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BioID: Evaluation of a new method for protein-protein interactions in *Trichoderma reesei*

ausgeführt am

Institut für Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften

der Technischen Universität Wien

unter Anleitung von

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Zusammenfassung

Der filamentöse Pilz *Trichoderma reesei* ist einer der wichtigsten Produzenten von (hemi)zellulolytischen Enzymen und spielt daher eine wichtige Rolle in unterschiedlichen industriellen Anwendungen, wie zum Beispiel in der Papier-, Lebensmittel-, Tierfutter- und Textilindustrie oder für die Saccharifikation von Lignozellulose-Biomasse zu einfachen Zuckern für die Herstellung von biobasierenden Produkten wie Biokraftstoffen. Obwohl *T. reesei* große Mengen an Zellulasen sekretieren kann, sind weitere Forschungen notwendig, um die Effizienz zu steigern und die Kosten der Zellulaseproduktion zu senken. Ein Weg die Kosten der Zellulaseproduktion zu minimieren sind Stammverbesserungsmethoden.

Ziel dieser Diplomarbeit war es, eine neue Methode, namens BioID, welche Protein-Protein Interaktionen detektiert, *in vivo* in *T. reesei* zu untersuchen. Nähe-basierende Biotin Identifikation (BioID) detektiert Proteininteraktionen in lebenden Zellen und verwendet dazu eine mutierte Biotinligase, BirA*, welche benachbarte Proteine biotinyliert. Um Interaktionspartner der beiden Zellulaseregulatoren XYR1 und LAE1 zu detektieren, wurde das BirA* Ligase kodierende Gen an das *xyr1* und *lae1* Gen fusioniert und mittels homologer Rekombination an ihren entsprechenden Genlokus integriert.

Phenotypische Analysen der *birA** getaggten *xyr1* und *lae1* Stämme zeigten, dass weder Wachstum und Sporulation, noch die Enzymproduktion beeinflusst wurden, was wiederum zeigt, dass beide Regulatoren in ihrer physiologischen Funktion nicht beeinträchtigt sind. Obwohl unterschiedliche Kohlenhydratquellen, Biotinkonzentrationen und Probenzeitpunkte getestet wurden, um neu-biotinylierte Proteine in den Proteinextrakten von *T. reesei* zu detektieren, konnte keine Biotinylierung nach einer Biotinzugabe beobachtet werden. Die getaggten XYR1 und LAE1 Proteine waren auch durch Western blotting nicht detektierbar, was auf eine niedrige Expression oder auf den Abbau des BirA*-Tags hindeuten könnte. Genexpressionsstudien zeigten, dass *lae1* auf Laktose nicht hochreguliert ist, im Vergleich zur reprimierenden Kohlenhydratquelle Glukose, hingegen zeigte *xyr1* eine starke Hochregulierung unter denselben Bedingungen.

Diese Ergebnisse zeigen, dass BioID nicht ohne weiteres zur Detektion von Protein-Protein Interaktionen in *T. reesei* verwendet werden kann und weitere Experimente nötig sind um die Aktivität, Expression und Stabilität von BirA* zu testen.

Abstract

The filamentous fungus *Trichoderma reesei* is one of the most important producers of (hemi)cellulolytic enzymes and plays an important role in different industrial applications including the paper, pulp, food, animal feed and textile industry or for saccharification of lignocellulosic biomass into simple sugars for the production of bio-based products including biofuels. Although *T. reesei* is able to secrete high level of cellulases further efforts are necessary to maximize efficiency and yield and minimize costs of enzyme production. One way towards lower costs of cellulase production is via strain improvement.

Aim of this thesis was to evaluate the novel method BioID to study protein-protein interactions *in vivo* in *T. reesei*. Proximity-based biotin identification screens for protein interactions in living cells and uses a mutated biotin ligase BirA*, which biotinylates proximal proteins. This enables their isolation by biotin-affinity capture. To screen for interaction partners of the two cellulase-regulators XYR1 and LAE1 the BirA* ligase encoding gene was fused to *xyr1* and *lae1* and the different gene fusion constructs were targeted to their respective gene locus by homologous recombination.

Phenotypical analyses of the *birA** tagged *xyr1* and *lae1* strains showed that tagging did not influence growth, sporulation or enzyme production indicating that the two regulators are not impaired in their physiological function. Different cellulase inducing carbon sources, biotin concentrations and sampling time points were tested to detect newly biotinylated proteins in the protein extracts of *T. reesei.* However, no biotinylation was detected following biotin addition. The tagged XYR1 and LAE1 proteins were also not detectable by western blotting, indicating low expression or a degradation of the BirA* tag. Gene expression studies showed that *lae1* was not upregulated on the cellulase expressing carbon source lactose in comparison to the repressible carbon source glucose while *xyr1* was highly upregulated.

These results show that BioID cannot be readily used to detect protein-protein interactions in *T. reesei* and further experiments are required to test the activity, expression and stability of BirA*.

List of abbreviations:

A, mA	ampere, milliampere
AB	antibody
AP-MS	affinity purification coupled to mass spectrometry
bp	base pairs
CBM	carbohydrate-binding module
Ct	cycle-threshold
DNA	deoxyribonucleic acid
g, mg, µg, