then were centrifuged at 4 °C and 25,000 g for 15 min supernatant was discarded. Pellets were washed with 70 % ethanol and samples centrifuged at 4 °C and 25,000 g for 5 min. Supernatants were discarded and the DNA pellet dried in a speedvac. DNA was resuspended in 30 μl water.

DNA-sequencing

DNA-samples were sequenced by SEQLAB (Göttingen) or GATC (Konstanz). *E. coli* mutant S1039 [199], [84] was sequenced by Prof. Dr. Thomas Eitinger (Humboldt-Universität zu Berlin, Institut für Biologie/Mikrobiologie) who kindly shared the sequences with us.

Codon optimization of *yigM*

The *yigM*-ORF was codon-optimized for expression in *E. coli*. Optimization and and synthesis of the optimized gene $yigM_{CO}$ were performed by GENEART Regensburg.

2.2.4 Methods with proteins

S. cerevisiae protein extracts

For preparation of *S. cerevisiae* protein extracts all steps were performed on ice and with cold solutions and centrifuges. 5 OD_{600} of cells were harvested and washed with TE-buffer (25 mM Tris pH 7.0, 5 mM EDTA) and transfered to a 1.5 ml tube. Supernatant was discarded and about 3 g glass beads (diameter 0.5 mm), 100 μl SDS-sample buffer and 1 μl 1 M PMSF (in DMSO) added. Cells were broken in a ribolyser (30 sec, level 5). Subsequently again 1 μl 1 M PMSF (in DMSO) was added and samples boiled for 2 min at 95 °C. For analysis of membrane-proteins

(Vht1P, Bio2P) samples were only heated to 42 °C for 2 min. After centrifugation (1 min, 13,200 g) supernatant was removed and used for further analysis.

Preparation of *S. cerevisiae* nuclear extracts

Yeast nuclear extracts were prepared with slight modifications according to [176]. All steps were carried out on ice and with cold buffers and centrifuges. 50ml YPD were inoculated to an OD_{600} of 0.1 from an overnight culture and grown at 30 °C with shaking until OD_{600} reached 0.6. Cells were washed with water, three times with buffer A and were transfered to a 1.5 ml tube. After resuspension in 350 μl buffer A and addition of 350 μl glass beads (diameter 0,75 - 1 mm) cells were broken in a ribolyser (30 sec, level 5). A hole was drilled into lid and bottom of the 1.5 ml tube and the tube placed onto a 15 ml Falcon tube. Nuclear extracts were collected by centrifugation (2000 g, 2 min), resuspended, transfered to a new 1.5 ml tube and again centrifuged (13,200 g, 2 min). Supernatant was discarded. Nuclear extracts were washed with 1 ml buffer A and used immediately for further analysis or frozen in liquid nitrogen and stored at -80 °C.

SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE)

Proteins were analysed by (SDS-PAGE) as described previously [107]. Gels were run with 120 V until the dye had reached the seperating gel and subsequently run with 200 V. Molecular weight marker was purchased from New England Biolabs (Protein Marker Broad Range). Staining of gels with Coomassie was performed as described [173].

Determination of protein concentrations

The Bradford protein assay was applied for spectrometric protein concentration measurements. In a cuvette 1 ml Bradford solution, 90 μl water and 10 μl protein were mixed for concentration measurements. The sample was incubated for 5 min at 25 °C and afterwards absorption was measured at 595 nm. As reference 1 ml Bradford solution with 100 μl water were applied. Protein concentration was determined with the help of a calibration curve which was established with known protein concentrations of BSA.

Western-blot analysis

Immunologic determination of proteins was performed by western-blot analysis. After separation by SDS-PAGE proteins were transfered to nitrocellulose membranes by wet-blot for 25 min at 360 mA. Membranes were stained with Ponceau S for loading control and detection of protein molecular weight maker. Membranes were blocked for 1 h in PBST with 1 % (w/v) skim milk powder. Antibodies/antisera or streptavidin-peroxidase were incubated for 1 h at room temperature or overnight at 4 °C in PBST with 1 % (w/v) skim milk powder in following dilutions (see tab. 2.10). Membranes were subsequently washed 3x 15 min with PBST with 1 % (w/v) skim milk powder. If necessary secondary antibodies were incubated 1 h at room temperature on the membranes and again washed 3x 15 min with PBST with 1 % (w/v) skim milk powder. Finally membranes were washed for 5 min with PBS and detected with home made chemiluminescence-solutions (1 ml of ECL1 and 1 ml ECL2 mixed for each membrane) or SuperSignal West Dura Extended Duration Substrate (Pierce) on X-ray films.

antibody/serum	dilution	source
primary:		
anti-HA polyclonal (sc-805)	1:2500	Santa Cruz Biotechnology
anti-Bio2p	1:2500	U. Mühlenhoff and R. Lill; Marburg
anti-Vht1p	1:1000	[144]
penta-His	1:2500	Quiagen
anti-biotin-peroxidase	1:2500	Sigma-Aldrich
secondary:		
anti-rabbit IgG	1:5000	Sigma-Aldrich
anti-mouse IgG	1:5000	Sigma-Aldrich
streptavidin-peroxidase	1:5000	Pierce

Table 2.10: **Antibodies, antisera and strepatvidin-peroxidase**

Expression and purification of BirA-6His

BirA-6His was expressed from a pQE60-vector under control of a lac-inducable promoter. 500 ml 2TY medium containing 100 μM biotin were inoculated with an OD_{600} of 0.1 and incubated at 25 °C until an OD_{600} of 0.6 was reached. Induction was accomplished by addition of 0.1 mM IPTG and cells were further incubated at

25 °C for 4 h. After expression the 500 ml cell culture were transfered into 50 ml tubes and centrifuged at 3,500 g and 4 °C for 15 min. Supernatants were discarded and the pellets were stored at -20 °C. Pellets of 500 ml culture were resuspended in 50 ml ice cold buffer (20 mM KPO_4 pH 7.0, 500 mM NaCl, 30 mM imidazole) and centrifuged at 4 °C and 3,500 g for 10 min at 4 °C. The pellet was again resuspended in 50 ml ice cold buffer (20 mM KPO_4 pH 7.0, 500 mM NaCl, 30 mM imidazole, 1 mg/l lysozyme) and divided into two 50 ml tubes and cells kept on ice for 15 min. Cell lysis was performed with an ultrasound processor with pulses of 0.5 s and amplitude of 0.6 for 30s on ice. This procedure was repeated a several times until turbidity of the cell suspension disappeared. Lysates were centrifuged twice for 15 min at 10,000 g, supernatant collected and filtered through a filter with 45 μm pore size. His-tagged protein was purified by affinity chromatography with a Ni-Sepharose column (Ø 0.9 mm) on an Amersham ÄKTA-FPLC. Column was washed with buffer (20 mM KPO_4 pH 7.0, 500 mM NaCl, 30 mM imidazole) and protein was eluted with the same buffer containing 300 mM imidazole. Protein expression and purification was carried out with assistance of Carina Mann de Oliveira during her Bachelor thesis.

2.2.5 Reporter-genes

Luciferase measurements

3 ml M9 minimal medium with appropiate biotin concentraions were inoculated to an OD_{600} of 0.1. Cells were grown under shaking at 37 °C to an OD_{600} of 0.7, harvested (13,200 g for 30 sec) and transferred to a 1.5 ml tube. Cell pellets were washed by resuspending the pellet in 1 ml uptake buffer followed by centrifugation as above. Cells were then resuspended in uptake buffer at desired OD_{600} for bioluminescence measurements. Detection was performed in a white 96 well polystyrene plate with a transparent flat bottom and each well contained 100 μl sample. Samples were mearsured in a TECAN Multimode Microplate Reader Infinite 200. Plates were shaken for 10 s with a 2 mm amplitude before readout of the OD_{600} and luminescence with an integration time of 6000 ms. Luminescence values are given as luminescence per OD_{600}.

GFP-reporter assays

A 63 bp sequence of the *VHT1*-promoter inserted in a pMELβ2-minimal promoter as described [183] was used for driving expression of GFP. Cells containing GFP-reporter plasmids were inoculated in MMA minimal medium with indicated biotin concentrations from an overnight culture in MMA + 2 mg/l biotin to an OD_{600} of 0.1. After growth for 6 h under shaking at 30 $^\circ$C cells were harvested (3 min, 3500 g), washed with water and adjusted to an OD_{600} of 10 in water. GFP-fluorescence of 100 μl of these cells were measured in 96 well polystyrene plates in a Varioscan plate reader. Fluorescence was excited with light of 396 nm and emission measured at 518 nm. Signals are given in fluorescence per OD_{600}. Cells with an empty vector were substracted as control.

β-galactosidase- (lacZ)-reporter assays

LacZ-reporter assays were performed after modifications of Kaiser [94] as originally described previously [123]. A 63 bp sequence of the *VHT1*-promoter inserted in a pMELβ2-minimal promoter as described [183] was used for driving expression of the *lacZ*-reporter-gene. Corresponding yeast strains from an overnight culture in MMA minimal medium with 2 mg/l biotin were inoculated in MMA minimal medium and different biotin concentrations (as indicated) to an OD_{600} of 0.1. After incubation under shaking for 6 h at 30 $^\circ$C cells were harvested. β-galactosidase-activities were measured from yeast protein extracts that were prepared as described before, unless cells were broken in KPO_4-buffer and extracts not boiled. Protein concentrations of extracts were determined with Bradford-assays as described. For β-galactosidase acitivity measurement 800 μl Z-buffer, 200 μl ONPG (4 mg/ml in Z-buffer) und 100-x μl KPO_4-buffer were mixed in 1.5 ml tubes and preincubated for 5 min at 37 $^\circ$C. Tests were started with addition of x μl protein extract and incubated at 37 $^\circ$C until solutions turned to yellow. Reactions were stopped by addition of 400 μl 1 M Na_2CO_3 and samples centrifuged (1 min, 13,200 g). Absorptions of supernatants were measured at 420 nm in a photometer. One unit (U) of β-galactosidase acitivity is defined as hydrolysis of 1 μmol ONPG per min and mg protein bei 37 $^\circ$C. Activity was calculated according to Lambert Beer's law. Therefore reaction volumes of 1.5 ml and the molar extinction coefficient of ONP (ϵ_{420} = 4500 $M^{-1}cm^{-1}$) were taken into account.

2.2.6 Electrophoretic mobility shift assays (EMSA)

EMSA was performed in a 12 % polyacrylamide gel in TBE buffer (see tab. 2.11 for composition of gels).

Component	Ammount
TBE	3 ml
30 % acrylamide	1.8 ml
80 % glycerol	187.5 μl
water	840 μl
10 % APS	45 μl
TEMED	3 μl

Table 2.11: **Composition of native gels for EMSA**

For binding of BirA to promoter fragments indicated ammounts of promoter-DNA and BirA-6His-protein were used. Binding buffer contained final concentrations of 10 mM Tris/HCl pH 7.5, 200 mM KCl, 100 μM biotin, 1 mM ATP, 2.5 mM $MgCl_2$ according to the protocol of Abbott and Beckett [1]. Samples were incubated on ice for 45 min before addition of 10x loading buffer (40 % glycerol, 0.35 % bromophenol blue 250 mM Tris/HCl pH 7.5). 20 μl of samples were loaded on each lane and electrophoresis was performed at a constant voltage of 200 V at 4 $^\circ$C for 1.5 h. DNA was stained with ethidiumbromide on the shaker for 20 min and afterwards gels were destained with water. Bands were detected under UV light.

2.2.7 Pyruvate carboxylase activity measurements

Pyruvate carboxylase activities were determined from *S. cerevisiae* according to Duggleby *et al.* [54]. Yeast protein extracts were prepared as described. Buffer used for activity tests contained final concentrations of 100 mM Tris/HCl pH 7.5, 2.5 mM ATP, 7 mM $MgCl_2$, 10 mM pyruvate, 20 mM $NaHCO_3$, 0.24 mM NADH and 1.2 mU malate dehydrogenase (MDH). 950 μl of this mixture were mixed with 50 μl of protein exctract in a polystyrene cuvette and degradation of NADH determined by measuring absorption at 340 nM every 20 sec for 20 min at room temperature. Slopes of linear parts of the resulting diagramms were calculated. Controls for internal oxidation of NADH by other factors that pyruvate carboxylases that are present in yeast cell extracts were made by repressing biotin dependent acitvity of pyruvate carboxylases with avidin. Therefore each extract was in parallel to standard assays preincubated with avidin (1U per 50 μl extract) for 5 min, NADH oxidation

monitored as described and slopes for this curves substracted from slopes for corresponding standard assays. Pyruvate carboxylase activites were calculated according to Lambert-Beer's law directly from these corrected slopes with taking into account the molecular extinction coefficient of NADH (ϵ_{340} = 6,000 $M^{-1}cm^{-1}$. Acitivties are given in units (1 U = synthesis of 1 μmol oxaloacetate per min) per mg protein.

2.2.8 Biotin uptake experiments

Overnight cultures with *E. coli* wt or *yigM*Δ cells (containing expression plasmids) were grown im M9 minimal medium without biotin (containing appropriate antibiotics) under shaking at 37 °C. Cultures for uptake experiments were inoculated from these overnight cultures in M9 minimal medium without biotin to an OD_{600} of 0.1 and grown under shaking at 37 °C until and OD_{600} of 0.7 was reached. If necessary cultures containing *yigM* on a pET24-plasmid and controls were induced with 0.5 mM IPTG at OD_{600} of 0.2. Cells were pelleted at 4 °C for 3 min at 3500 g, washed with cold water and cold uptake buffer and pelleted again as before. Subsequently they were resuspended in uptake buffer to an OD_{600} of 10 and stored on ice. Uptake assays were performed in uptake buffer in volumes of 500 μl with an appropriate ammount of cells. Tubes were incubated under stirring at 37 °C for 1 min before adding 1 mM glucose to energize cells. After one more min tests were started by addition of 100 nM $^{[14]}$C- or $^{[3]}$H-biotin if not stated otherwise. Samples of 60 μl were taken at indicated timepoints, cells filtered on GN-6 Metricel membranes (pore size 0.45 μm), washed with 100 mM NaCl and filtered again. Radioactivity was determined with a liquid scintillation counter.

2.2.9 Isolation of membrane fractions of *E. coli* and reconstitution in membrane vesicles

Cells with appropriate strains were grown overnight in M9 minimal medium without biotin under shaking at 37 °C. Cultures for membrane fraction isolation were inoculated to an OD_{600} of 0.05 im M9 minimal medium without biotin. Expression of *yigM* from pET24 was induced with with 1 mM IPTG at an OD_{600} of 0.1 and cells further incubated in the shaker at 37 °C until OD_{600} of 1 was reached ($\approx$ 3 - 4 h). Cells were harvested (4500 g, 10 min, 4 °C) and washed with lysis-buffer (10 mM Tris pH 6.5, 1 mM DTT, 0.5 mM EDTA, 10 mM PMSF). Subsequently cells were resuspended in lysis buffer, 1 mg/l lysozyme added and cells kept on ice for 15 min. Lysis of cells was accomplished by sonification (0.5 sec pulses, amplitude 0.6) until

lysates became clear. Membranes were seperated by centrifugation (30 min, 110,000 g, 4 °C), washed with lysis buffer and centrifuged as before. Supernatant was discarded and membranes resuspended in 10 mM Tris pH 6.5, 20 % glycerol, 1 mM DTT and protein concentrations determined with Bradford-reagent as described.

To generate a proton gradient, prepared membranes were fused with proteoliposomes containing cytochrome c oxidase ([52], [187]). Cytochrome c oxidase (1 mg, purified from bovine heart) was reconstituted with 40 mg *E. coli* lipids (total extract, Avanti Polar Lipids, Alabaster, AL, USA) as described previously [77].

These proteoliposomes with cytochrome c oxidase, or E. coli lipids without cyotochrome c oxidase were mixed with *E. coli* membrane preparations (2 mg lipid + 100 μg membrane protein) a final volume of 250 μl. 1 mM $MgSO_4$ was added and samples freezed in liquid nitrogen and thawed at room temperature. Subsequently sonication for 3 sec at low energy followed. Uptake assays were performed with 500 μl samples (proteoliposomes diluted in uptake buffer) at 37 °C under stirring. After 1 min of preincubation uptake was started by addition of 100 mM (final concentration) radiolabeled $^{[3]}$H-biotin if not stated otherwise.

In case of use of proteoliposomes with cyotochrome c oxidase proton gradients were established by addition of 20 mM ascorbate, 200 μM TMPD (N,N,N',N',Tetramethyl-p-phenylenediamine) and 20 μM cytochrome c (from equine heart, Sigma), which energizes the cytochrome c oxidase to transport H^+ outside of vesicles. Only cytochrome c oxidase inserted in the right orientation will interact with the cytochrome c, which is applied outside. Fresh ascorbate was added (1:50) when the assay solution had turned blue due to oxidized TMPD.

Aliquots of 25 μl were drawn at indicated timepoints, diluted with 2 ml 100 mM LiAc and filtered on GN-6 Metricel membranes (pore size 0.45 μm). Filters were washed again with 2 ml 100 mM LiAc and radioactivity counted with a liquid scintillation counter. Uptake rates are given in pmol biotin per mg membrane protein.

2.2.10 Statistics

Biotin uptake experiments were analyzed by non-linear regression. Ammounts of uptaken biotin from 0.5 to 2 min of each experiment were tested for linearity ($\geq$ 95%) and the resulting uptake velocities (represented by the slopes of uptake curves) analyzed for significant differences. Values from reporter assays with GFP were tested on signifcancy by t-test or one-way ANOVA (Tukey-test).

3 Results

3.1 Biotin uptake in *E. coli*

To identify candidate genes for the *E. coli* biotin transporter, homology searches with genes encoding biotin transport systems from other organisms like SMVT from mammals, *VHT1* from *S. cerevisae*, *vht1+* from *Sz. pombe* in the *E. coli* genome were performed. Searches did not lead to high-scoring homologues.

For modular transport systems that were identified among many species in gram-positive bacteria and archea no homologues in the subdivisions of β-, γ-, δ- and ϵ-proteobacteria (including *E. coli*) could be found [157]. Homology search with *B. subtilis bioY* in *E. coli* did not lead to genes with high homology. In conclusion this results suggested, *E. coli* must contain a new transport system for biotin.

3.1.1 Candidate genes

Eisenberg *et al.* were able to generate four classes of *E. coli* mutants resistant to α-dehydrobiotin, a toxic structure analogon of biotin via random mutagenesis with nitrosoguanidine [55]. One group showed derepressed levels of the biotin biosynthetic enzymes, the second one showed lesions in the *bio*-operon and a third one general permeability defects and weak growth. The fourth one was affected in biotin uptake and desiganted *bioP*. Mapping by P1-phage transduction experiments revealed the *bioP*-mutation lying at 75 min on the *E. coli* chromosome between the *ilv*-operon and the *metE*-gene, but much closer to *metE* [55]. So a search for genes encoding proteins with at least 10 predicted transmembrane-domains as candiadate-genes for the biotin-transport protein in the *ilv-metE* region was carried out. Transmembrane domains were predicted by TOPCONS. As shown in fig. 3.1 and tab. 3.1 three candidate genes were identified.

To analyze if these candidate genes have any influence on biotin transport, uptake experiments with $^{[14]}$C-labeled biotin and knockout-mutants for the three mentioned genes and a corresponding wt-strain were performed. In this experiment *yifK*Δ and

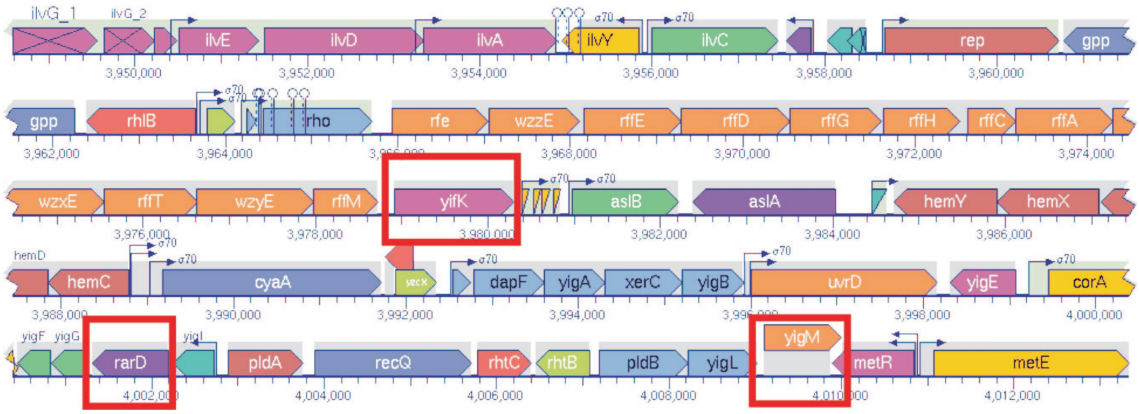

Figure 3.1: **Region between *ilvG* and *metE* on the *E. coli* chromosome.** Open reading frames between *ilvG* and *metE*. Candiate genes for the biotin transport protein with 10 or 12 TMDs are marked with a red box.

gene name	TMDs	annotation on ECOCYC
yifK	10	uncharacterized member of the APC superfamily of amino acid transporters
rarD	10	predicted chloramphenicol resistance permease
yigM	10	putative uncharacterized transport protein

Table 3.1: **Candidate ORFs for the *E. coli* biotin transporter.** Candidate genes from the *ilv-metE* region with their predicted TMDs and annotated predicted gene functions from the ECOCYC-database (www.ecocyc.org)

rarDΔ showed similar uptake to the wildtype, whereas biotin uptake in *yigM*Δ, that lies closest to *metE*, was abolished (see fig. 3.2).

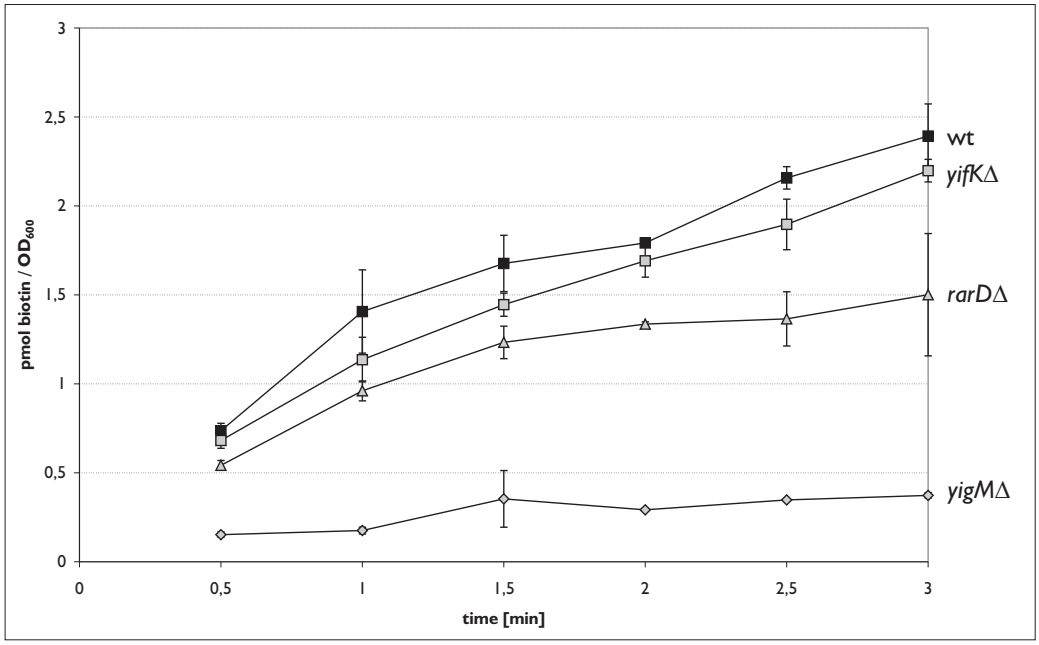

Figure 3.2: **Uptake experiments with *E. coli* wt cells and single knockout mutants of the candidate genes.** Biotin uptake in *E. coli* wt, *yifK*Δ, *rarD*Δ and *yigM*Δ mutants cells has been measured. The experiments were performed in 50 mM KP_i-buffer pH 6.0 with 10 mM $MgCl_2$ and with 100 nM $^{[14]}$C-biotin. Tests were started by adding biotin and aliquots taken every 30 seconds for 3 min. The mean values of three independent experiments are shown. Bars indicate standard deviations. Mutants were purchased from Coli Genetic Stock Center (CGSC).

3.1.2 *In silico* analysis of YigM

The YigM-protein consists of 299 amino acids with a molecular weight of 33727 Da. The 12 negatively and 20 positively charged amino acids result in an isoelectric point of 9.64. 211 of the 299, representing 71 % of all amino acids are part of the 10 predicted transmembrane domains. YigM C- and N-terminus are predicted to be located in the cytoplasm (see fig. 3.3) and the protein contains only very small hydrophilic streches. No similarity of YigM to other known biotin transport-proteins were found. There are no motifs that could give hints to the function of the protein. One domain that can be found is a domain of unknown function (DUF6) and the protein can be classified into the subfamiliy of carboxylate/amino acid/amine transporters (CAAT family TC 2.A.78) [172]. CAATs are one of ten subfamilies belonging to the family of secondary amino acid transporters found exclusively in bacteria [172]. Members of the CAAT family represent integral membrane proteins with sizes from 287 to 310 amino acids and 10 putative TMDs. CAAT proteins can be found in phylogenetically divergent bacteria and archaea, e.g. in *E. coli*, *B. subtilis* and *Aspergillus fulgidus* multiple paralogues are present. Representatives of the CAAT family show low degrees of sequence similarity with members of the ubiquitous L-rhamnose transporter (RhaT) family (TC 2.A.9) and with the eukaryotic triose phosphate transporter family (TC 2.A.50) [172].

The CAAT family probably arose from an internal gene-duplication event as the first halves of these proteins are homologous to the second halves. None of these prokaryotic proteins of CAATs is functionally characterized. Nevertheless functions have been ascribed to some members like the MttP protein of the *Methanosarcina barkeri* that is supposed to transport methylamine [60] and YtfF of *Chlamydia trachomatis* that may transport basic amino acids [182]. Additionally, MadN that is part of the malonate utilization operon of *Malonomonas rubra* and was speculated to be an acetate-efflux pump [13], as well as PecM of *Erwinia chrysanthemi* controlling pectinase, cellulase and blue-pigment production are members of CAATs [155].

Homologous genes of *yigM* can be found in different *Shigella*, *Citrobacter*, *Salmonella*, *Klebsiella* and *Yersinia*-strains (Fig. 3.4). These are mostly annotated as (inner) membrane or putative transport proteins, but the function of none of them has been experimentally shown.

The *yigM*-open reading frame can be found as a single gene on the chromosome and is not part of an operon (see fig. 3.1).

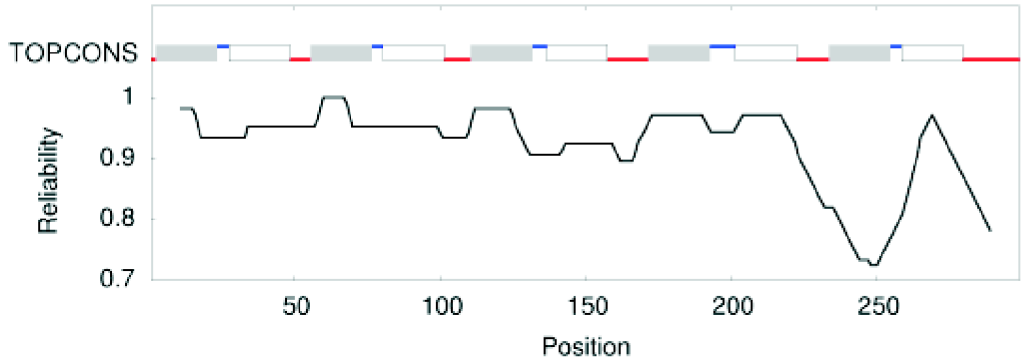

Figure 3.3: **Predicted transmembrane-domains of *yigM*.** Predictions were made by TOPOCONS (http://topcons.net/), that gives consensus predictions of five single topology prediction programs. Boxes indicate transmembrane regions, red lines predicted cytoplasmic and blue lines predicted periplasmic parts of the protein.

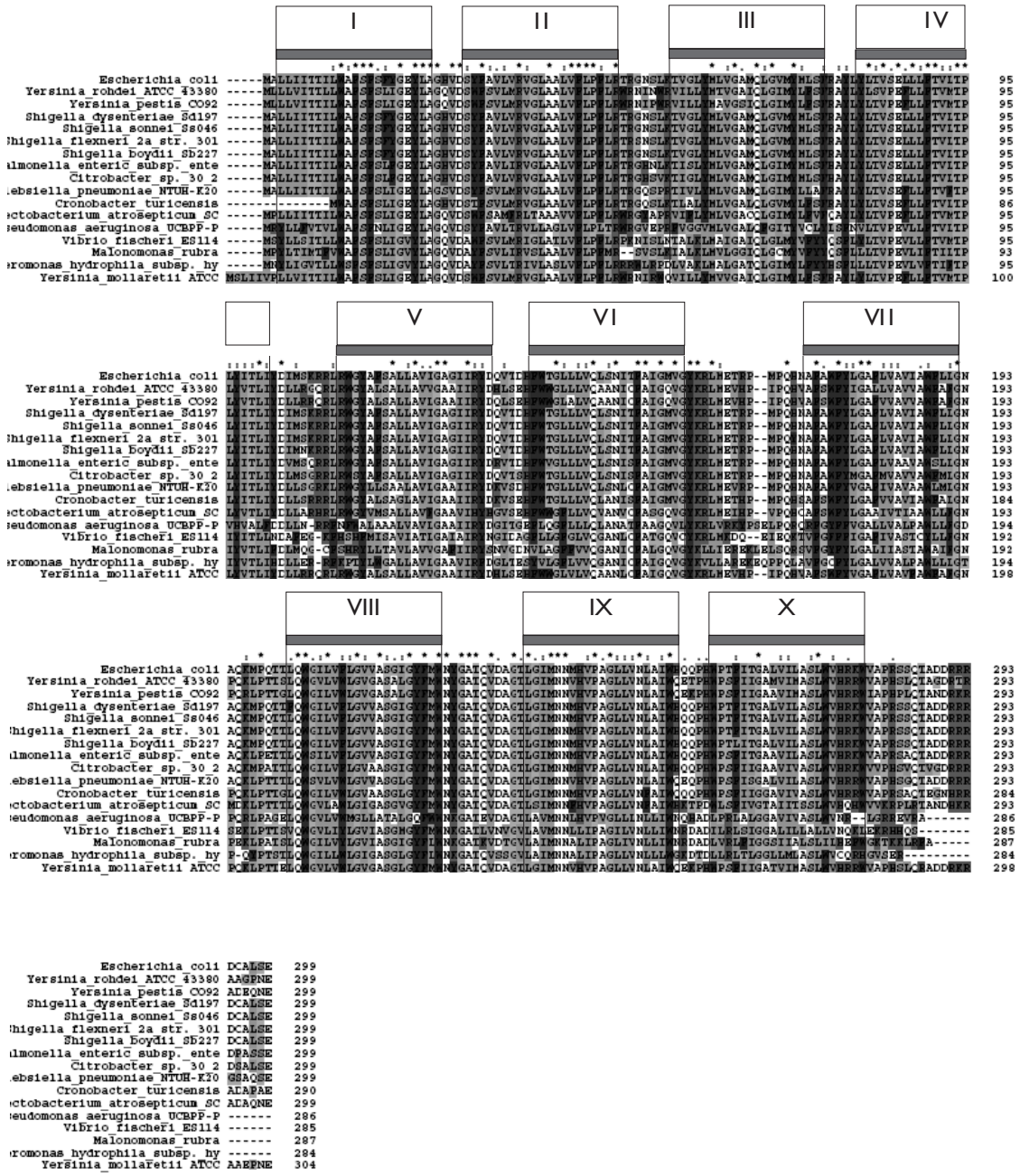

Figure 3.4: **Alignment of YigM-homologous proteins from different bacteria.** Predicted transmembrane domains for *E. coli* YigM are indicated by boxes with roman numbers. Predictions were made by TOPOCONS (http://topcons.net/).

3.1.3 Immunological detection

As YigM seemed to be the most promising candidate it was cloned into a pBS vector under control of the constitutively active P_R-promoter of the phage λ. Versions of YigM with a C-terminal or N-terminal HA-epitope-tag have also been cloned in this vector for detection of the protein on a western-blot (see fig. 3.5). These plasmids were transformed in wt- and *yigM*Δ-strains and uptake experiments performed in intact cells (see fig. 3.6).

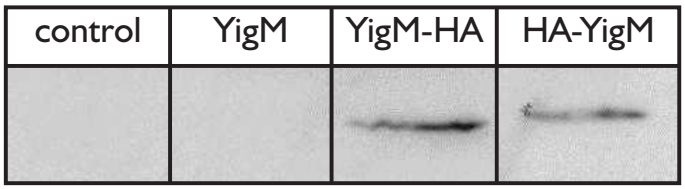

Figure 3.5: **Western blot of *yigM*-with HA-epitope.** Membrane-proteins of *yigM*Δ-cells with empty pBS-P_R (control) and with pBS-P_R-*yigM*, -*yigM*-HA and -HA-*yigM* were run on a 15 % SDS-gel, western blotted and detected with an α-HA-antiserum.

The western-blot in fig. 3.5 showed expression of both tagged versions of YigM. Bands appeared at the expected molecular weight of about 35 kDa. The western-blot shows similar expression of HA-YigM and YigM-HA, independent on which end of the protein the tag is added. The untagged protein YigM has as expected not been detected.

3.1.4 Uptake experiments

The uptake experiments showed the unlabeled version is able to significantly enhance uptake in wt-cells in comparison to cells with an empty vector by a factor of 2. Both versions with the HA-tag showed smaller uptake rates than YigM expressed without HA-epitope, but still significantly higher uptake rates than the wt cells with empty plasmid (factor 1.5). Concerning the *yigM*Δ-mutant similar results were observed. All three plasmids expressing YigM showed significantly higher uptake rates that the control with the empty vector. Differences in uptake rates were bigger than in wt with 3.5-fold increase of uptake velocity in pBS-P_R-yigM , 2-fold in pBS-P_R-yigM-HA and 3-fold in pBS-P_R-HA-yigM compared to control, respectively. Thus overexpression of YigM leads to increased uptake rates in both, wt and the *yigM*Δ-mutant. The acitvity of YigM is decreased by the fusion of the HA-epitope to the protein, no matter on which end of the protein the tag is added (see fig. 3.6).

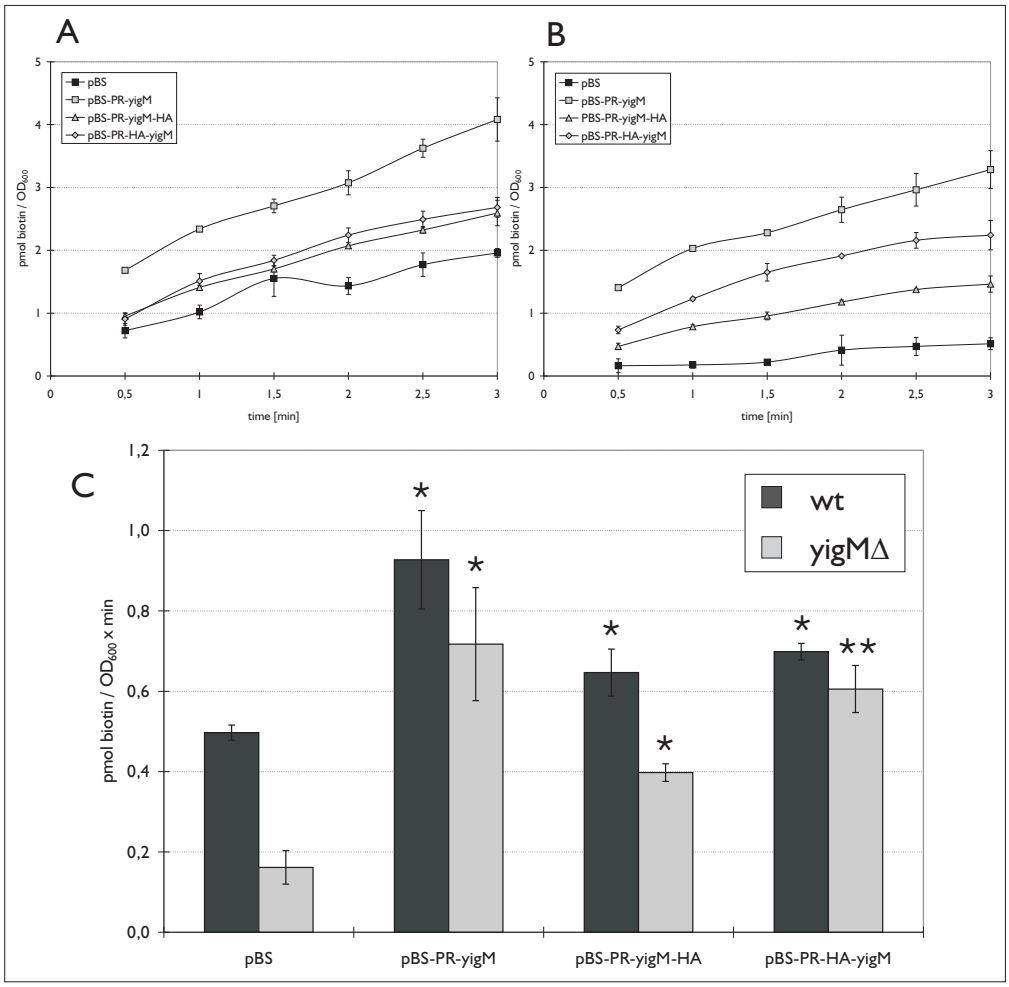

Figure 3.6: **Uptake experiments with tagged and untagged *yigM* in pBS-$\mathbf{P}_R$.** The experiments were performed in 50 mM KP_i-buffer pH 6.0 with 10 mM $MgCl_2$ and with 100 nM ^{14}C-biotin. Tests were started by adding biotin and aliquots taken every 30 seconds for 3 min. The mean values of three independent experiments are shown. Bars indicate standard deviations. A: wt; B: *yigM*Δ; C: uptake velocities. **: P < 0,001, *: P < 0.05 vs corresponding pBS, by ANOVA.

One general result of these experiments was that *yigM* is not strongly expressed from the plasmids. Due to the weak expression and increase of uptake rates a *yigM* version with codons optimized for expression in *E. coli* ($yigM_{CO}$) was tested. Uptakes assays again were performed in intact cells, but a markedly increased uptake rate also has not been observed with the codon-optimized gene. So finally, another expression system was tested and *yigM* was cloned into pET24 under control of a lac-inducible T7-promoter.

3.1.5 Expression of a codon-adapted *yigM* from pET24

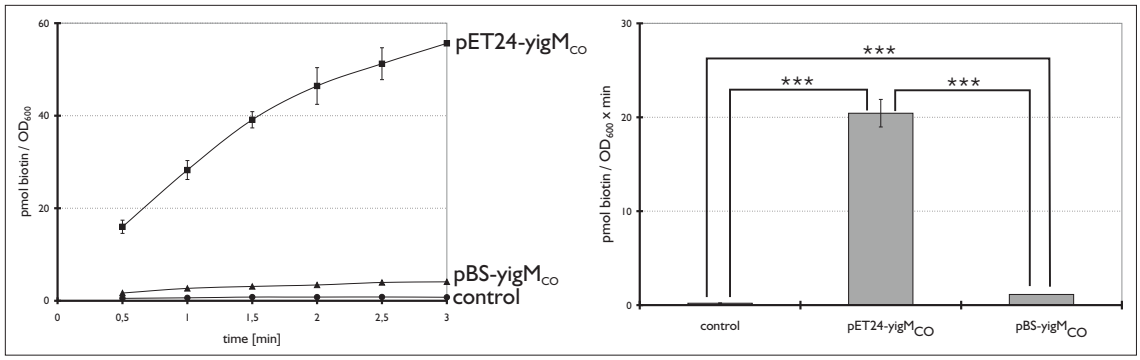

Figure 3.7: **Uptake experiments with *yigM* in two different expression vectors.** Uptakes were measured in *E. coli* BL21(DE3) with an empty pET24 vector (control), pET24-*yigM*$_{CO}$ (pET24-yigM$_{CO}$) and pBS-P$_R$-*yigM*$_{CO}$ (pBS-yigM$_{CO}$). A: Uptake 0.5 until 3 min, B: Uptake velocities. The mean values of three independent experiments are shown. Bars indicate standard deviations. ***: $P < 0.001$ by ANOVA.

The overexpression of *yigM* in pET24 showed highly significant and 18-fold higher v_{max} compared to expression from pBS-P$_R$ (see fig. 3.7). Biotin uptake in the control with an empty vector is neglegible although experiments have not been performed in a *yigM*-knockout-strain but in wt-cells. So the (over)expression of *yigM* seems to be the limiting factor for biotin uptake in *E. coli*.

3.1.6 K$_M$-value of YigM for biotin uptake

To compare the properties of YigM to the published data for biotin uptake in *E. coli* K12 wt-strains the K_M-value and v_{max} were determined. In these experiments *yigM*Δ-mutant with pBS-P$_R$-*yigM* were used.

From this experiments a K_M of 74 ± 14 nM and a v_{max} of 2,3 pmol/min for 1 OD_{600} that correspondes to 7,0 pmol/min for 1 mg dry weight of cells was measured (see fig. 3.8). Additional experiments to characterize substrate specificity as well as pH- and temperature-optimum of the transport were not performed. This is due to the fact that many previously used substrate analogons are not commercially available and that biotin uptake in *E. coli* wt cells, which is shown here to be solely dependent on YigM, has been extensively studied before [138], [142], [143]

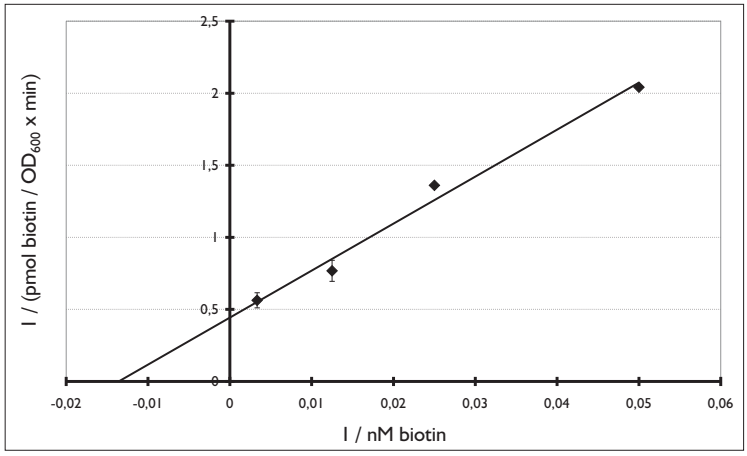

Figure 3.8: **Determination of the K_M-value of YigM.** Uptakes were measured in *E. coli yigM*Δ-cells with pBS-P_R-yigM$_{CO}$. Uptakes were performed at biotin concentrations of 20, 40, 80 and 300 nM of $^{[3]}$H-biotin and uptake velocites determined from 0.5 until 2 min after starting the experiment. The mean values of two independent experiments are shown. Bars indicate standard deviations.

3.1.7 Energetization of biotin transport

One still open question is the energy dependence of the transport system, because of contradictory results of different groups in the 70s and 80s. Piffeteau and coworkers reported energy dependent uptake of biotin and proposed a proton-symport mechanism for transport [143], [142]. In contrast Cicmanec and Lichstein claimed no energy requirement [35]. So further efforts were made to clarify this point and gain insight into the transport mechanism. Uptake experiments with BL21(DE3) carrying pET24-*yigM*$_{CO}$ in absence of glucose, the presence of the protonophors CCCP and FCCP and the respiratory chain inhibitor NaN_3 were performed. The uptake rates were lowered to about 60 % of the levels of standard assasy with addition of CCCP and FCCP and to 80 %, but not significantly with addition of NaN_3 or without glucose compared to the standard assay (see fig. 3.9). All values differ highly significant form the control assay ($P < 0.0001$). This results suggest biotin is transported via facilitated diffusion.

Another possibility, beyond a H^+-gradient to energize transport in bacteria is a sodium-symport mechanism that is e.g. used for the uptake of proline, melibiose, glutamate, serine and threonine, citrate or branched chain amino acids [209]. Sodium-cotransport would probably not directly be affected by the protonophors

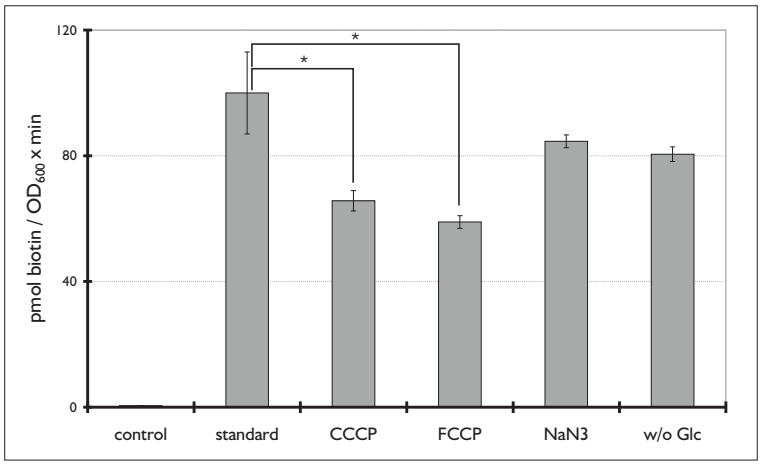

Figure 3.9: **Effects of uncouplers on biotin uptake over YigM.** Uptakes were measured in *E. coli* BL21(DE3) with an empty pET24-vector (control), pET24-$yigM_{CO}$ (standard) with standard uptake assays as described in materials and methods. CCCP and FCCP were added in a concentration of 100 μM, NaN_3 1 mM. One series of experiment has been performed without addition of glucose (w/o glucose) and cells were incubated for 3 min before starting the experiment by adding radiolabeled biotin. The mean values of three independent experiments are shown. Bars indicate standard deviations. *: $P < 0.05$ by ANOVA.

and NaN_3 during the short time uptake-assays are performed. Therefore generating an artificial sodium gradient by adding different concentrations of NaCl (10, 50, 100 and 200 mM) to the uptake buffer was tried, but no effects on biotin uptake were observed (data not shown). Also changing the uptake-buffer from 50 mM KP_i pH 6 to 50 mM NaP_i pH 6 did not bring different results (data not shown). Additionally countertransport assays showed there is an exchange of biotin between the cells and the medium. For this assay cells were incubated for 30 min with 50 nM $^{[3]}$H-labeled biotin. After this period, the cells were washed and then competed with 500 nM unlabelled biotin after this 30 min. Radioactivity in the cells goes back to about 20 % after competition with unlabelled biotin compared to the maximum before adding the unlabelled substrate. Alternatively, cells preincubated for 30 min with 50 nM unlabelled biotin, washed and shifted to 50 nM $^{[3]}$H-biotin demonstrated rapid uptake of the radiolabelled substrate (see fig. 3.10). These results also support a facilitated diffusion mechanism.

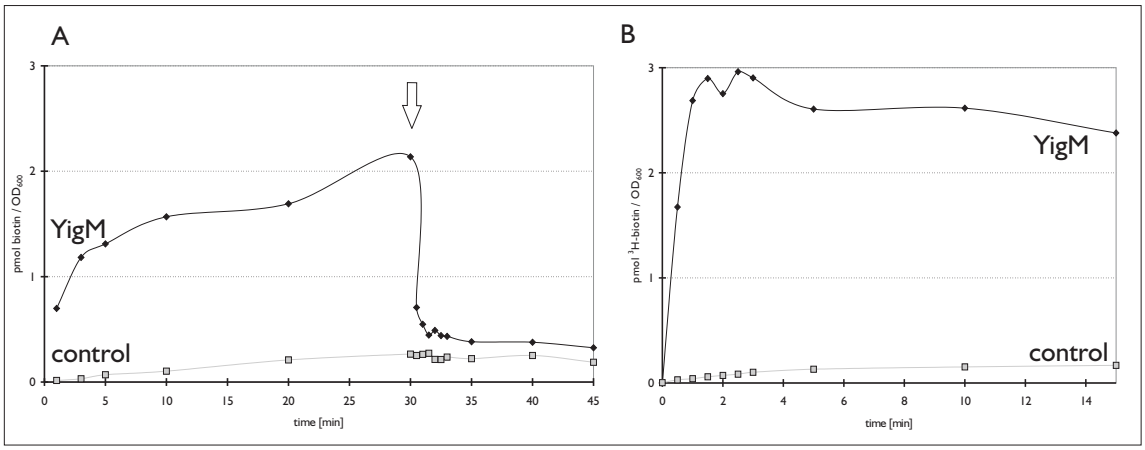

Figure 3.10: **Counterflow experiments with YigM.** Experiments were performed in wt cells with pBS (control) and with pBS-P_R-yigM (YigM). A: Cells were incubated with 50 nM $^{[3]}$H-biotin from 0 to 30 min. At 30 min 500 nM non-radioactive biotin was added (indicated by the arrow). B: Cells were preincubated for 30 min with 50 nM non-radioactive biotin. At time t=0 min 50 nM $^{[3]}$H-biotin was added. Figures show representative results from one of three independent experiments.

3.1.8 Uptake experiments in membrane vesicles

To circumvent perturbing influences like unknown internal biotin concentrations, attachment of biotin to biotin-proteins and metabolic state of cells that all might influence uptake in intact cells, experiments with membrane vesicles were carried out. A further advantage of the *in vitro* system is the ability to generate a completely artificially energized system and to start in an unenergized situation.

These vesicles were prepared by breaking of cells, isolating the membrane fraction by ultracentrifugation and fusing these membranes with artificial *E. coli* membrane lipids. Energy in form of a proton gradient and a membrane potential can be supplied by using membranes containing purified cytochrome c oxidase and addition of ascorbate, TMPD and cytochrome c. The whole procedures are described in more detail in chapter 2 of this work.

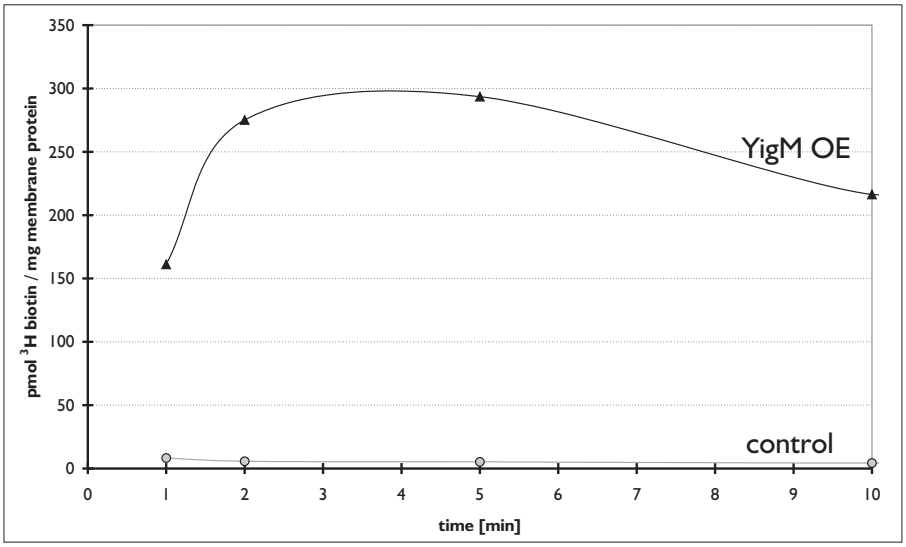

Figure 3.11: **Uptake experiments in membrane vesicles.** Membrane vesicles were prepared and uptake experiment performed as described in "Material and methods" for membranes overexpressing YigM from pET24-*yigM* (YigM OE) and for a strain carrying an empty pET24-plasmid (control). At time t=0 min 50 nM ^{3}H-biotin was added. The figure shows representative results from one of three independent experiments.

For overexpression of YigM rapid increase of radioactivity and saturation at about 4 min after starting the experiment has been observed (see fig. 3.11). The control-experiment did not show an increase of radioactivity over the same time range. Energizing membrane vesicles with cytochrome c oxidase did not reveal different

results (data not shown).

In summary, the uptake experiments in intact cells and membrane vesicles suggested biotin is taken up by facilitated diffusion. Destroying proton gradients by protonophores CCCP and FCCP, depleting cells of energy by blocking the respiratory chain with NaN_3 or detaining glucose did not show markedly effects on biotin uptake rates in intact cells. Membrane vesicles showed uptake of biotin without energization and uptake did not increase after supplying energy. So energy seems not to be necessary to take up biotin via YigM.

3.1.9 Sequence of YigM from the *E. coli* biotin transport mutant S1039

Another evidence that YigM is the essential factor for biotin uptake was shown through *E. coli* strain S1039 [199], [84]. This strain originates from random mutagenesis and lacks intrinsic high-affinity biotin-uptake activity. As this strain was not available to us, but was used in previous experiments by the group of Prof. Dr. Thomas Eitinger, Humboldt-Universität zu Berlin, Institut für Biologie/Mikrobiologie, Prof. Dr. Eitinger did the sequencing and kindly shared the results with us. Sequencing revealed the *yigM*-open reading frame of this strain contains two point mutations (AGC to AGA and CCG to TCG in nucleotides 85 and 729) that lead to two amino-acid exchanges (S28R and P243S, respectively) (see fig. 3.12).

These two resisues are well conserved in most homologues of *yigM* (Fig. 3.12). To find out which of both mutations was responsible for the decrease in transport activity they were separately introduced in pBS-P_R-*yigM* and uptake assays were carried out.

The experiment showed that both single mutations lead to a severe and significant reduction of biotin uptake. The remaining activites were 25 % or 27 % for S28R or P243S compared to the wt, respectively. Additionally the mutations show synergistic effects when they are both present in the protein and reduce transport activity significantly in comparison to the single mutations. Transport activity for the double mutant does not differ significantly from control (see fig. 3.13).

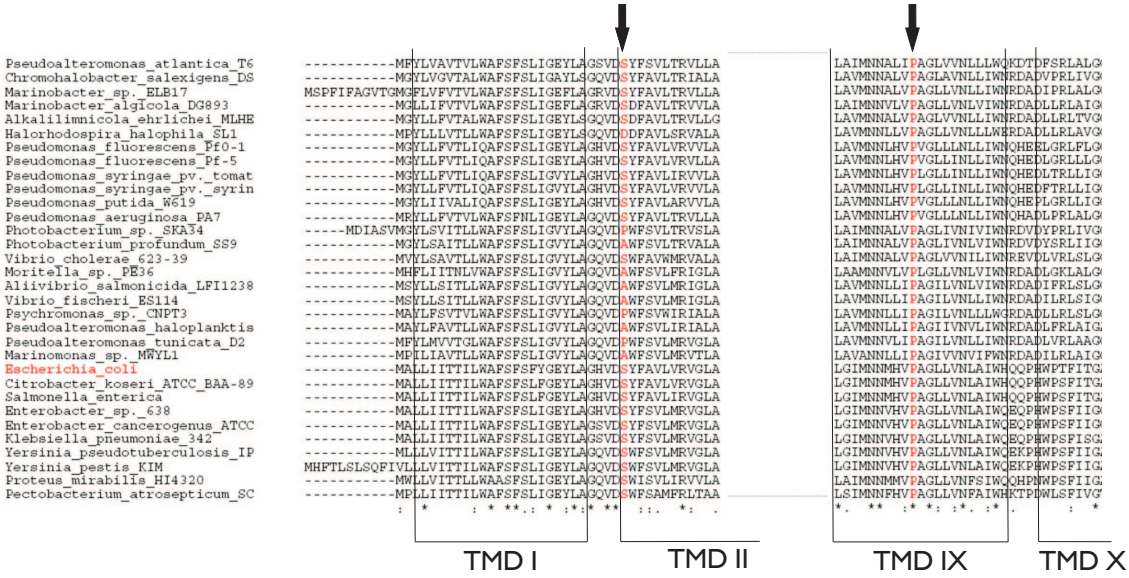

Figure 3.12: **Sequence alignment of *yigM* amino acid sequences of different bacteria.** The alignment shows that S28 is very well conserved except for some of the shown organisms and P243 is perfectly conserved in all of them (S28 and P243 marked with arrows). Transmembrane domains predicted by TOPCONS (http://topcons.cbr.su.se/) for *E. coli* YigM are indicated by boxes and roman numbers.

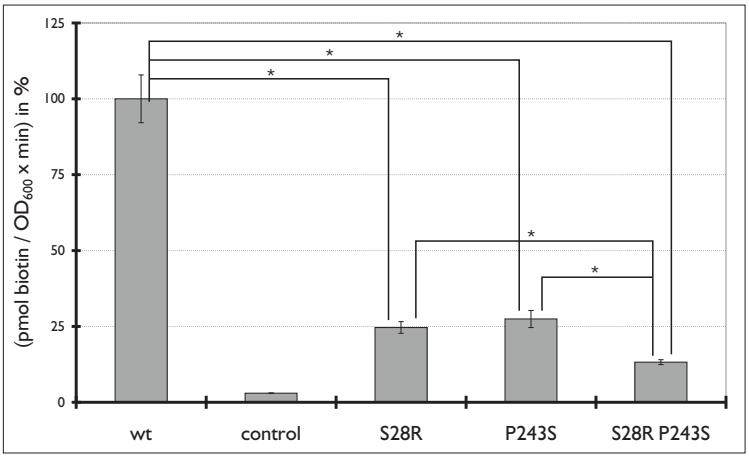

Figure 3.13: **Biotin uptakes in yigMΔ cells expressing YigM the mutations S28R and P243S.** Standard uptake experiments in *E. coli yigM*Δ with wt, *yigM* (control), *yigM*S28R (S28R), *yigM*P243S (P243S) and *yigM*S28R,P243S (S28R P243S) in pBS-P_R and an empty pBS-P_R-vector (control). Values indicate uptake velocities in % with wt *yigM* set to 100 %. The mean values of three independent experiments are shown. Bars indicate standard deviations. *: $P < 0.05$ by ANOVA.

3.1.10 C-terminal truncation of yigM

The reduction of transport activity in the HA-tagged versions of *yigM* suggested that the termini of the protein might be essential for its function. The predicted topology of the protein suggests that the first transmembrane domain begins with amino-acid two or three. Thus an impact on function by adding an epitope-tag right before is not astonishing. At the C-terminus of the *E. coli* protein about 20 amino acids can be found in the cytoplasm according to topology-predicitions. Some YigM orthologs have shorter C-termini (see fig. 3.4), suggesting this part of the protein might not be essential for the activity. Also striking is the quite uncommon accumulation of seven charged amino acids among the last eleven in the protein (see fig. 3.14).

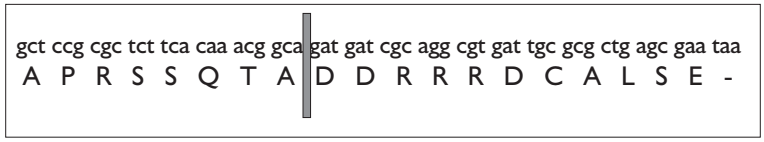

Figure 3.14: **Truncation of *yigM* at the C-terminus.** The open reading frame of *yigM* was truncated by 33 bps resulting in loss of 11 amino acids, corresponding to the length of the shorter homolouges. The truncation-site is indicated by the grey bar.

To get evidence for an implication of the C-terminus uptake experiments with YigM truncated by eleven amino acids were performed.

Truncation of the C-terminal end of the protein results in a significant reduction of transport-activity by 70 % (see fig. 3.15), similar to the findings made with a C-terminal HA-epitope tag added to the full-length protein (see fig. 3.6).

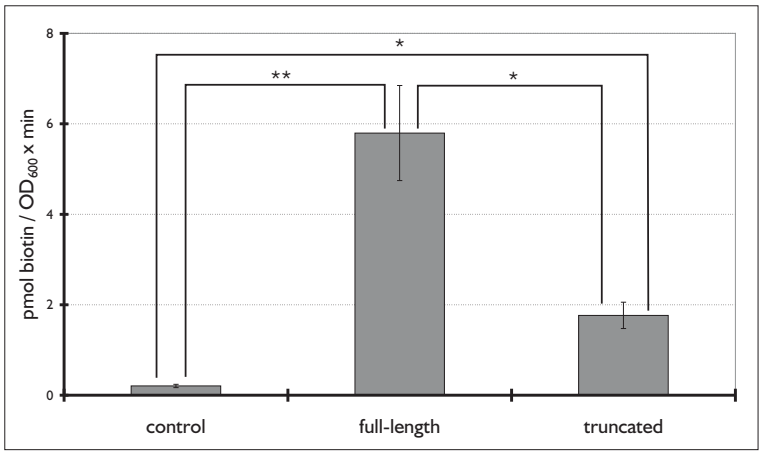

Figure 3.15: **Uptake experiments with full-length and C-terminally truncated YigM.** Standard uptake experiments in *E. coli yigM*Δ with empty pET24 (control), full-length YigM_{CO} (full-length) and C-terminally truncated YigM_{CO} (truncated) expressed from pET24. Values indicate uptake velocities. The mean values of three independent experiments are shown. Bars indicate standard deviations. *: $P < 0.05$, **: $P < 0.001$ by ANOVA.

3.1.11 Gene regulation of *yigM*

Experiments of Piffeteau and Gaudry showed that biotin uptake activity is inversely correlated with the biotin concentration in the medium [142]. Prof. Dr. Thomas Eitinger found that the promoter region of *yigM* contains a sequence rather similar to the binding site of BirA in the biotin operator of the bio-operon (see fig. 3.16). Due to this observation a regulation of the *yigM*-promoter by BirA seems plausible. Sequence comparisons of promoters from *E. coli yigM* and homologues showed at least partial conservation of the BirA-binding sites (see fig. 3.16).

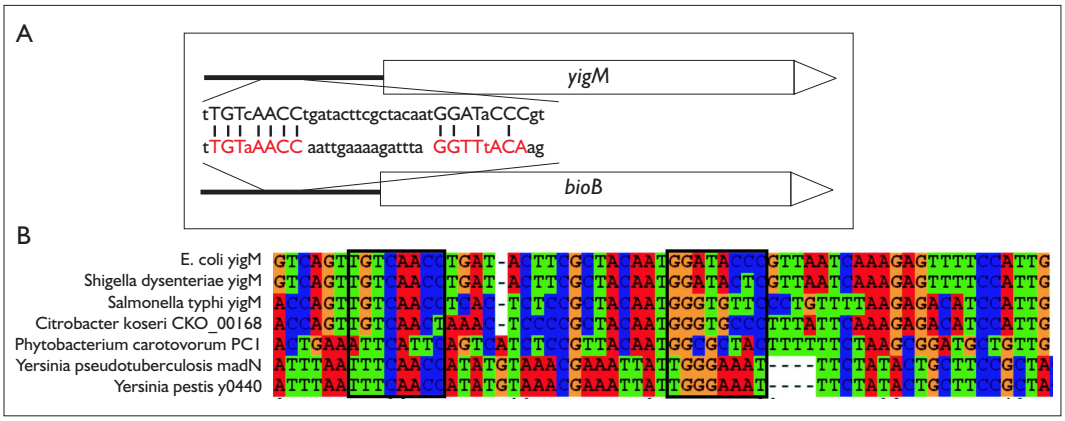

Figure 3.16: **Promoters of *bioB* and *yigM*.** A: The BirA-binding site in P*bioB* is marked in red, conserved bases shown in upper case. Corresponding bases in P*yigM* are also shown in upper case. B: Sequence alignment of promoter regions of *yigM* homologes. Potential BirA-binding sites are indicated by boxes.

3.1.12 Luciferase-reporter constructs

Luciferase-reporter-constructs for both promoters (P*bioB* and P*yigM*) were cloned to investigate regulation of these genes at various biotin concentrations. To get evidence if both promoters are regulated by biotin luciferase activities in wt-strain, the biosynthesis-mutant *bioB*Δ and the transport-mutant *yigM*Δ were measured. Cells carrying reporter plasmids were grown in liquid cultures in M9 minimal medium without biotin overnight with shaking at 37 °C. Freshly from thes overnight cultures inoculated cultures in M9 containing indicated concentrations of biotin were inoculated to an OD_{600} of 0.1 and incubated in the shaker at 37 °C until an OD_{600} of approx. 0.7 was reached. Cells were washed with PBS and OD_{600} and luminescence

for each biotin concentration measured in 96-well plates. Reporter acitivities were calculated as luminescence/OD_{600}.

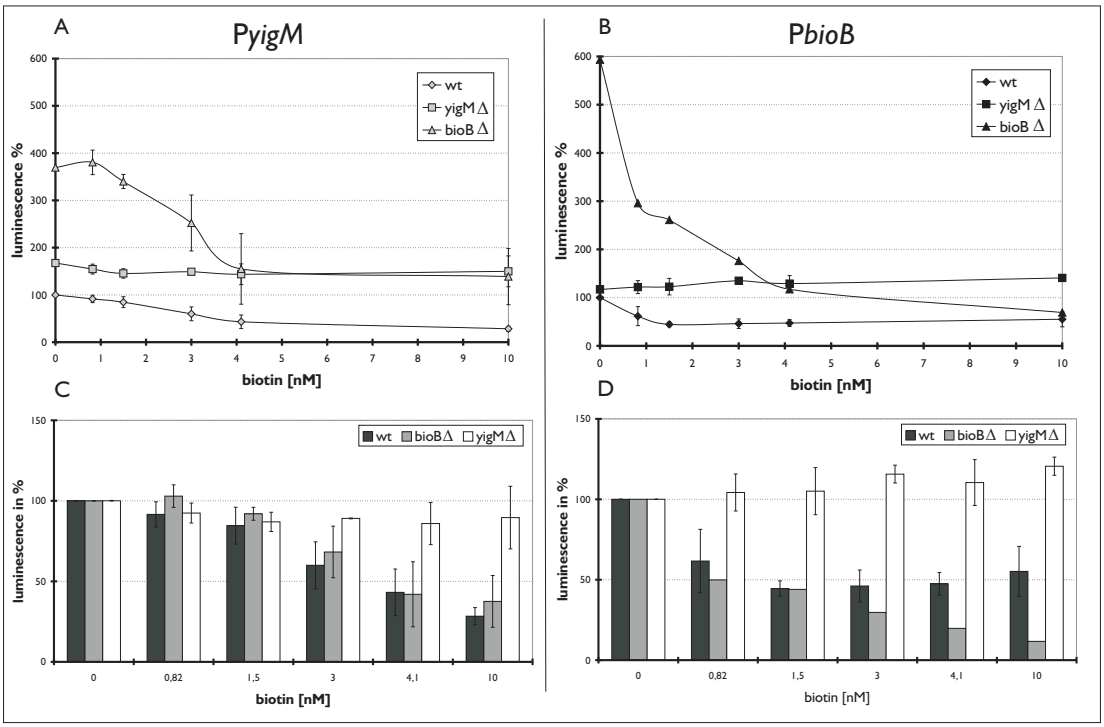

Figure 3.17: **Measurements of luminescence in wt, *bioB*Δ and *yigM*Δ.** Expression of luciferase was driven by the promoter of *bioB* (P*bioB*) or *yigM* (P*yigM*). Luminescence values are given in %. For A and B values of wt at 0 nM biotin were set to 100 %. For C and D values at 0 nM biotin were set 100 % for each of the three strains. The mean values of two independent experiments are shown. Bars indicate standard deviations. Luciferase values for *bioB*Δ with P*bioB* have only been measured once.

Fig. 3.17 shows that luminescence decreases with rising concentrations of biotin for both promoters in wt and *bioB*Δ. In *yigM*Δ luminescence is more or less constant over the biotin-concentration range tested for both promoters. Concerning P*bioB* at 0 nM biotin about 6-fold higher values for *bioB*Δ in comparison to wt and *yigM*Δ was observed achieved. The expression levels for wt and *bioB*Δ at 10 nM are equal and about 2-fold lower than for *yigM*Δ at this concentration, as well as for wt at 0 nM biotin. Promotor activites are highest in *bioB*Δ at 0 nM biotin with a two- to threefold higher expression compared to *yigM*Δ and wt for P*yigM*. For *yigM*Δ luminescence is 1.5-fold higher without biotin than for wt. At 10 nM biotin *bioB*Δ and *yigM*Δ both reach a level that is about threefold higher than for wt. But for both, wt and

*bioB*Δ expression from P*yigM* at 10 nM is about the half compared to 0 nM biotin. In conclusion regulation of both promoters is similar in all three tested strains. A difference between P*bioB* and P*yigM* is, that lower concentrations of biotin are sufficient to repress expression from P*bioB*. Reporter activity decreased to about 50 % of maximal activity at 0.82 nM biotin and stays at that level until up to 10 nM in wt. In *bioB*Δ cells luminescence drops to approximately 10 % of the maximum. Expression from P*yigM* in contrast is downregulated at higher biotin concentration, still keeps 80 to 90 % of its maximal activity in 1.5 nM and 0.82 nM biotin, respectively and reporter acitivty decreases to 30 % at 10 nM. This is true for both wt and *bioB*Δ cells. The results of the luciferase reporter assays showed that P*bioB* and P*yigM* both are regulated by biotin. Dependence of gene expression on the strain background was demonstrated, too. This aspect will be further discussed in chapter 4 of this work.

In addition to the experiments described above different previously characterized BirA-mutants [7], [57] were tested with the luciferase reporters (see fig. 3.18). These analyses were carries out to test if expression from P*yigM* is also regulated by BirA as for P*bioB*. That seem likely because the BirA-binding site is in parts present in P*yigM* and both promoters are regulated by biotin, as shown before. As *birA* is essential for *E. coli* and no knockout for the gene is available, the tested strains all carry point-mutations in the *birA* gene.

Again luciferase-tests with P*yigM* and P*bioB* were carried out to see if both promoters are regulated in a similar manner or with the same tendencies in the BirA-mutants and in contrast to the measurements in a wt background.

The results of these experiments demonstrated reporters with P*yigM* show the same tendency of activation in both BirA-mutants just like P*bioB*. Overall levels for activation are higher for P*bioB* compared to P*yigM* in all three tested mutants. However results suggest BirA-dependent regulation of P*yigM*.

3.1.13 Electrophoretic mobility-shift assays

To directly test a possible binding of BirA to P*yigM*, as it was shown for P*bioB* [106], electrophoretic mobility-shift assays were performed. Fragments of about 100 bps from P*bioB* and P*yigM* that were also used as promoters for the luciferase reporter plasmids and different concentrations of purified BirA-6His-protein were tested. BirA-6His was purified in coopreation with Carina Mann de Oliveira as described in chapter 2. To ensure the presence of biotinyl-AMP that is a prerequesite

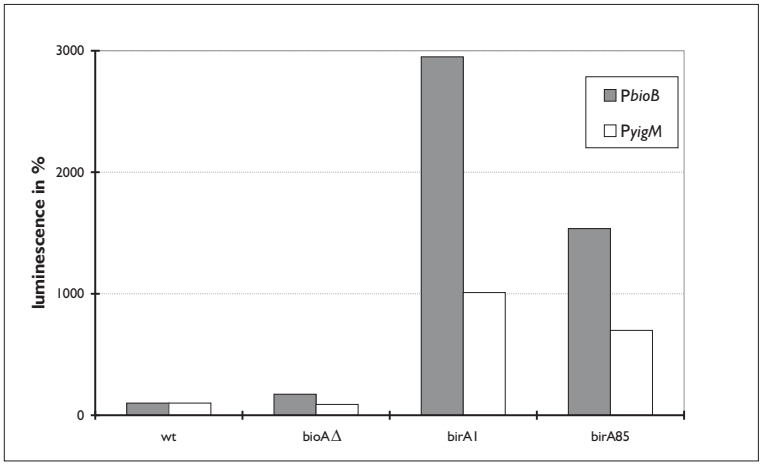

Figure 3.18: **Luciferase activities of P*bioB* and P*yigM* in wt, *bioA*Δ and *birA*1- and *birA*85-mutant.** Expression of luciferase was driven by the promoter of *bioB* (P*bioB*) or *yigM* (P*yigM*). Cells were grown for 6 h in 10 nM biotin as this biotin concentration was the lowest one where all tested strains were able to grow. Luminescence values are given in %. Values of wt were set to 100 %.

for BirA-binding to DNA, experiments in presence of biotin and ATP as described in [188] as well as in absence of both as negative control were carried out. The assays contained 12 nmole (800 ng) of each promoter fragment and protein from 6 nmole to 70 nmole (800 to 2400 ng). The molar ration (protein : DNA) varied between 0.5 and 5.8. As an additional negative control the promoter of the yeast biotin-transporter *VHT*1 was used, that did not show a shifted band with the highest protein concentration used for P*bioB* and P*yigM* (data not shown). After incubation on ice for 45 min samples were loaded on the native gel. For P*bioB* beginning at a ratio 2:1 protein:DNA a shift of promoter-DNA was observed. For P*yigM* no shift was detectable even with higher protein concentrations of BirA-6His (see fig 3.19).

To detect the BirA-6His-protein the EMSA gels were western-blotted followed by detection with α-His-antibody. Here again a clear band for P*bioB*-BirA-complex was observed, but a P*yigM*-BirA-6His-complex has not been detected. The BirA protein stays in the wells of the gel.

Thus the experiments confirmed that BirA binds to P*bioB*, whereas a direct interaction of P*yigM* and BirA was not detected under the conditions used here.

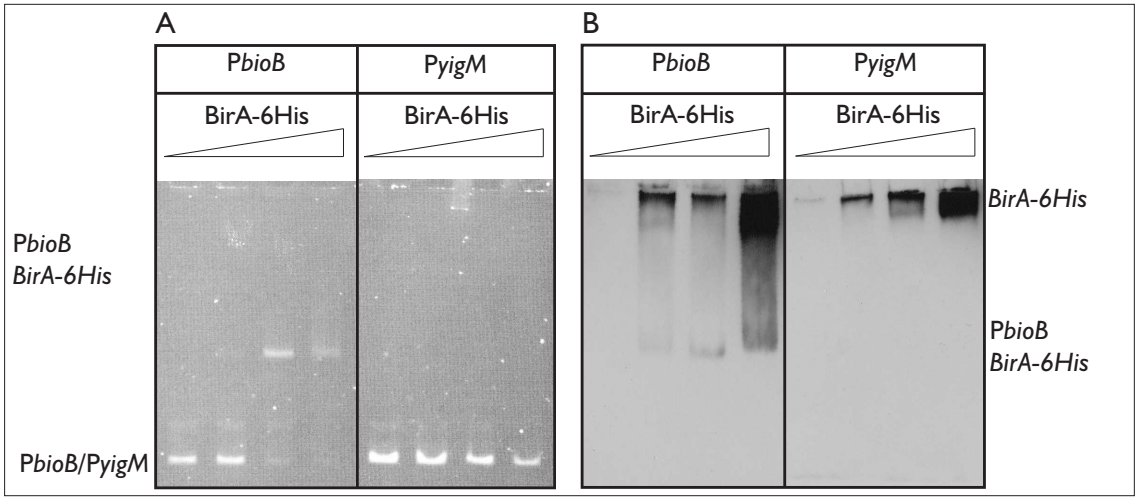

Figure 3.19: **EMSA with P*bioB*, P*yigM* and BirA-6His.** A: EMSA with P*bioB* and P*yigM* (12 nmole) and rising concentrations of BirA-6His (0 nmole, 6 nmole, 15 nmole, 70 nmole), gel stained with ethidiumbromide; B: Western blot of the EMSA gel, detected with α-His-antibody.

3.2 Biotin sensing in *S. cerevisiae*

It was extensively shown by several groups that *S. cerevisae* is able to sense the biotin status and to react with enhanced expression of genes involved in biotin biosynthesis and transport when a lack of the vitamin is detected [210], [185], [144], [201]. However we are far from understanding the whole process of biotin sensing. In this work two different experimental strategies were used to find missing parts of the pathway.

One strategy started with *VHR1* that was shown to be involved in biotin sensing and was proposed to be a candidate for the transcription factor [201]. With the second approach different biotin-proteins, especially the pyruvate carboxylases *PYC1* and *PYC2* were investigated in more detail.

To find a suitable timepoint and biotin concentration when cells still grow normaly, but show an undersupply of biotin, samples of yeasts grown in "normal" biotin concentrations (2 mg/l) and in low biotin medium (0,02 mg/l) were collected in a time series every two hours. Cells were broken and protein preparations were analyzed for protein biotinylation by western-blot analysis. Detection of biotinylation was performed with streptavidin-peroxidase (strep-PO) (see fig. 3.20).

Growth appeares to be normal up to 10 h in medium with 0,02 mg/l biotin (data not shown). Weaker biotinylation of Acc1p and Hfa1p, Dur1,2p can already be observed after 2h. At this time Arc1p is not detectable on the blot any more. A decrease in biotinylation of Pyc1p and Pyc2p appears at 4 h and becomes more evident after 6 and 8 h. For further experiments the cells were grown for 6 h and used for subsequent analysis.

In general there are two different ways to detect yeasts response to decreased biotin concentrations. Two assays to analyze biotin sensing were established in this work. One assay detects expression of transport- and biosynthesis-genes that increases in low biotin concentrations in wt cells and can be detected by higher abundance of the proteins on a western-blot. This analysis in the following will be shown for the Bio2 protein. Reporter constructs with β-galactosidase (lacZ) or GFP under the control of a minimal-promoter containing the BRE-element plus further about 20 bps on each side of the *VHT1*-promoter [183] were performed as a second tool for visualizing biotin sensing. This analysis allows a more quantitative analysis of the cellular reaction to different biotin concentrations.

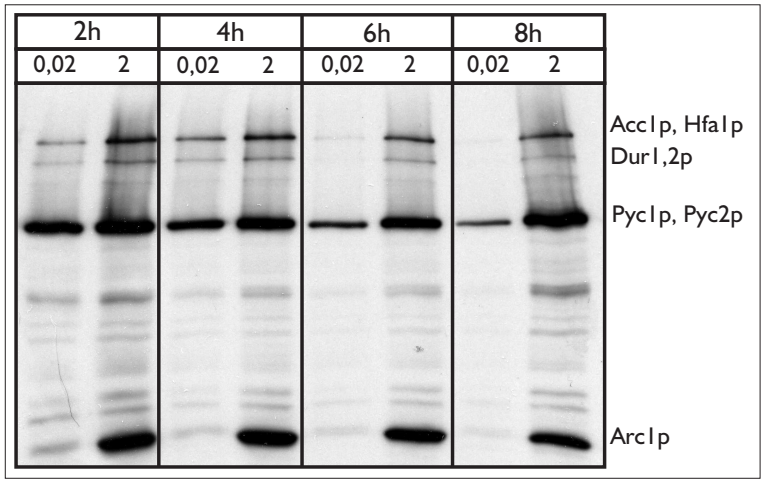

Figure 3.20: **Western-blot of biotinylated proteins in *S. cerevisiae* W303.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken at indicated time points and whole-cell-protein extracts prepared. Extracts from 0.5 OD_{600} per lane were run on a 10 % SDS-gel and western-blots were performed. Blots were incubated with streptavidin-peroxidase. Bands on the blot represent the biotinylated proteins Acc1p, Hfa1p, Dur1,2p, Pyc1p, Pyc2p and Arc1p as indicated.

3.2.1 *VHR1* and biotin sensing

With *VHR1*, as the transcripion factor, taking a central role in biotin sensing further efforts were made based on the findings of Weider *et al.* [201]. First of all growth experiments with wt-cells and a *vhr1*Δ-mutant were performed in medium with low (0.02 μg/l) and normal (2 μg/l) biotin concentration, but a difference in growth was not observed (data not shown). *vhr1*Δ-mutants were tested with the BRE-lacZ-reporter-assays and on Bio2p-expression by western-blot. These experiments showed that an increase in expression of Bio2p (see fig. 3.21) or increased reporter gene expression (see fig. 3.22) in low biotin concentrations can not be observed in a *vhr1*Δ mutant in contrat to wt cells and confirmed the findings of Weider *et al.* [201].

A complementation of the *vhr1*-knockout by expressing the protein from a single-copy (YCplac) and an multi-copy (YEplac) vector under control of the endogenous promoter was successful as shown by increased expression of Bio2p in cells grown in low biotin medium (see fig. 3.22). However, according to results of the GFP-reporter-tests (see fig. 3.23) wt-levels were not achieved.

For further analysis like detection by antibodies or purification, epitope tags were

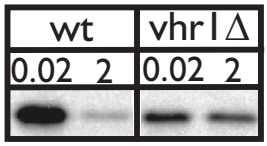

Figure 3.21: **Western-blot of W303 wt and *vhr1*Δ with anti-Bio2p.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken after 6 h for whole-cell-protein extracts. Extracts from 0.5 OD_{600} per lane were run on a 10 % SDS-gel and western-blots performed. Blots were detected with anti-Bio2p-antibody.

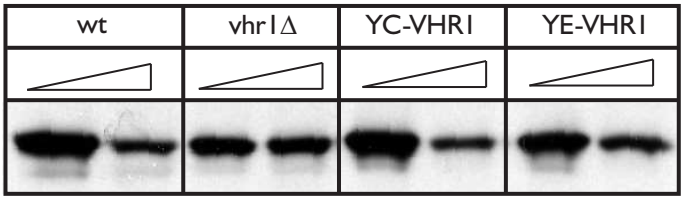

Figure 3.22: **Complementation of *vhr1*Δ-mutant.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken after 6 h for whole-cell-protein extracts. Extracts from 0.5 OD_{600} per lane were run on a 10 % SDS-gel and western-blots performed. Blots were detected with anti-Bio2p-antibody. W303 wt (wt), *vhr1*Δ (*vhr1*Δ), single-copy plasmid YCplac33-VHR1 (YC-VHR1) and multi-copy plasmid YEplac-VHR1 (YE-VHR1).

fused to the protein. As BLAST searches indicated the C-terminus of the Vhr1 protein to be less conserved than the N-terminus a HA-, a ZZ- tag and GFP were fused to the C-terminal side of the protein. To provide the natural expression level of the protein the fusions were made in the genome, so that the promoter and the environment of the *VHR1*-gene are not changed. Functionality of the tagged protein was tested by western blot and lacZ-reporter assays. Both experiments revealed that the function of Vhr1p was compromised when the protein contained an epitope tag (see fig. 3.24). Reporter acitivtiy in wt was increased significantly in cells from 0.02 mg/l compared to 2 mg/l. Neither in *vhr1*Δ cells, nor in any of the three tagged versions lacZ-activities increased significantly in cells from 0.02 mg/l compared to 2 mg/l. Reporter activities for wt cells grown 0.02 mg/l biotin differ highly significant from all other strains grown in the same biotin concentration, whereas values of cells grown in 2 mg/l biotin do not differ significantly for any of the tested strains. Altogether results of this experiment indicated all three tagged Vhr1p-versions are

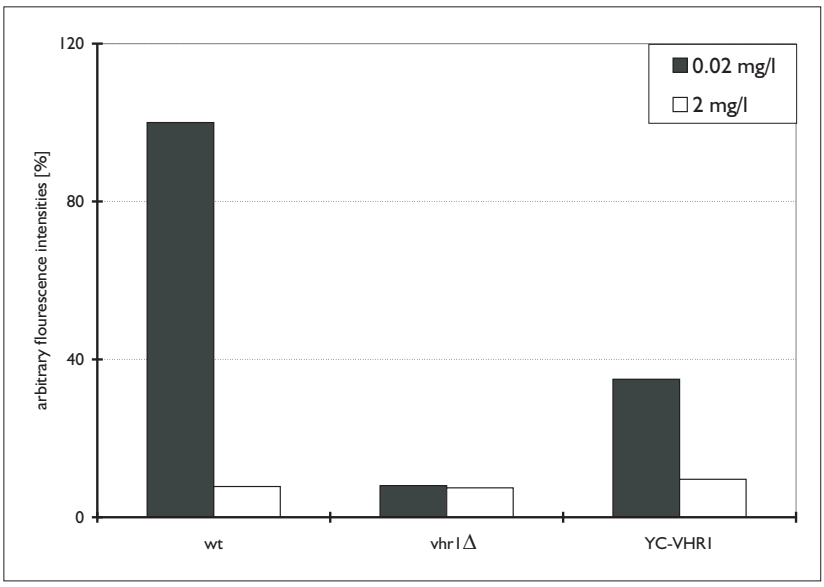

Figure 3.23: **Fluorescence measurements with a GFP-reporter plasmid of the complementation of *vhr1*Δ.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken after 6 h and GFP-fluorescence measured as described in "Materials and methods". GFP-fluorescence is given in %. Values for wt from 0.02 mg/l biotin were set to 100 %. Values for W303 wt (wt), *vhr1*Δ (*vhr1*Δ) and single-copy plasmid YCplac33-VHR1 (YC-VHR1) are shown.

not functional in sensing. Some results of Weider *et al.* [201] and [202] are based on fusing Vhr1p with epitope tags or protein domains and so have to bee seen critical as findings made in this work showed. Discussion in this context is provided in chapter 4 of this work.

In spite of higher conservation of the N-terminus, fusions with a 3HA-epitope were additionally constructed at this side of the protein. Expression of the constructs from the low-copy-plasmid pRS416 under control of the Met25-promoter again showed loss of function for 3HA-Vhr1p in β-galactosidase reporter assays (see fig. 3.24), as 3HA-Vhr1p is not able to induce lacZ-expression of the reporter in low biotin concentrations.

The experiments show that obviously the integritiy of both termini of Vhr1p is essential for correct function. Thus, tagging of the protein on one of the termini while preserving the biotin sensing activity seems to be impossible. Due to the limited options with the analysis of Vhr1p further experiments focussed on the biotin proteins, especially Pyc1p and Pyc2p.

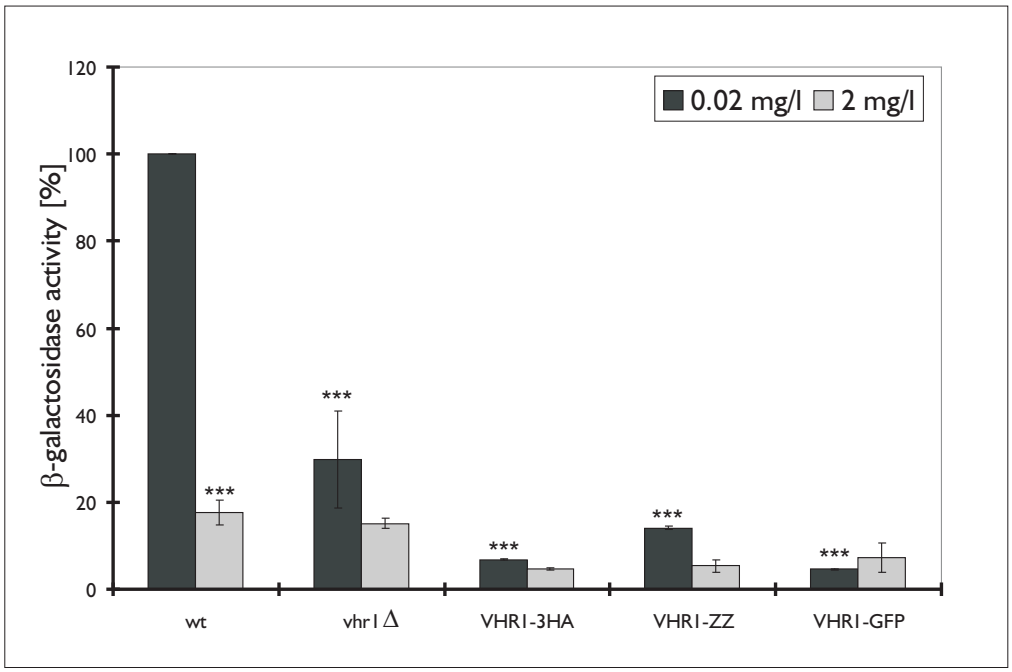

Figure 3.24: **Measurements of β-galactosidase-activities with a BRE-lacZ-reporter in wt, *vhr1*Δ VHR1-3HA, -ZZ and -GFP.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken after 6 h and β-galactosidase-activities measured as described. Activities are given in %. wt from 0.02 mg/l biotin was set 100 %. The mean values of two independent experiments are shown. Bars indicate standard deviations. ***: $P < 0.0001$ vs. wt at 0.02 mg/l by ANOVA.

3.2.2 The function of pyruvate carboxylases in biotin sensing

Beyond the known functions of Vhr1p and Bpl1p in biotin sensing it is not yet clear if the proteins carrying biotin as a cofactor are involved in sensing. To adress this question single knockouts of *PYC1*, *PYC2*, *HFA1*, *DUR1,2* and *ARC1* were tested with a GFP-reporter on sensing low biotin (see fig. 3.25). Similar experiments have been carried out by Pirner and Stolz with a β-galactosidase-reporter construct [144]. They observed reduced biotin sensing in *arc1*Δ and *pyc2*Δ cells. *ACC1* was not included in the experiments in this work. The *ACC1* gene is essential and a knockout is lethal. Effects of Acc1p on biotin sensing have already been investigated in a strain expressing *ACC1* from the galactose inducible *GAL1*-promoter but only weak impact on biotin sensing could be found [144]. In parallel protein extracts of wt-cells were made and analysed by western-blot to detect protein biotinylation (see fig. 3.25).

The measurements show a clear and significant induction of reporter activity for wt, *dur1,2*Δ and *hfa1*Δ cells grown in low biotin medium. Also for *arc1*Δ a significant induction at low biotin concentrations compared to normal concentrations can be observed, although the absolute values are smaller than for the wt and the mutants mentioned above. A different reaction can be seen in *pyc*Δ cells. Induction in low biotin is much smaller but still significant for *pyc1*Δ but not significant in *pyc2*Δ. Fluorescence values for cells from medium with 0.02 mg/l biotin all differ highly significant except between wt and *dur1,2*Δ, whereas values from cells grown in 2 mg/l biotin do not differ significantly. Induction factors of fluorescence at 0.02 mg/l compared to the mean values of all six tested strains at 2 mg/l biotin were calculated:

Strain	Induction factor
wt	31
arc1Δ	7
dur1,2Δ	31
hfa1Δ	18
pyc1Δ	7
pyc2Δ	0

Table 3.2: **Reporter gene activities in biotin protein knockout mutants.** Induction factors of fluorescence values for cells grown in 0.02 mg/l and the mean fluorescence values of cells grown in 2 mg/l biotin are indicated.

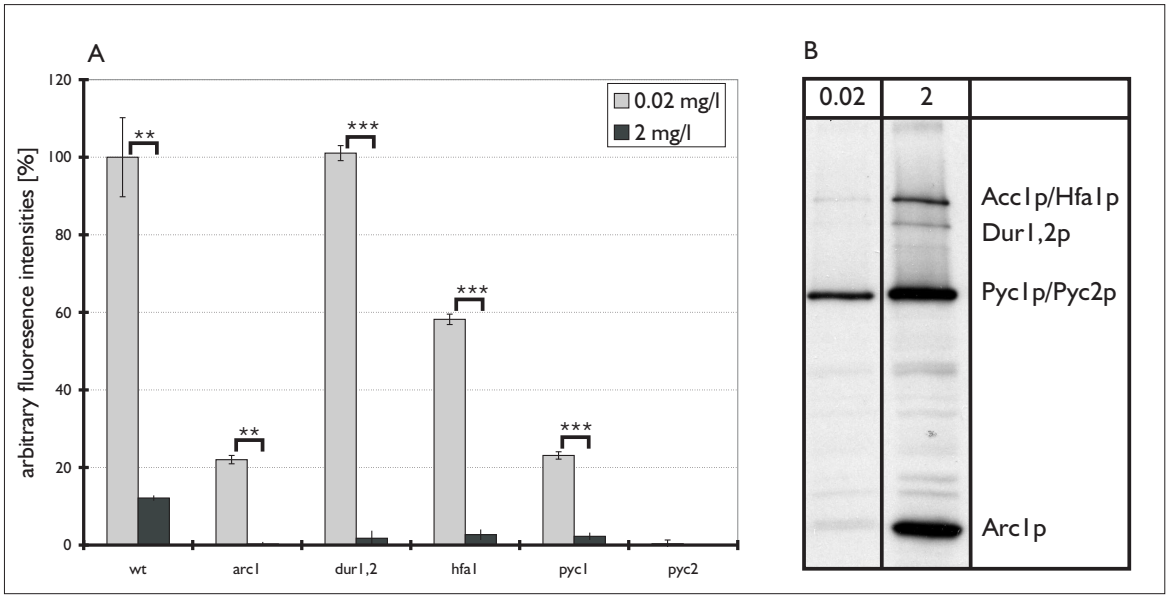

Figure 3.25: **Measurements of fluorescence with a BRE-GFP-reporter in wt and knockouts of biotin proteins in *S. cerevisiae*.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken after 6 h for A: GFP-fluorescence measurements. Fluorescence values are given in %. wt from 0.02 mg/l biotin has been set to 100 %. Mean values of three independent experiments for wt, arc1Δ (arc1), dur1,2Δ (dur1,2), hfa1Δ (hfa1), pyc1Δ (pyc1) and pyc2Δ (pyc2) are shown. Bars indicate standard deviations. (***: $P < 0.0001$, ** $P < 0.001$ by t-test). B: Whole-cell-protein extracts from 0.5 OD_{600} wt-cells per lane were run on a 10 % SDS-gel and western-blots performed. Blots were detected with streptavidin-peroxidase.

3.2.3 Single and double knockouts of *PYC1* and *PYC2*

To get further insight into the function of *PYC*s in biotin sensing double knockouts of the two genes were made. The double mutants require aspartate for growth as a source for oxaloacetate, because this cannot be generated from pyruvate any more. Pyruvate carboxylases catalyze carboxylation of pyruvate to oxaloacetate that is funneled into the citric acid cycle. There is serves as precursor for gluconeogenesis and anaplerotic purposes. Biotin sensing in the *pyc1pyc2* double mutant is completely abolished and cells show the same phenoptype as *vhr1*Δ-mutants, that means no reaction on low biotin concentrations. One explanation for this observation are higher cytoplasmic biotin levels caused by a knockout of the two Pyc proteins that are major targets of biotinylation. To adress this possibility *arc1*Δ*pyc1*Δ- and *arc1*Δ*pyc2*Δ-mutants were generated. As Arc1p is the most abundant biotinylated protein in the cells, a knockout should result in higher cytoplasmic biotin content and reduce the response to low biotin. This indeed could be observed in reporter gene assays (see fig. 3.25). Bio2p-expression was detected by western-blot as an indicator for biotin sensing. The *arc1*Δ*pyc*Δ-mutants do not show the same pattern as wt and are still affected in biotin sensing. Again, as already seen in single knockouts for *pyc1* and *pyc2* the effect is more pronounced in a *pyc2*Δ-mutant. *Pyc1*Δ*pyc2*Δ-mutants show the most severe phenotype and no difference between low and high biotin concentrations.

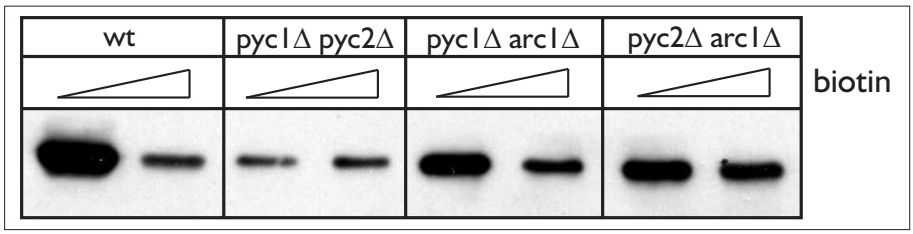

Figure 3.26: **Western-blot for Bio2p-expression in wt, *pyc1*Δpyc2Δ, *pyc1*Δ*arc1*Δ and *pyc2*Δ*arc1*Δ.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken after 6 h for whole-cell-protein extracts. Extracts from 0.5 OD_{600} per lane were run on a 10 % SDS-gel and western-blots performed. Blots were detected with anti-Bio2p-antibody.

Additionally a complementation of *pyc1*Δ*pyc2*Δ-mutants by expressing one of the proteins from a plasmid was tested. The two genes including their endogenous promoters were cloned on the low-copy-plasmid pRS316. Western-blots were de-

tected with α-Bio2p-antiserum. Again cells with the *pyc1*Δ-genotype (*pyc1*Δ*pyc2*Δ pRS316-PYC2) showed a milder phenotype than *pyc2*Δ-cells (*pyc1*Δ*pyc2*Δ pRS316-PYC1)(see fig. 3.27).

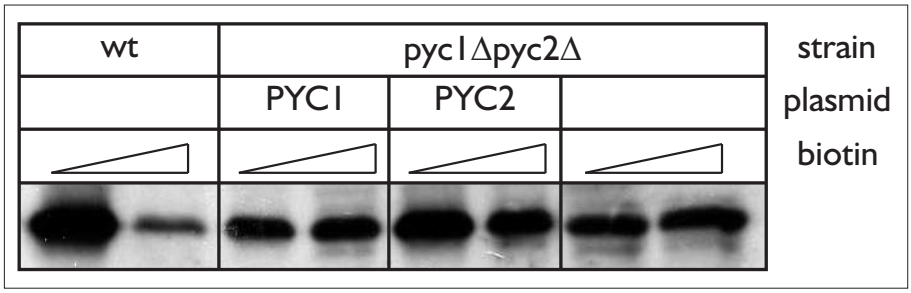

Figure 3.27: **Western-blot for Bio2p-expression in wt, *pyc1*Δpyc2Δ and doubleknockout with plasmids pRS316-*PYC1* and -*PYC2*.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken after 6 h for whole-cell-protein extracts. Extracts from 0.5 OD_{600} per lane were run on a 10 % SDS-gel and western-blots performed. Blots were detected with anti-Bio2p-antiserum.

3.2.4 Complementation of *pyc1*Δ*pyc2*Δ with *pyr1+* from *Schizosaccharomyces pombe*

The fisson yeast *S. pombe* contains only a single pyruvate carboxylase gene, *pyr1+*. A complementation-experiment in a *S. cerevisae pyc1*Δ*pyc2*Δ-mutant was performed, by transforming the double-knockout with *pyr1+* expressed from a low-copy-number plasmid under the control of the Met25-promoter (p416-MET25P-*pyr1+*). The resulting strain was tested for biotin sensing by western-blot with anti-Bio2p antiserum and for biotinylation with streptavidin-PO (see fig. 3.28). The transformed cells were able to grow in the absence of aspartate, indicating the *S. pombe* protein was functional and catalytically active in *S. cerevisiae*. *pyc1*Δpyc2Δ cells were not able to grow in minimal medium without supplementation of aspartate. Biotinylation of the Pyr1 protein has been detected with streptavidin-PO. Cells were not responding to low biotin concentrations by increasing expression of Bio2p. Thus, the defect in biotin sensing was not complemented by expression of Pyr1p, suggesting biotinylation and enzymatic activity of pyruvate carboxylase can not be crucial for biotin sensing. *S. cerevisiae* Pyc2p so must have other features that contribute to functional sensing.

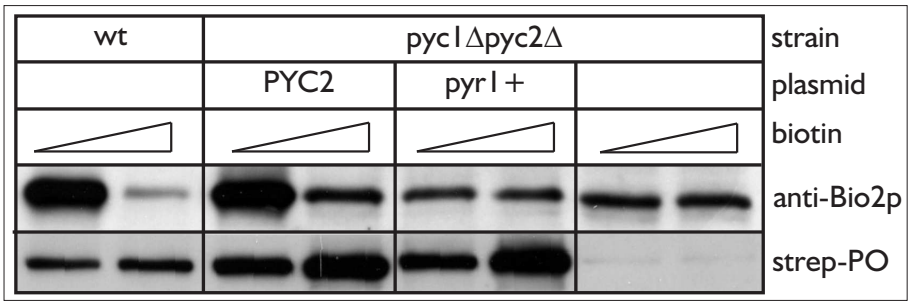

Figure 3.28: **Western-blot for Bio2p-expression and protein biotinylation in wt, *pyc1*Δ, *pyc1*Δ*pyc2*Δ*pyr1*+ and *pyc1*Δ*pyc2*Δ.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken after 6 h for whole-cell-protein extracts. Extracts from 0.5 OD_{600} per lane were run on a 10 % SDS-gel and western-blots were performed. Blots were detected with anti-BIO2-antiserum and strep-PO.

3.2.5 Truncation of Pyc2p C-terminus

For further analysis of Pyc1p and Pyc2p the proteins were tagged with a 3HA-epitope-tag. As the C-termini of the proteins seemed to be less conserved (see fig. 3.31) these parts were selected for the fusion with the tags. PYCs were expressed under control of their endogenous promoters on single-copy plasmids YCplac. Requirement for aspartate of the *pyc1*Δ*pyc2*Δ was abolished by expression of Pyc1-3HAp as well as Pyc2-3HAp. Functionality in biotin sensing was tested with GFP-reporter-assays. In the test both tagged Pyc-proteins show fluorescence values like the *pyc1*Δ*pyc2*Δ-mutant at low biotin concentrations, meaning the 3HA-tag somehow inhibits the function of both proteins in biotin sensing. This loss of function has to be due to the tag, because the two proteins expressed under the same promoter and on the same plasmid show functional sensing (data not shown). Missing expression or biotinylation has been excluded by western-blot analysis and detection with strep-PO where both proteins were detected(see fig. 3.29).

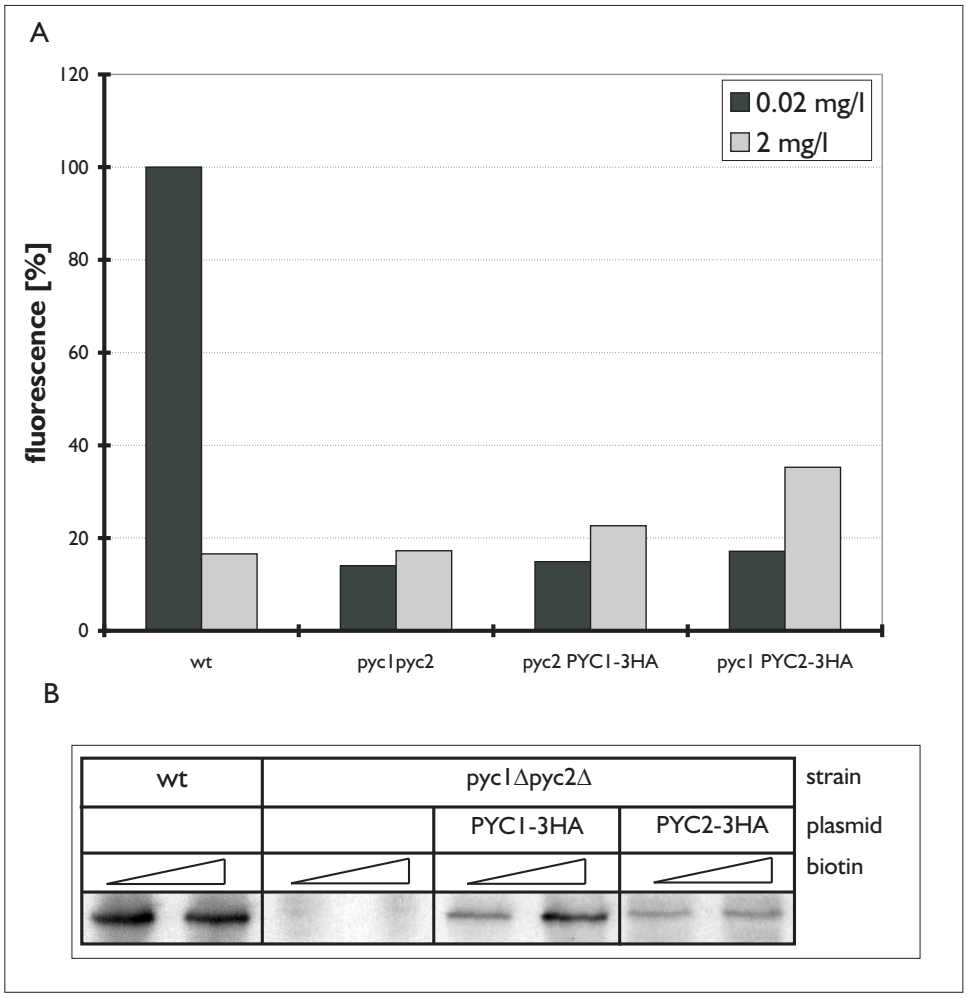

Figure 3.29: **Measurements of fluorescence intensities with a BRE-GFP-reporter and western blot for cells with C-terminally tagged *PYC*s.** A: Fluorescence values are given in %. Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken after 6 h for GFP-fluorescence measurement. wt from 0.02 mg/l biotin was set to 100 %. Values for wt, 3HA-PYC1, 3HA-PYC2, *pyc1*Δ, *pyc2*Δ and *pyc1*Δ*pyc2*Δ are shown. B: Western blot detected with strepavidin-PO for wt and *pyc1*Δ*pyc2*Δ-strains with empty vector (*pyc1*Δ*pyc2*Δ), YCplac-PYC1-3HA (PYC1-3HA*pyc2*Δ) and YCplac-PYC2-3HA (*pyc1*Δ)PYC2-3HA.

As C-terminal tagging negatively affected biotin sensing, N-terminally tagged versions under control of the *MET25*-promoter on the low-copy vector pUG36 were created (pUG36-*MET25*P-3HA-*PYC*). This plasmid also complements aspartate requirement of *pyc1*Δ*pyc2*Δ mutants. GFP-reporter assays showed tagging on this terminus has weaker influence on biotin sensing than with tagged C-terminus. Highly significant differences in fluorescence between cells grown in 0.02 mg/l and 2 mg/l biotin can only be observed for wt. Nevertheless an increase in reporter activity in cells from low biotin is clearly visible for both N-terminally tagged Pyc-proteins like for wildtype, indicating functional sensing (see fig. 3.30).

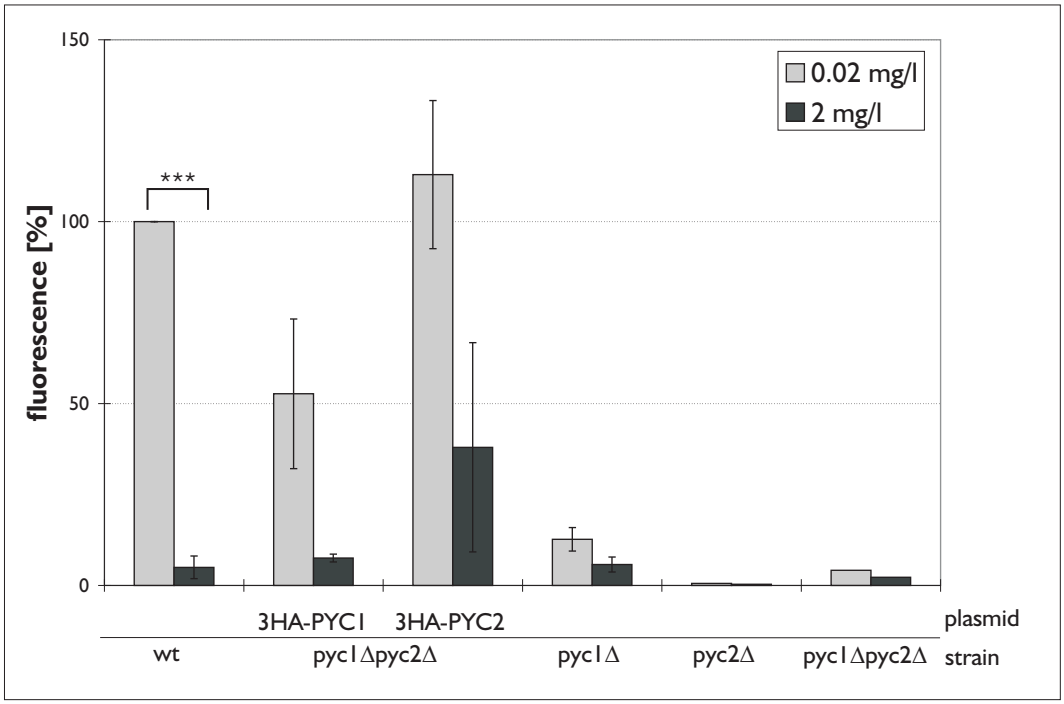

Figure 3.30: **Measurements of fluorescence intensities with a BRE-GFP-reporter in cells with N-terminally tagged *PYC*s.** Fluorescence values are given in %. Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were taken after 6 h for GFP-fluorescence measurement. Values for wt from 0.02 mg/l biotin have been set 100 %. The mean values of three independent experiments are shown for wt, 3HA-*PYC1*, 3HA-*PYC2*, *pyc1*Δ, *pyc2*Δ and *pyc1*Δ*pyc2*Δ. Bars indicate standard deviations. ***: $P < 0.0001$ by t-test.

Functional N-terminally and infuntional C-terminally tagged pyruvate carboxylases suggest an involvement of the Pyc C-termini in biotin sensing. Sequence align-

ments with Pycs from other organisms showed that the *S. cerevisiae* Pyc-proteins contain additional 9 or 10 amino acids compared to mammalian and 7 or 8 amino acids compared to *Sz. pombe* and *Bacillus* pyruvate carboxylases for Pyc1p and Pyc2p, respectively (see fig. 3.31). So C-terminal truncations in two- or four-amino-acid steps as indicated were created to find residues essential for sensing. The truncations were only generated for Pyc2p as this protein appeared to have the bigger impact on biotin sensing in all previous assays.

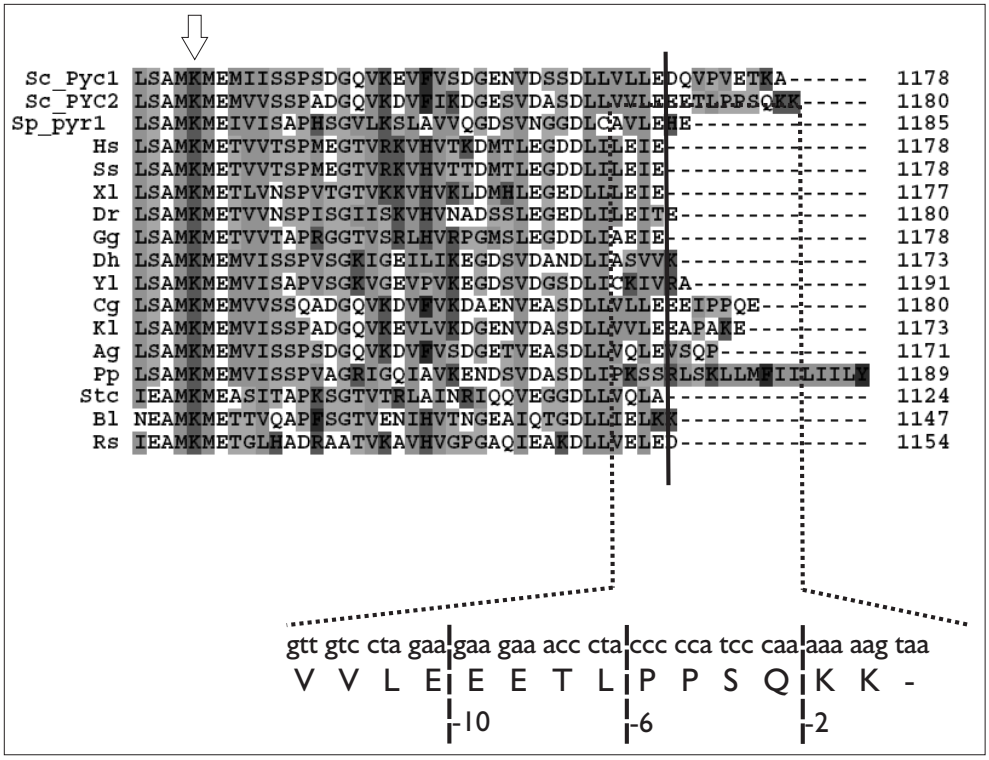

Figure 3.31: **C-terminal ends of different pyruvate carboxylases and truncations in *S. cerevisae* Pyc2p.** Sequence alignments of pyruvate carboxylases from Sc (*Saccharomyces cerevisiae*), Hs (*Homo sapiens*), Ss (*Sus scrofa*), Bl (*Bacillus licheniformis*), Gg (*Gallus gallus*), Stc (Streptomyces coelicolor), Xl (Xenopus laevis), Dr (Danio rerio), Rs (Rhodobacter sphaeroides), Kl (*Kluyveromyces lactis*), Dh (*Debaryomyces hansenii*), Cg (*Candida glabrata*), Yl (*Yarrowia lipolytica*), Ag (*Ashbya gossypii*), Sp (*Schizosaccharomyces pombe*), Pp (*Pichia pastoris*). The arrow indicates lysine residues modfied with biotin in holoproteins. In Pyc2p from *S. cerevisae* three different truncations lacking 2 (-2), 6 (-6 and 10 (-10)) amino acids as shown were constructed.

Truncations were made in the context of the N-terminally tagged Pyc2 protein analyzed before. Again GFP-reporter-assays were utilized to determine the sensing

ability and measurements indicated an increased induction of fluorescence the shorter the C-terminus of Pyc2p was. Differences for cells from 0.02 mg/l biotin were not significant from wt for 3HA-PYC2-full-length and 3HA-PYC2-(-2), but significantly higher fluorescence values were observed for 3HA-PYC2-(-6) and 3HA-PYC2-(-10) (see fig. 3.32). To exclude impact of the truncations on expression and biotinylation of Pyc2p western-blots were made and detected with α-HA-antibody and strep-PO. No difference in biotinylation or expression of full-length and any of the truncated Pyc2p-versions was obvious (see fig. 3.32) .

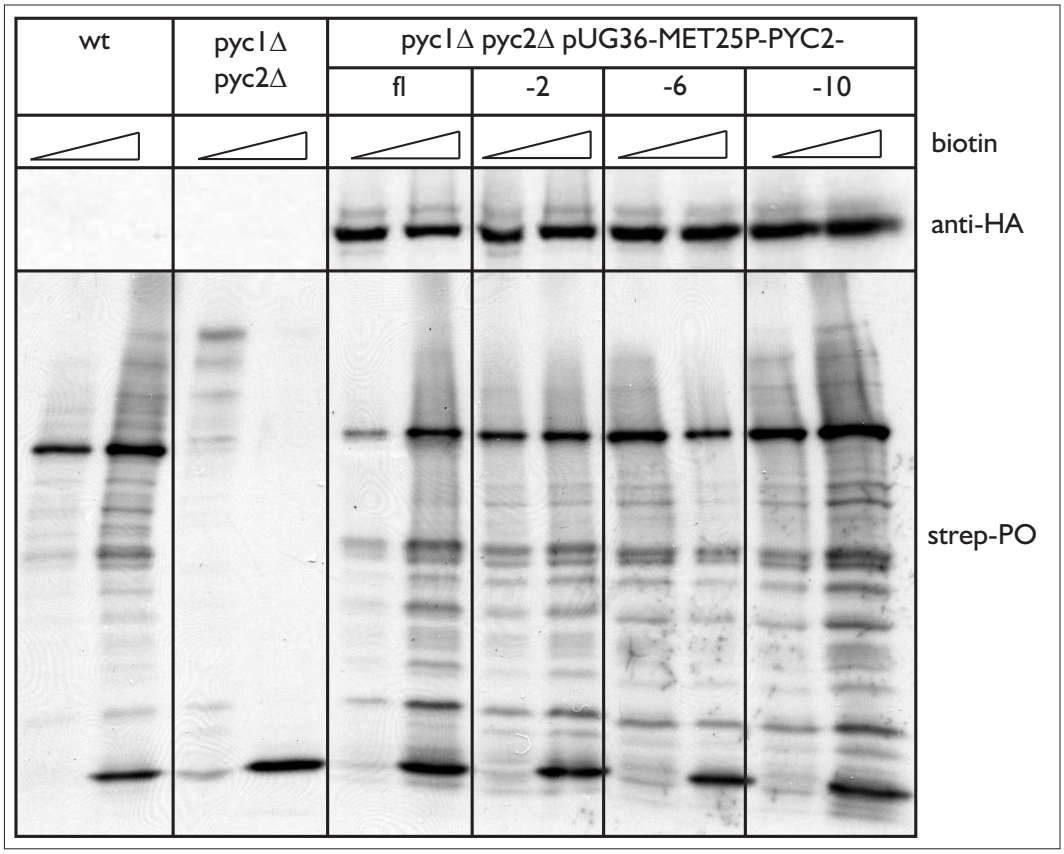

Figure 3.32: **Expression level and biotinylation of full-length and truncated Pyc2p.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were collected after 6 h for whole-cell-protein extracts. Extracts from 0.5 OD_{600} per lane were run on a 10 % SDS-gel and western-blots been performed. Samples for wt, *pyc1*Δ*pyc2*Δ without plasmid and with pUG36-MET25P-PYC2-full length (fl) and truncated by 2 (-2), 6 (-6) and 10 (-10) amino acids were analyzed. Upper part detected with anti-HA-antibody. Lower part detected with strep-PO.

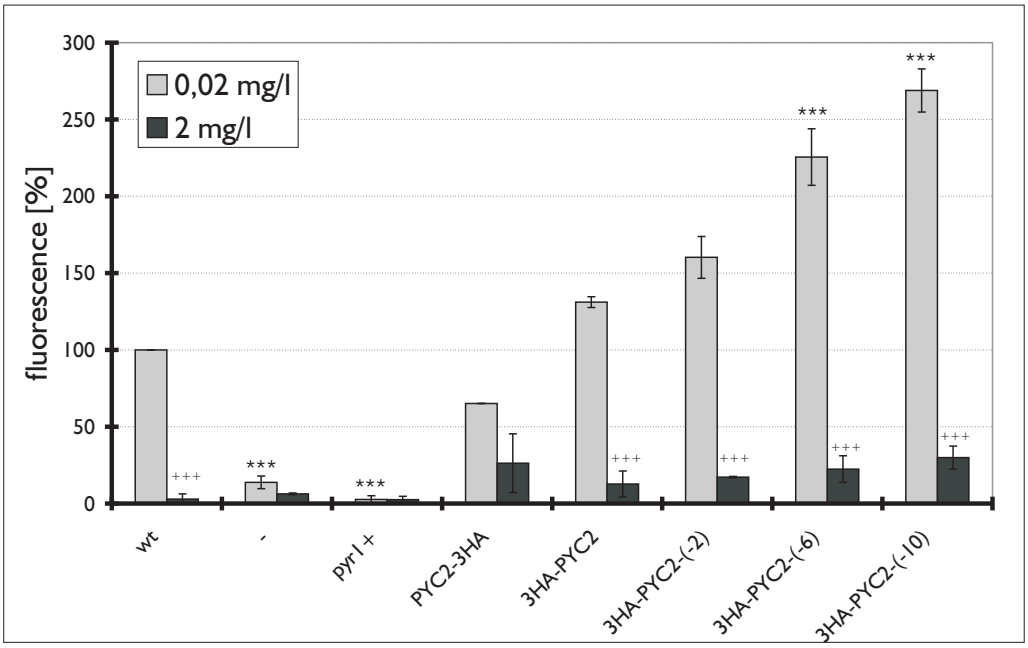

Figure 3.33: **Measurements of fluorescence intensities with a BRE-GFP-reporter in cells with truncated Pyc2p.** Fluorescence values are given in %. Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 and 2 mg/l biotin. Samples were collected after 6 h for GFP-fluorescence measurement. wt from 0.02 mg/l biotin has been set to 100 %. The mean values of three independent experiments are shown for *pyc1*Δ*pyc2*Δ without plasmid (-) and with plasmids pRS416-*pyr1+* (pyr1+), YCplac33-*PYC2*-3HA (PYC2-3HA) and pUG36-MET25P-3HA-*PYC2* in full length (3HA-PYC2), and truncated by 2 (3HA-PYC2-(-2)), 6 (3HA-PYC2-(-6))and 10 (3HA-PYC2-(-10))amino acids. Bars indicate standard deviations. ***: $P < 0.0001$ vs wt 0.02 mg/l by ANOVA, +++ : $P < 0.0001$ value for 0.02 mg/l vs value for 2 mg/l for each plasmid by ANOVA.

Additionally, pyruvate-carboxylase-activities of *pyc1*Δ*pyc2*Δ double-knockouts expressing full-length and truncated Pyc2p-versions were measured. Therefore protein extracts of yeasts grown for 6 h in minimal medium with low biotin concentration (0.02 mg/l) were made. Protein concentrations were determined and enzyme activities measured in a coupled enzyme test with malate dehydrogenase [54]. Finally oxidation of NADH in the test was measured photometrically and pyruvte carobxylase activites calculated in units/mg yeast protein extract.

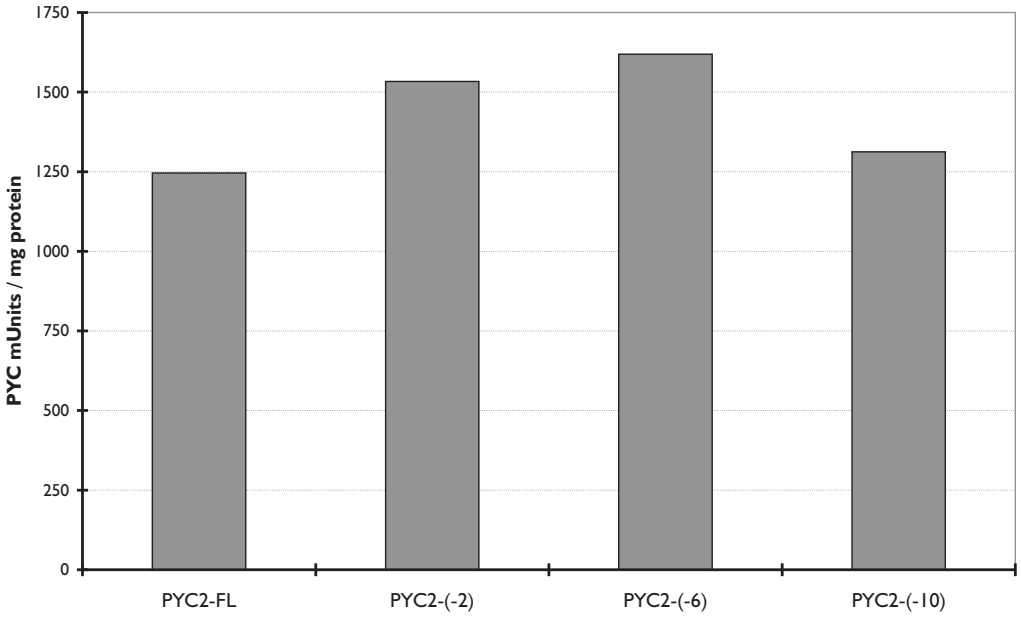

Figure 3.34: **Pyruvate carboxylase enzyme activity measurements with truncated versions of Pyc2p.** Cells from an overnight culture with 2 mg/l biotin were inoculated in 0.02 mg/l biotin. Samples were collected after 6 h for sample preparation. Values for *pyc1*Δ*pyc2*Δ with plasmids pUG36-MET25P-3HA-*PYC2* in full length (PYC2-FL), and truncated by 2 (PYC2-(-2)), 6 (PYC2-(-6))and 10 (PYC2-(-10))amino acids are shown.

No clearly different pyruvate carboxylase activities were measured for the four tested plasmids, suggesting that the truncations do not affect enzyme activity. Thus, the effects observed in sensing seem not to be caused by a change in enzyme activity (see fig. 3.34).

3.2.6 Co-immunoprecipitation of Pyc2p

To identify possible interaction partners of Pyc2p that might play a role in biotin sensing, a co-immuno precipitaion was carried out. To avoid possible interfering protease acitivity the strain c13-ABYS-86 that carries deletions of four proteases was used. The strain was transformed with pUG36-Met25P-*PYC2* and pUG36-Met25P-*PYC2*(-10) and grown in medium containing 2 mg/l and 0.02 mg/l biotin. 3HA-Pyc2p was purified from the cell extracts with monoclonal anti-HA-antibodies and bound to a protein-A-column to which the F_C-region of the antibodies binds. Wt-strain was used as control. Elution of all binding proteins was performed by boiling with SDS-loading-buffer and the eluates were analysed on a 17 % SDS-gel (see fig: 3.35).

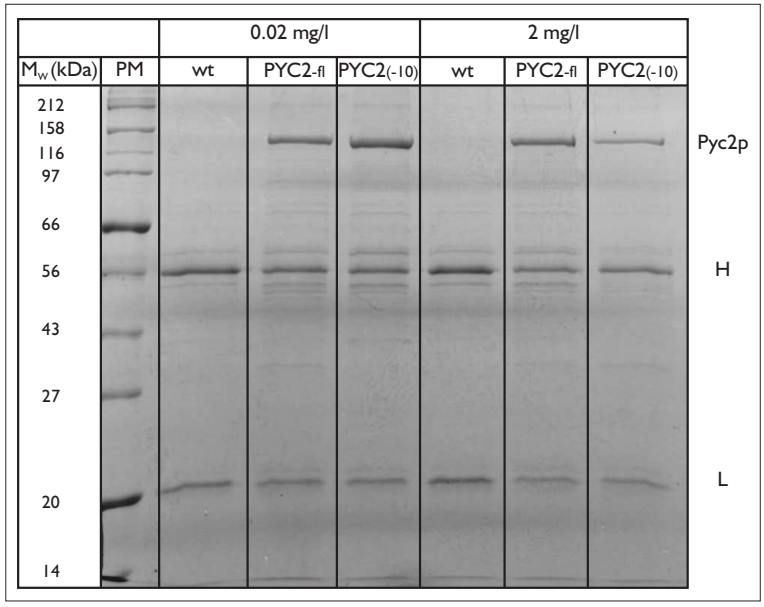

Figure 3.35: **Co-immunoprecipitation of full lenght and (-10) Pyc2p.** Coomassie-stained gel with protein extracts from c13-ABYS-86 cells grown in 0.02 mg/l and 2 mg/l biotin. Cells contained no plasmid (wt) or pUG36-MET25P-3HA-*PYC2*- full length (PYC2-fl) or truncated by 10 amino acids (PYC2(-10)). Samples were prepared as described in the text.

As expected the bands for 3HA-Pyc2p and 3HA-Pyc2p-(-10) appear at a molecular weight about 130 kDa. The other prominent two bands at 56 and 27 kDa correspond to the heavy and light chain of the anti-HA-antibody used for purification, respectively. Apart from these three bands no other protein-band that might represent an interaction partner of Pyc2p was obvious. All other bands were rather

weak compared to Pyc2p and appeared in all lanes, so they most likely represent proteins binding unspecifically tho the column .

3.3 Histone biotinylation in *S. cerevisiae*

Biotinylation of histones has been shown in different mammalian cells and seems to fulfill several functions [14], [32], [91]. As *S. cerevisae* is also a eucaryotic organsim it is conceivable that histones might be biotinylated here, too. To proove this hypothesis, strains with chromosomally 3HA-tagged histones H2B, H3 and H4 were used. Fusion proteins additionally contained a MNase on the C-terminal end of the histones, followed by the 3HA-epitope. Strains were generated by Katharina Merz [122] and kindly provided for analysis in this work. Nuclear extracts were isolated as described in material and methods and samples analysed by SDS-page and western-blot. Blots were detected with anti-HA- and anti-biotin-antibody and with strep-PO.

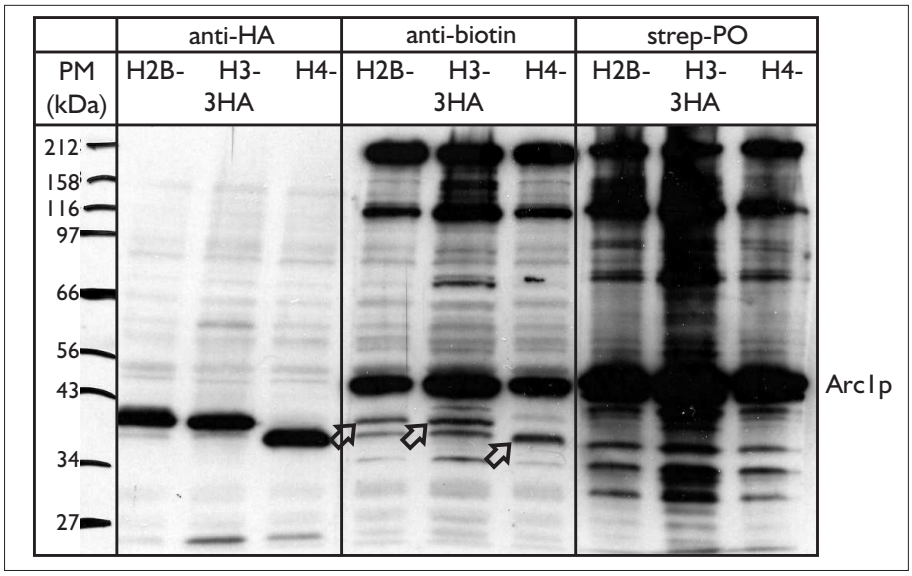

Figure 3.36: **Western-blot of yeast nuclear extracts with 3HA-tagged histones H2B, H3 and H4.** The blot was detected with anti-HA-, anti-biotin-antibody and strep-PO. Bands on the blot detected with anti-biotin -antibody that show the same molecular weight as bands for histones detected with anti-HA-antibody are marked with arrows.

Additionally to the known biotin-proteins in the extracts, bands corresponding to all three tested histones could be detected with anti-biotin-antibody (marked in fig. 3.36), but not with strep-PO. In general more unspecific reactions were present on the blot detected with strep-PO in comparison to anti-biotin-antibody (see fig. 3.37).

To avoid contamination with other biotinylated proteins (especially Arc1p) that

also are present on the western-blots (fig.3.36) the histones were purified by making use of the 3HA-tags. Nuclear extracts were incubated with anti-HA-antibody, purified over protein-A sepharose column and eluated fractions subsequently again analysed by SDS-PAGE and western-blot detected with anti-HA- and anti-biotin-antibody.

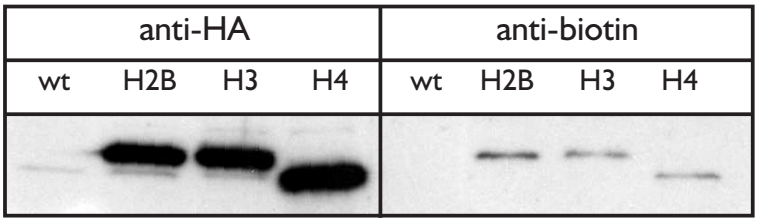

Figure 3.37: **Western-blot of purified 3HA-tagged histones.** Nuclear extracts with 3HA-tagged histones were purfied via their epitope-tags as indicated in the text and analysed by western-blot with anti-HA- and anti-biotin-antibodies.

Bands corresponding to histones H2B, H3 and H4 were detected with anti-biotin antibody. For detection with strep-PO no signal was found (data not shown).

Additionally a second approach was used to provide evidence for the biotinylation of histones. Nuclear extracts were purified over a monomeric avidin column, where all biotinylated proteins bind and the eluted fractions were analyzed on a western-blot with anti-HA antibody. This experiment led to the detection of bands corresponding to H2B-3HA, H3-3HA and H4-3HA on a western-blot with eluated fractions from the avidin-column detected with anti-HA-antibody (fig. 3.38).

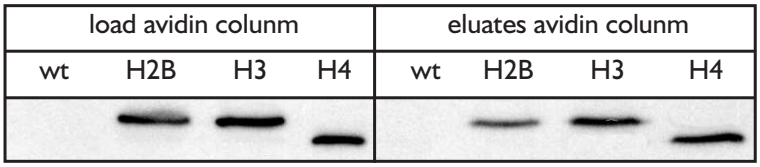

Figure 3.38: **Western-blot of 3HA-tagged histones after pruification over an avidin column.** Nuclear extracts with 3HA-tagged histones were purfied over an avidin column and analysed by western-blot. Blots were detected with anti-HA-antibody.

The above experiments clearly demonstrated that histones are biotinylated in *S. cerevisiae*. To find the amino acids that are biotinylated *in vivo* histone purification via monomeric avidin was upscaled. To avoid protein degradation during purification, yeast strain c13-ABYS-86, lacking 4 proteases in comparison to W303 wt was

used. Eluated fractions of the avidin-column were run on a 17 % SDS-gel (see fig. 3.39). The gel was stained with colloidal coomassie and bands in the M_W-range of histones (H1: 27.80 kDa, H2A: 13.99 kDa, H2B: 14.95 kDa, H3: 15.356 kDa, H4: 11.37 kDa) cut and analysed by fingerprint sequencing (performed by Prof. Dr. Axel Imhof, Zentrallabor für Proteinanalytik, LMU München).

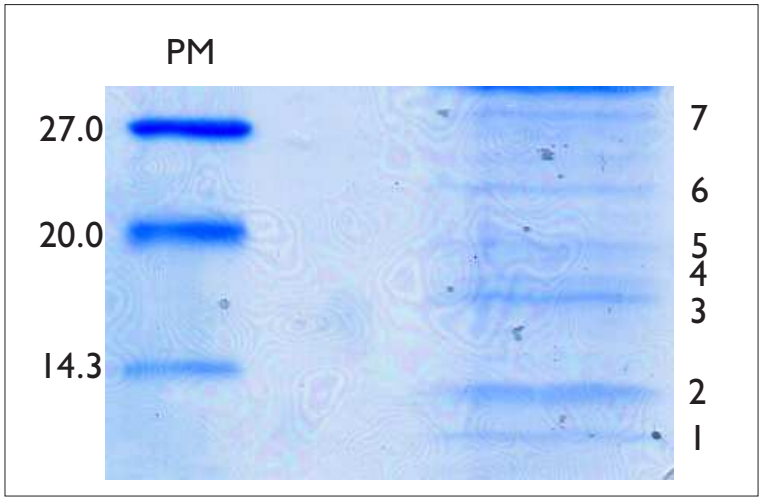

Figure 3.39: **Coomassie-gel of purified proteins for mass fingerprinting.** Nuclear extracts were purified over an avidin column and eluates run on an SDS-gel. Visible bands from the M_W-range smaller than 27 kDa were cut and analysed by fingerprint sequencing. PM: protein molecular weight marker. Molecular weights are indicated in kDa. Indicated bands (1 - 7) were cut and analyzed by mass fingerprinting.

As shown in table 3.3 no histones were identified among the analyzed bands from the gel. The identified proteins are not known to be biotinylated or to bind biotin.

To increase the yield and purity of histones in cell extracts different protocols like precipitation with 0.5 M HCl or 33% TCA and high salt extraction [178] were tried but this did not result in better detection of biotinylated histones (data not shown).

band	gene (systematic) name	annotated function at SGD	M_W
1	*RS21A* (YKR057W)	40S ribosomal protein S21-A, cytosolic	9.7 kDa
2	*COX6* (YHR051W)	subunit VI of cytochrome c oxidase	17.3 kDa
	SUI1 (YNL244C)	translation initiation factor eIF1	12.2 kDa
3	*CPR6* (YLR216C)	peptidyl-prolyl cis-trans isomerase (cyclophilin)	42.1 kDa
4	avidin		
5	*SOD1* (YJR104C)	cytosolic copper-zinc superoxide dismutase	15.9 kDa
6	*TSA1* (YML028W)	thioredoxin peroxidase	21.6 kDa
	GUK1 (YDR454C)	guanylate kinase	20.5 kDa
7	*EGD2* (YHR193C)	nascent polypeptide-associated complex α-polypeptide	18.7 kDa
	SBA1 (YKL117W)	co-chaperone that binds to and regulates Hsp90 family chaperones	24.1 kDa

Table 3.3: **Proteins identified by mass fingerprinting from purification of nuclear extracts over an avidin column**

4 Discussion

On the one hand the "classical" functions of biotin as a cofactor in carboxylases are long established and in most parts well understood. Recently other functions of the vitamin get into the focus of research. Important roles in cell signalling processes and histone modification affecting gene expression are ascribed to biotin and some biotin catabolites.

4.1 Biotin transport

4.1.1 The *E. coli* biotin transporter represents a new class of bacterial biotin transporters

Most higher organisms depend on effective uptake mechanisms for biotin as they are not capable of biosynthesis. Different plasma membrane transporters that have different evolutionary backgrounds have been characterized. The sodium dependent multivitamin transporter SMVT seems to take the major role for biotin transport in mammals [152], [219], [95]. MCT1 (monocarboxylate transporter 1) is also proposed to transport biotin in some tissues or cell types [43]. Both transporters share no significant homology. Homology of SMVT1 to the sodium dependent pantothenate transporter PanF of *E. coli* gives a hint that transporting this substance could have been conserved, and the ability to transport also the in parts structurally related biotin and lipoic acid gained later [92]. This might also explain why SMVT transports pantothenate with higher affinity (K_M = 4.9 μM) than biotin (K_M = 15.1 μM) and lipoic acid [152]. Transport of biotin by MCT1 seems to be a complete new feature, as the standard or main substrates for other members of this transporter family are lactate and pyruvate, aromatic amino acids and thyroid hormones [121], [59].

Biotin transporters that show no structural or sequence homologie to MCT1 and SMVT1 can be found in yeasts. Vht1p in *S. cerevisae* [185] and Vht1p in *Sz. pombe* [184] share almost identical biological properties like affinities in the submicromolar range and similar substrate specificties [165]. Both have 12 transmembrane domains, are members of the family of allantoate transporters, but are only distantly

related. The closest homolog to *S. cerevisiae* Vht1P in *Sz. pombe* is the pantothenate transporter Liz1p. Plants that are able to synthesize biotin *de novo* transport the vitamin via sucrose-H^+-symporters as shown for *Arabidopsis thaliana* AtSUC5 [113]. The affinity of the *A. thaliana* transport system could not be determined as a saturation was not possible up to 2 mM biotin, that is at the upper limit of biotin solubility in water.

In addition to their ability to synthesize biotin *de novo*, several microorganisms have the capacitiy to take up the vitamin from the invironment. In procaryotes a mechanistically new class of modular transporters was published by Rodionov *et al.* in 2008 [157]. These occur in more than 400 modules from over 200 species. Among them are transporters for metal ions (cobalt and nickel), amino acids (methionine and precursors, tryptophane), vitamins (biotin, thiamine, cobalamin, riboflavin, folate, niacin, pyridoxine, lipoate, pantothenate) and other substances (queosine, thiazole) can be found. The transport systems contain three different components. The substrate specificity is mediated by a distinct integral membrane protein (S-component) with 155-230 amino acids and six transmembrane domains. This protein is able to bind the substrate and in some cases can alone facilitate diffusion of the substrate. An ATPase (A) subunit supplies the transport process with energy and a special transmembrane protein (T) mediates the interaction between the S- and A-components. A- and T-subunits can be specific for only one substrate and interact with one S-component, or are shared by various S-proteins for different substrates in one organism. Shared AT-copmlexes are called ECF (energy coupling factor). This organization of a tripartite transport system has experimentally been shown for the *Rhobobacter capsulatus* biotin uptake system [84]. Substrate affinity for biotin was described to be 250 nM without ECF and 5 nM in presence of the ECF-components. Comparative genome analysis predicted 54 biotin transport systems with dedicated AT-modules and 81 with shared ECFs in different species from *Firmicutes*, *Thermotogales*, *Proteobacteria*, *Archaea*, *Cyanobacteria*, *Actinobacteria* and some others [157]. Biotin transport systems of this class are present in various organisms mentioned above, mainly in gram-positives among them, but can not be found in most of β-, γ- and ϵ-proteobacteria, including *E. coli*. Candidate genes for the *E. coli* biotin transporter have not been found by homology searches with any of the known transport proteins. This makes it likely that (a) new protein(s) that is/are not related to any of the known biotin transport systems enables biotin transport in *E. coli* and most likely other gram-negative bacteria.

4.1.2 *yigM* encodes the *E. coli* biotin transport protein

The experiments in this work were based on the findings of Eisenberg *et al.* who mapped mutations that drastically reduce biotin uptake and decrease the sensitivity of *E. coli* cells towards the toxic analogon α-dehydrobiotin between the *ilv*-operon and *metE* on the chromosome [55]. Annotated functions, as well as surrounding genes did not give hints for or against a role in biotin transport of one of the three candidate genes *yifK*, *rarD* and *yigM* identified in this region.

Several lines of evidence confirm the role of YigM as the plasmamembrane biotin transport protein of *E. coli*:

A *yigM*-knockout strain does not show biotin uptake. Thus, YigM has to be an essential component for biotin uptake. Additionally the experiments with a knockout-mutant showed that YigM is the only high affinity uptake system for biotin in the bacterium.

Overexpression of YigM led to increased uptake of biotin in *E. coli* cells, indicating YigM is the limiting factor for transport. Expression from two different vectors and promoters further showed dose dependency of uptake.

The *yigM*-gene of the biotin uptake deficient strain S1039 [199] contains two mutations. These mutations result in the exchange of the well conserved serine at position 28 just at the beginning of TMD2 against the basic amino acid arginine and of the highly conserved proline at position 243 in TMD9 against serine. Both mutations on their own were sufficient to decrease the biotin uptake activity significantly to about 25 % of the wildtype level. Both mutations together even showed a stronger, although not completely synergistic but significant effect compared to the single mutations.

Kinetic parameters determined for YigM in this work are consistent with earlier published data. Prakash and Eisenberg measured a K_M-value of 140 nM and a v_{max} of 6.6 pmol per min and mg dry cells [151] in comparison to a K_M of 74 $\pm$ 14 nM and v_{max} of 2.3 pmol per min and OD_{600}, corresponding to 7.0 pmol per min and mg dry cells measured in this work. Results of Piffeteau and coworkers (K_M-value of 50 nM and a v_{max} of 7 pmol per min and mg dry cells for a biotin deficient mutant and 27 nM and 6.8 pmol per min and mg dry cells for a K12 wt) also showed similar values [143], [142]. This makes it very likely YigM is the protein responsible for biotin transport observed by the groups mentioned above. The only values that are different from the ones presented here were published by Cicmanec and Lichstein with a v_{max} of 0.020 $\pm$ 0.016 pmol per min and mg dry cells [35]. This difference

might be explained by the experimental conditions used by these authors. They measured uptake up to 150 min and samples from 5 to 40 min were used to calculate uptake rates. In contrast Prakashs and Eisenbergs, Piffeteaus *et al.* and the values determined in this work are based on samples taken in a time range up to 5 or 10 minutes.

4.1.3 Transport mechanism of YigM

The energy requirements of biotin transport in E. coli were controversally discussed. Again Cicmanecs and Lichsteins [35] proposals are not consistent with the results of most of the other groups. Altough Cicmanec and Lichstein expected the transport to require energy as they found accumulation of biotin in the cells, they did not see any decrease of biotin uptake in experiments made without glucose, or addition of inhibitors like NaN_3, iodoacetate, sodium fluoride, sodium cyanide, sodium arsenate and 2,4-dinitrophenol.

Other publicatons reported biotin uptake is energy dependent: Prakash and Eisenberg [151] as well as Piffeteau [143], [142] describe a twofold reduction in transport velocity and a 35% reduction in the uptake plateau in the absence of glucose compared to standard experiments in wt, as well as in a biosynthesis mutant. Piffeteau also demonstrated biotin uptake to be sensitive to the uncoupler CCCP if the substance was added before biotin uptake experiments were started. Addition of CCCP to preloaded cells resulted in biotin efflux [143], [142]. These results could not be verified in this work. Addition of the protonophores CCCP and FCCP at the same concentrations used by Piffeteau (10 μM) only resulted in a slight decrease of uptake rate to about 60 or 70 %. The effect of NaN_3 was even weaker and not significant. Excluding glucose from the uptake assay resulted in a 20% and not significant reduction in transport acitivity. However effects are much weaker than that observed by the other groups and suggest a facilitated diffusion mechanism rather than active uptake by a proton-symport mechanism as proposed by Prakash and Piffeteau. Findings consistent with Piffeteaus data were observed in counterflow experiments. These results also rather suggest a facilitated diffusion mechanism than active uptake by proton symport, as further uptake or at least retension of biotin than exchange would be expected after addition of unlabelled biotin to radioactively preloaded cells, if cells would take up the vitamin actively against a concentration gradient. Because Piffeteau [143], [142] and Prakash [151] propose a proton gradient dependent transport, that could not be verified by the uptake experiments

in intact cells, uptake assays were also performed with membrane vesicles. The system was supplied with energy by reconstitution of bovine cyotchrome c oxidase that produces a membrane potential and a proton gradient. Membranes from cells overexpression YigM (BL21 with pET24-yigM) were compared to membranes from a knockout strain (*yigM*Δ). The rapid influx of radioactivity before energization of the vesicles indicates influx of biotin via facilitated diffusion. After energizing the system by addition of cytochrome c, no further increase in radioactivity was observed. Preincubation of the vesicles with the protonophore FCCP or creating an artificial Na^+-gradient had no influence on the transport processes. All these results suggest biotin uptake by facilitated diffusion and not via an active H^+-dependent transport.

In general there are several possibilities for bacteria to energize transport across biological membranes. One rather special case is the phosphotransferase system that is used almoust exclusively for sugar and sugar alcohol uptake, is restricted to bacteria and could not be identified in archaea or eukaryotes [6], [47]. There are no hints that biotin could be transported by this mechanism.

Beyond the phosphotransferase system bacteria transport some substrates in a sodium dependent manner. These transporters primarily appear in thermophilic and alcaliphilic bacteria [126]. Substrates of these transporters include melibiose, proline, glutamate, serine and threonine, pantothenate, citrate and branched chain amino acids [209]. Except for the *E. coli* serine and threonine transporter SstT that has 8 or 9 predicted TMDs [98], [132] all other mentioned systems contain 12 transmembrane-helices. In general sodium dependent transporters are classified into eleven families [171], [154]. This families display only weak sequence similarities towards each other, making it very difficult to predict if YigM is acting in a sodium dependent way. Experiments to explore the sodium dependency of transport by YigM gave no hints that biotin might be symported with sodium.

The third option for energizing transport is a proton-symport mechanism and most active transport processes in bacteria use the proton motive force as energy source [93]. Experiments with protonophores that destroy the proton gradient did only show weak effects on biotin uptake so it seems unlikely YigM transports biotin via a proton-symport mechanism, although this was proposed by several groups before [151], [143], [142]. As biotin uptake across YigM by a phospho-transferase-system seems to be very unlikely and all experiments with energizing transport in membrane

vesicles by a proton-, as well as a sodium-gradient did not give positive results, an uptake by facilitated diffusion is proposed. This mechanism is also consistent with the results from experiments in intact cells.

Taken together, there are two possible scenarios how biotin transport by YigM can work mechanistically. The rising radioactivity in experiments with membrane vesicles prior to energization with cytochrome c oxidase might reflect uptake that is not energy dependent and occurs via facilitated diffusion or simply binding of biotin to YigM. The missing effects after addition of protonophores, provision of an artificial sodium gradient and omission of glucose from the uptake experiments support this model. The effects of uncouplers and missing glucose on biotin transport reported by Prakash and Piffeteau might be secondary effects reflecting an unfavourable energy state of the cells. If the substances would really have a direct effect on biotin transport by YigM an amplification of these effects would be expected in cells overexpressing YigM in contrast to wt cells used by other groups, but that was not observed in this work.

A second possibility is that YigM intercts with an unknown protein which changes its transport properties. In this scenario YigM alone could act as a biotin binding protein or a facilitator. This might explain why overexpression of YigM results in increasing radioactivity in uptake experiments. However, in these experiments it cannot be discriminated if biotin is taken up into the cells or merely bound at the cell surface. This might also explain why no energy dependency was observed in the experiments adressing this question. A second protein could be necessary for energizing transport. If the concentration of this protein is limiting for energy dependent uptake the overexpression of YigM would overlie the effects of protonophores or missing glucose in the uptake experiments, as binding to or facilitated diffusion over YigM would not to be energy dependent. Evidence the protein alone is sufficient for the uptake of biotin has not finally been shown, as heterologous expression of YigM in a *S. cerevisae vht1*Δ biotin transport-mutant was not able to complement the growth defect of the yeast on medium with low biotin concentrations. This can have different reasons like unproper folding or membrane targeting of the bacterial protein in the eucariotic cells. Expression studies with a GFP-fusion protein have not been made, as YigM already lost about 50 % of its biotin transport ability with a relatively small HA-tag. Retaining functionality could not be expected with the much bigger GFP, so localization of the GFP-fusion protein could show artefacts.

Another reason for missing activity in *S. cerevisae* might of course also be the lack of a further essential component. Another organism where heterologous expression could be examined is *Bacillus subtilis*, as biotin uptake and biosynthesis are characterized in this this bacterium [17], [157]. Heterologous expression should ideally been tried in a mutant defective in biosynthesis and transport in this bacterium.

4.1.4 Homologues of *yigM* might represent biotin transporters of other gram negative bacteria

Some of the homologues of *yigM* are designAted *madN* and annotated as putative acetate efflux pumps. This annotation originates from a gene cluster encoding proteins responsible for malonate decarboxylation in the anaerobic bacterium *Malonomonas rubra* [13]. The cluster consists of 14 genes (*madABCDEFGHKLMNYZ*) and functions of the genes *A* to *F* have been identified (see fig. 4.1). *MadA* encodes the acyl-carrier-transferase (reaction 2), *madB* the integral membrane protein carboxybiotin decarboxylase (reaction 4), *madC* and *madD* two subunits of the carboxyltransferase (reaction 3), *madE* the acyl-carrier-protein and *madF* the biotin-protein. The functions of *madK*, *Y*, *Z* and *madN* are still unknown [13]. Taking into account that the decarboxylation reaction depends on biotin as a cofactor, presence of a biotin transporter gene in the cluster makes sence from a biological standpoint. MadN as a homolog to YigM might be this gene.

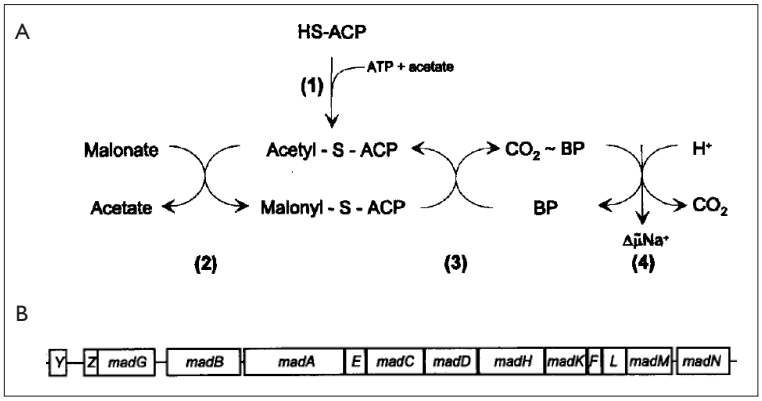

Figure 4.1: **The *mad* gene-cluster of *Malonomonas rubra* (from [13]).** A: Reactions involved in generating a Na^+-gradient from decarboxylation of malonate in *M. rubra*. B: *mad*-operon of *M. rubra*. The enzymes encoded by the genes in the operon and the reactions they catalyze are described in the text.

Homologues of *yigM* can be found, apart from other *E. coli* strains, in other

gram-negative bacteria like *Salmonella*, *Klebsiella*, *Shigella*, *Citrobacter* and *Yersinia* (fig.3.4). The genes are all annotated as putative inner membrane proteins of the 10 TMS Drug/Metabolite Exporter (DME) family or as carboxylate/amino acid/amine transporters, but none of them has been experimentally characterized.

4.1.5 Expression of *yigM* is regulated by biotin

Dependency of biotin transport on biotin concentration in the medium where cells were grown before the uptakes experiments have already been reported in 1974 by Prakash [151] and 1985 by Piffeteau and Gaudry [142]. No biotin uptake could be measured in cells grown in rich medium in this work (data not shown). Measurements with luciferase reporter-constructs under control of the promoters of *bioB* and *yigM* both show biotin dependent regulation. Results of the luciferase assays with reduced uptake to about 50 % at 10 nM biotin in a wt-strain are comparable with measurements of Prakash [151] and Piffeteau and Gaudry [142]. Higher concentrations of biotin were necessary to repress expression from P*yigM* than from P*bioB*. Similar findings have already been made by Prakash and were confirmed in this work [151]. With proposing different repressors for regulation of transport and biosynthesis, Prakash, Eisenberg and Pai might be right, as well [138]. Promoter analysis of *yigM* and *bioB* showed that a putative BirA binding-site is present in P*yigM*, however it contains only one half of the palindrome. Luciferase-reporter-assays in strains with mutations in BirA show not only an impact on P*bioB* that was known before [112], [1]. Also the activity of P*yigM* was changed. Expression from both promoters increased. So BirA is not only involved in repression of biosynthesis genes, but also of the transporter YigM. Evidence for binding of BirA to both promoters was sought using EMSA. Binding of BirA to P*bioB* has been shown over a range of different BirA-concentrations and a clear shift of the *bioB*-promoter in a distinct band was obvious. Localization of BirA in this band was verified by western-blot. In the same experiments no binding of BirA to P*yigM* was observed. Serveral reasons for this are conceivable. Results of the reporter-assays described above suggest different binding-behaviour of BirA to the two promoters with repression of P*yigM* at higher biotin concentrations. Simulation of these conditions was tried by adding higher amounts of biotin and ATP to the binding-buffer for the EMSA but no difference has been observed (data not shown). Involvement of BirA in the repression of P*yigM* is out of doubt as shown by reporter assays with BirA-mutants. If the increase in reporter-activity is caused by missing binding of BirA, or by the lower abundancy of biotinyl-AMP that might affect another transcription factor, remains unclear from these experiments. Reduction of luminiscence levels with lower biotin concentrations for P*bioB* compared to P*yigM* reported before already suggested differences in regulation of both promoters. If these are caused by different affinities caused by different promoter sequences, especially for one half site of the BirA-binding se-

quence in P*yigM* or by involvement of a second protein can not be answered finally.

4.2 Biotin in *S. cerevisiae*

Gene regulation by biotin is a well known and in some organisms good understood process. Expression of biotin biosynthesis genes from the *bio*-operon in *E. coli* is the best characterized example. The central role here is occupied by the BirA protein that is not only the enzyme modifying the biotin carrying subunit of acetyl-CoA-carboylase, but also the transription factor repressing expression of biosynthesis genes at high biotin concentrations in the medium [22], [40], [12], [10], [11]. In mammalian cells evidence for a role of soluble guanylate cyclase and a cGMP-dependent protein kinase have been shown [162], [135], [95]. Leon-Del-Rio published decrease of mRNA levels for SMVT after biotin starvation in rat liver and kidney, while expression of the gene in the brain was not affected [109]. The author suggests this regulation guarantees a sufficent supply of the brain with biotin. Regulation was also studied in human-derived cell lines. In HepG2 cells biotin starvation leads to decrease in SMVT mRNA [136], [135]. Jurkat cells from different biotin concentrations showed no differences in abundance of SMVT protein [114].

In the present work unknown parts of the biotin sensing pathway in *S. cerevisae* were investigated. Several observations about regulation of genes involved in biotin metabolisms were already made. Expression of the *S.pombe* biotin transporter Vht1p increases with decreasing concentration of the vitamin in the medium and leads to higher uptake rates in cells from low biotin concentrations [184]. Higher biotin uptake rates have also been measured in *S. cerevisiae* cells from low biotin concentrations compared to normal biotin concentrations, although here expression of the *VHT1* mRNA of the transporter decreases with decreasing biotin concentrations [185]. Previously reported results of Pirner and Stolz [144] and Weider *et al.* [201] demonstrated the existence of a UAS for biotin dependent regulation called BRE or VHRE. Six genes of biotin metabolism (*VHT1*, *BIO2*, *BIO5* and *BPL1*) contain this regulation element in their promoters [144], [201]. Weider and coworkers additionally found the transcription factor *VHR1*. These results were the basis for the experiments presented here.

Altough above mentioned results shed some light on the sensing-pathway, essential parts are still not known. What is still missing for complete understanding is the low biotin signal Vhr1p recognizes. Further accordance of biotin proteins in sensing could not be demonstrated, nor completely excluded. Comprehensive understanding of the whole pathway could not be achieved within the scope of the present work,

but evidence for an involvement of the biotin-proteins pyruvate-carboxylase 1 and especially 2 in biotin sensing was provided.

4.2.1 *VHR1* and biotin sensing

As reported by Weider *et al.*, *VHR1* plays an essential role in the biotin sensing process [201]. According to the authors Vhr1p is necessary to induce expression of *VHT1* and *BIO5* of yeasts growing in low concentrations of biotin and that there is no enhanced level of the corresponding mRNAs in a *vhr1*-mutant. Further the group showed a predominatly nuclear localization of the protein, but no dependence of localization on biotin levels. Yeast one hybrid experiments were performed with the N-terminal part of Vhr1p that contains a predicted helix-turn-helix motif and with the transactivating domain of Gal4p. These fusion protein was able to induce expression from a promoter containing the BRE indicating the presence of an DNA-binding domain in this part of the protein. Vice versa one hybrid assays with a fusion protein consisting of the DNA-binding domain of Gal4 in combination with the C-terminal part of Vhr1p resulted in reporter expression, suggesting presence of an activation domain. As *VHR1* could not be grouped into a class of already described transcription factors and homologues of the protein are only present in closely related yeast species the authors proposed a new class of transcription factors in *Saccharomycetales* involved in biotin sensing. Direct binding of Vhr1p to DNA could not be shown with co-immunoprecipitation experiments by Weider [202]. The signal recognized by Vhr1p is still unknown.

Experiments in this work revealed that the deletion of *VHR1* resulted in a lack of response to low biotin concentrations, shown by unaffected expression of Bio2p in low biotin medium in a *vhr1*Δ-mutant in contrast to increased expression of the protein in wt (see fig. 3.21). Expression of Vhr1p from a plasmid under control of the endogenous promoter resulted in at least partial restoration of biotin sensing ability, confirming the results of Weider [201]. Further experiments with the protein showed a negative influence of fusing the protein with different epitope tags (3HA, ZZ) at the N- as well as the C-terminal end. These fusion proteins were not able to complement the biotin sensing defect of a *vhr1*Δ mutant. An explanation for an impact of epitope tags might be the highly conserved amino acids 11 to 117 within the DUF6 domain of Vhr1p at the N-terminus, as well as the highly conserved last 10 amino acids at the C-terminal end of the protein [202]. This fact limits possibilities for other important experiments such as purification of the protein and the search

for possibly interacting proteins. Localization of Vhr1p in the nucleus was shown with a GFP-fusion of the protein by Weider *et al.* [201]. According to results in the present work this localization has to be seen critical as this fusion protein was not functional in our hands and results with the unfunctional protein would not be meaningful. Functionality of the fusion protein was not shown by Weider *et al.*. So further details about the mechanism of Vhr1p, like the signal for low biotin status sensed by the protein and if or how binding to the BRE takes place are still unclear. Different scenarios are possible. The simplest one would be recognition of biotinyl-AMP concentrations. This in *E. coli* is the critical substance indicating biotin status of the cell and is bound by the bifunctional BirA [11], that mediates expression or repression of biotin regulated genes. Another one would be the interaction with one or different biotinylated proteins. The fact that Bpl1p is essential for a correct sensing process [144] supports both possibilities, but more complex scenarios can not be excluded.

Also, it is not known if Vhr1p binds directly to the BRE. This question could be adressed by EMSA. One further interesting aspect are interactions between Vhr1p and Snu13p, as well as Bpl1p an Snu13p found in a high throughput tandem affinity purification assay by Krogan *et al.* [104]. Snu13p is annotated as an RNA binding protein and part of U3 snoRNP involved in rRNA processing, as well as part of U4/U6-U5 tri-snRNP involved in mRNA splicing [51], [116]. Snu13p is an essential protein but a strain with the protein under control of a galactose inducible promoter was provided to us by Dr. Ray O'Keefe (School of Biological Sciences, University of Manchester, Manchester, M13 9PT, UK). Growing cells in medium with galactose medium with normal expression of Snu13p and in glucose medium leading to downregulation of Snu13p did not show any differences in biotin sensing (data not shown).

Another possibility for further investigating Vhr1p would be to generate an antibody against the protein. This tool would enable experiments like monitoring expression level or detecting the protein in EMSAs by a supershift.

4.2.2 Pyruvate carboxylases and biotin sensing

Pyruvate carboxylases are essential for biotin sensing

Defects in biotin dependent reporter-expression was found in deletion mutants lacking *ARC1* and especially *PYC1* and *PYC2*. The effect in the *arc*1Δ mutant

can be explained by the fact that deletion of the protein leads to a higher cytoplasmic biotin concentration, thus buffering the effect of low biotin concentration in the medium [144]. In contrast the effect of deleting *PYC1* and *2* can not be explained by elevated biotin concentrations in the cytoplasm, as both enzymes seem to carry similar ammounts of biotin, but the response to low biotin concentrations in the single knockouts of the proteins differs clearly.

Pyruvate carboxylases 1 and 2 resulted of a gene duplication of the whole *S. cerevisae* genome [213]. The amino acid sequences show more than 90 % homology over the whole sequence but are less related at the C-terminal end where the biotin binding domain is located [190], [195]. Expression of both genes is not regulated by biotin. Regulation of both genes in general is different, as their promoters contain different regulatory elements and transcription factor binding sites [190], [19], [120]. What is out of doubt is that the two proteins, especially Pyc2p is essential for correct biotin sensing as shown by western blots detecting Bio2p, as well as by GFP-reporter assays in this work.

Pyruvate carboxylase biotinylation and enzyme activity are not critical for sensing

Results observed with pyruvate carboxylase of *Sz. pombe*, *pyr1+*, exclude a role for biotinylation and enzyme activity in sensing. *pyr1+* is functionally expressed in a *pyc1*Δ*pyc2*Δ doubleknockout mutant in bakers yeast as cells expressing Pyr1p do not require aspartate for growth as the mutant does. Biotinylation of Pyr1p was shown by western-blot detected with strep-PO. Defects in sensing in the double knockout mutant was not complemented by heterologous expression of Pyr1p. If biotinylation or enzyme activity would be the (only) essential criteria in biotin sensing, functional sensing should have been restored by heterologous expression. As this was not the case, additional or other properties of the enzyme seem to be required.

The C-terminus of Pyc2p plays a role in biotin sensing

As C-terminally tagged Pyc2p showed a defect in biotin sensing different C-terminally truncations were examined. These experiments were also inititated because the extreme C-termini of various pyruvate carboxylases indicated a high level of variability, both in length and sequence.

Striking is especially that both *S. cerevisiae* Pyc-proteins are about 10 amino acids longer than most orthologs. Even in the most homolog proteins to *S. cerevisiae* Pyc2p C-termini are in most cases shorter: e.g *Candida galbrata* 2 amino acids, *Vanderwaltozyma polyspora* 10 amino acids, *Lachancea thermotolerans* 2 amino acids, *Kluyveromyces lactis* 9 amino acids, *Zygosaccharomyces rouxii* 2 amino acids, *Ashbia gossypii* 10 amino acids, *Pichia angusta* 14 amino acids and *Pichia guliermondii* 9 amino acids. One C-terminus comparable in length can be found in *Pichia pastoris* that has only one pyruvate carboxylase gene [120]. An interesting experiment in this context would be heterologous expression of the *P. pastoris* pyruvate carboxylase in *S. cerevisiae*, as the C-terminus of this protein is comparable to the bakers yeast homologue. Truncations of the C-terminus indeed showed an effect on biotin sensing. The relative amounts of Pyc2p were not affected by truncations, shown by detection of the N-terminally tagged proteins by western-blot. Altered biotinylation might be caused by the truncations as the biotinlyted lysine residue can be found at the C-terminal part, 45 amino acids before the end of the full length protein were excluded by western-blots detected with strep-PO. Data of Polyak *et al.* support this finding, as the C-terminus does not lie in spatial vicinity to the biotinylated lysin residue that is located on the opposite side in the 3D-structure [147]. Conclusive analysis of Pyc2p biotinylation would require the quantitative analysis of unbiotinylated apo-Pyc2p and biotinylated holo-Pyc2p, as western-blots can hardly be quantified. It can be excluded enzymatic activity of pyruvate carboxylases causes different reactions on low biotin concentrations. Different activities for shorter versions of Pyc2p in comparison to the full length protein were not found. Data observed with the truncated versions of Pyc2p support the findings already made by complementation of a *pyc1*Δ*pyc2*Δ double mutant with *Sz. pombe pyr1+* and show requirement of factors beyond biotinylation, expression and ezyme activity for intact biotin sensing.

A possibility that would be consistent with the results in this work would be interaction of Pyc2p with a still unknown protein. The presence of different charged amino acid residues at the C-terminus (E1222, E1223, K1230 and K1231) suggests this part of the protein to be a potential site for ionic interactions. Promising candidates about know interaction partners from the *Saccharomyces* Genome Database (SGD http://www.yeastgenome.org/) that might be involved in the biotin sensing pathway could not be identified. Directly finding a possible interaction partner by co-immunopreciptiaton of Pyc2p did not meet with success, but this might have several

reasons like the growth conditions of cells or buffer composition during preparation and does not exclude the existence of an interaction. In any case, Pyc2p takes influence on cells response to low biotin concentrations and is an interesting and promising candidate for further experiments.

4.2.3 Histone biotinylation

Basis for histone biotinylation in *S. cerevisiae*

A relatively new field in research is covalent histone modification by biotinylation. Early evidence about nuclear localization of biotin was found by Dakshinamurti and coworkers in 1963, as they observed part of radiolabelled biotin injected to rats and chicken in the nuclei of cells [45]. Hymes *et al.* found that biotinidase can *in vitro* exchange biotin between biocytin and histones [89]. About 25 % of biotinidase can be found in the nucleus of mammalian cells but the functional appearence of this finding is unclear [145]. Stanley and coworkers detected that up to 1 % of the intracellular biotin can be found in the nucleus and additionally showed biotinylation of all five histone-proteins (H1, H2A, H2B, H3 and H4) from human lymphocytes by western-blot using streptavidin and anti-biotin-antibodies [181]. But also HCS is a candidate for histone biotinylation due to its nuclear localization that was observed by Narang et al. in HeLa, Hep2 and fibroblast cells with use of anti-HCS-antibodies in western-blots and with immuno-fluorescence [125]. They also showed that recombinantly expressed and purified HCS is able to transfer biotin to all 5 histone proteins in presence of ATP. As Narangs examination of samples from patients with MCD caused by defects in HCS showed reduced histone biotinylation, HCS seems to be the enzyme that biotinylates histones *in vivo*. For mammalian histones, modifications in H2A, H3 and H4 have been identified by *in vitro* enzymatic biotinylation of synthetic peptides by biotinidase and HCS and subsequent MS-analysis [80]. In *S. cerevisiae* data about cytoplasmic [105] and also nuclear localization of Bpl1p [88] provide a basis for histone biotinylation in yeast, so different efforts to clarify this point were made. Also the fact that a canonical biotinylation domain is not absolutely required for protein biotinylation [97] makes it possible that further yeast proteins are biotinylated. In this work evidence is provided that histones H2B, H3 and H4 of *S. cerevisiae* are biotinylated *in vivo*.

Histones H2B, H3 and H4 are biotinylated in *S. cerevisiae*

On the one hand 3HA-tagged histones H2B, H3 and H4 were immunoprecipitated and biotinylation detected by western-blot analysis with anti-biotin-antibody. On the other hand nuclear extracts were purified over an avidin column and eluted by boiling with SDS-sample buffer. The eluates were analyzed on western-blot with anti-biotin-antibody. Bands for histones H2B, H3 and H4 were detected. Together these results proove that *in vivo* biotinylation of histones in *S. cerevisiae* occurs. Attempts to identify biotinylated histones directly by MS have not been successful. This might be due to limited detection sensitivity in the coomassie stained gel with eluates of the avidin column in comparison to western-blot where detection was possible. Another reason could be insufficient purification of histones prior to loading them on the avidin column. In the experiments nuclear extracts were used that showed better results than whole cell extracts, but obviously further purification is necessary. Histone biotinylation seems to be a rare event with estimations of less than 0.03 % of all histone proteins being biotinylated [4]. So detection levels seemed to be a problem with the methods tried in this work. Another point is that yeast cells contain six other biotinylated proteins in much higher amounts that can disturb purification and detection of biotinylated histones. A big problem could be contamination of the nuclear extracts with proteins from other parts of the cells. Indeed several proteins were found in the eluates of the avidin column that are localized in cytoplasm (Rsa1p, Cpr6p, Tsa1p) or in mitochodria (Cox6p). Thus nuclear proteins or histones should be enriched from nuclear preparations for further experiments. Other methods to enrich histones from nuclear extracs like precipitation with H_2SO_4 [58], HCl (protocl from ABCAM; http://www.abcam.com/) acid- and high-salt-extraction [178] and an alternative protocol [200] did not lead to better results (data not shown). Another reason for identifying other proteins than histones might be these proteins are more highly abundant. An example for this problem seemed to be Sod1p that was described to be located amongst others in the nucleus and in about 500,000 copies per cell [65]. Histone proteins appear in 200,000 to 500,000 molecules per cell. Taking into account that only about 0.3 % of histones are estimated to be biotinylated, Sod1p is present in 300- to 500-fold higher concentrations in the cell and might unspecifically bind to the column. *In vitro* assays for biotinylation of synthetic peptides by *S. cerevisae* Bpl1p similar to those used in the identification of biotinylated amino acids in mammalian histones [25] have not been tried.

Detection and function of *in vivo* biotinylated histones

The biotinylation of histones is not discussed without controversy. Bailey and coworkers reported artifactual detection of histone biotinylation by streptavidin [4]. Healy *et al.* even claim biotinylation of histones is not a natural modification. They were able to confirm results for biotinylation of histones in *in vitro* assays that were used to identify biotinylated lysine residues, but were not able to detect *in vivo* biotinylation of histones with different methodes e.g. modification of histones with $^{[3]}$H-labelled biotin as a very sensitive method [83]. They showed that less than 1 in 100,000 histone molecules carry biotin as a modification *in vivo*. This is even a factor 30 below estimations of Bailey *et al.* [4] and again shows that detection of *in vivo* biotinylated histones is very challenging. Very sensitive methods have to be used in detecting *in vivo* histone biotinylation. This might be the reason why *in vivo* biotinylation of histones could not be shown directly by MS-analysis in this work. Current techniques detect *in vivo* biotinylation with avidin- or streptavidin-conjugates or specific antibodies against one distinct biotinyl-lysin-residue in a histone protein after a preceding histone enrichment, e.g. by acid precipitation [181], [140], [219], [82]. Detection of *in vivo* histone biotinylation with anti-biotin-antibody was also demonstrated for *S. cerevisiae* histones H2B, H3 and H4 in this work.

Nervertheless different functions are ascribed to biotinylated histones. In *Drosophila melanogaster* HCS mediated histone biotinylation is reported to be necessary for normal gene transcription patterns and life span, as well as heat tolerance [26], [29]. First results have also been published for mammalian cells, where biotinylation of K12 in histone H4 is more abundant in heterochromatin [27]. Biotinylation of the same lysine residue also decreased response to DNA double strand breaks [103]. In the context of DNA damage increased biotinylation of histones after UV-light treatment was shown in Jurkat cells [140]. Recently repression of transposable elements by histone biotinylation in human, mouse and *Drosophila melanogaster* cells has been described [34]. Another interesting finding is regulation of SMVT expression by chromatin remodeling [69]. Gralla and coworkers proposed a biotin dependent nuclear localization of HCS in Jurkat cells. Cells growing in 10 nmol/l biotin showed more HCS in the nucleus and more biotinylated K12 in histone H4 in the region of promoter 1 of SMVT compared to cells from 0.25 nmol/l biotin [153]. Biotinylation of H4 K12 turned out to decrease expression of SMVT by 86 %. Additionally biotin independent expression was shown to be driven by the transcription factors KLF-4 and AP-2 [153].

Taking together these results suggests new possibilities for biotin sensing in yeast. As first hints for histone biotinylation were provided in this work, further characterization of the biotinylated lysine residues should follow. Direct gene regulation, especially by silencing like shown in mammalian cells [69], [140], [27] through this modifications seems to be possible in yeast cells, too. Another point that needs to be addressed is if the biotin group can be removed from histones, as *S. cerevisiae* contains no biotinidase like mammalian cells, but the exchange of modified against unmodified histones seems to be a possible alternative. Besides for a requirement of Bpl1p [144] and Vhr1p [201] and an involvement of Pyc2p (this work), the biotin sensing process in *S. cerevisiae* is not fully understood and the signal the transcription factor Vhr1p recognizes is still unknown. Especially the fact that interactions of Vhr1p, histone H2A [104] and histone H2B.1 [63] were found supports a possible function of biotinylated histones in biotin sensing. In this scenario it is conceivable that Vhr1p binds to biotinylated histones, preventing the protein to act as a transcription factor at BRE containing promoters. Biotin depletion would reduce histone biotinylation and release Vhr1p from its immobilized, inactive state. There is no experimental evidence for this hypothesis, but it is at least compatible with the known function of Bpl1p and Vhr1p. Pyc2p for which an involvement in biotin sensing is without doubt is in fact not included in this hypothesis. This interaction of Vhr1p with the biotinylated histones might give hint to another important tessera in the sensing-puzzle.

5 Summary

Biotin transport in *E. coli* was already observed in 1972. Results in this work demonstrate *yigM* is the gene encoding the *E. coli* biotin transporter. The protein has 10 predicited transmembrane domains and shows homology to members of the subfamiliy of carboxylate/amino acid/amine transporters. Deletion of *yigM* led to complete loss, overexpression with different expression systems to a dose dependent increase of biotin uptake. The function of YigM was shown in whole cells and in reconstituted membrane vesicles. Weak inhibition of biotin uptake by protonophores, the respiratory chain blocker NaN_3, as well as uptake in not energized membrane vesicles suggest biotin uptake by a facilitated diffusion mechanism. *E. coli* cells overexpressing YigM displayed saturable biotin uptake kinetics (K_M of 74 $\pm$ 14 nM). This value is similar to previously reported measurements in wt cells, indicating that YigM is the only biotin transporter in *E. coli*. The set of plasmamembrane biotin transporters is extended by *yigM*, as transporters for mammals, eucaryotic unicellular organisms, gram-positive bacteria and archea have already been described before. Biotin dependent regulation of the *yigM*-gene was shown by experiments with a luciferase-reporter construct.

In *S. cerevisiae* evidence for a role of biotin dependent pyruvate carboxylases, especially pyruvate carboxylase 2 in biotin sensing was provided. Single-knockouts of of the two *PYC* genes reduce the response to low biotin concentrations, as shown by GFP-reporter-assays and western-blot. A double-knockout mutant for both pyruvate carboxylase genes even suppresses low biotin signals completely. The role of Pyc2p in biotin sensing was demonstrated to be independent of changes in expression, biotinylation and enzymatic activity. However experiments with fusing an epitope tag and truncation of the protein at the C-terminus revealed this end of the protein seems to influence sensing.

Additionally *in vivo* biotinylation of *S. cerevisiae* histones H2B, H3 and H4 was demonstrated. A possible role of biotinylated histones in biotin sensing is discussed.

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6 Appendix

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Abbreviations

°C	degree celcius
μg	microgram
μl	microlitre
μM	micromolar
Amp^R	ampicillin resistance gene
ATP	adenosinetriphosphate
BCCP	biotin-carboxyl-carrier-protein
bp	basepair
BPL	biotin-protein-ligase
BRE	biotin response element
CCCP	Carbonyl Cyanide m-Chlorophenyl-hydrazone
C-Terminus	carboxyterminus
DAPA	diaminopelargonic acid
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotide-5-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylendiaminetetraacetic acid
e.g.	for example (lat. exempli gratia)
EMS	ethylmethansulfonate
EtOH	ethanol
FCCP	carbonyl cyanide p-(tri-fluoromethoxy)phenylhydrazone
fig	figure
Gal	galactose
GFP	green fluorescent protein
Glc	glucose
h	hour
HA	hemagglutinin
HCS	holocarboxylasesynthetase
His	histidin
IPTG	isopropyl-β-D-thiogalactopyranoside
KAPA	7-keto-8-aminopelargonic acid
kb	kilobase

kDa	kilodalton
Km	Michaelis-Menten-constant
Km^R	kanamycin resistance gene
l	litre
LB	Luria Bertani (medium)
lacZ/LacZ	β-galactosidase
min	minute
ml	milliliter
mM	millimolar
MMA	synthetic minimal medium
mRNA	messenger ribonucleic acid
NAD(P)+/NAD(P)H	nicotinamidadenindinucleotide (phosphate), oxidized/reduced
Ni-NTA	nickel-nitrilotriacetic acid matrix for affinity chromatography of proteins with a 6His-tag
N-Terminus	aminoterminus
OD_X	optical density at wavelenght λ = x nm
ONPG	ortho-nitrophenyl-β-galactopyranoside
ORF	open reading frame
PAGE	polyacrylamide-gelelectrophoresis
PCC	propionyl-CoA-carboxylase
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
PYC, Pyc	pyruvatcarboxylase
RNaseA	ribonuclease A
rpm	revolutions per minute
RT	room temperature
SAM	S-sdenosylmethionine
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium Ddodecylsulfate
sec	second
SMVT	sodium-dependent multivitamin transporter
Strep-PO	streptavidin-peroxidase conjugate
Sz. pombe	*Schizosaccharomyces pombe*
tab	table
TEMED	N,N,Nt',Nt'-tetramethylethylendiamin

Tris	tris-(hydroxymethyl)-aminomethane
UAS	upstream activating sequence
U	unit (enzyme activity)
TMD	transmembrane domain
UV	ultra violet
v/v	volume/volume
Vit	vitamin
VHRE	vitamin H response element
wt	wildtype
w/v	weight/volume
w/o	without
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YNB	yeast nitrogen base
ZZ	binding domain of *Staphylococcus aureus* protein A, binding to Fc-domains of IgG in two copies used as epitope

amino acids					
A	ala	alanin	M	met	methionin
C	cys	cystein	N	asn	asparagin
D	asp	aspartate	P	pro	prolin
E	glu	glutamate	Q	gln	glutamin
F	phe	phenylalanin	R	arg	arginin
G	gly	glycin	S	ser	serin
H	his	histidin	T	thr	threonin
I	ile	isoleucin	V	val	valin
K	lys	lysin	W	trp	tryptophane
L	leu	leucin	Y	tyr	tyrosin

nucleobases		
A	ade	adenin
C		cytosin
G		guanin
T		thymin
U	ura	uracil

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Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, den 23. Februar 2010

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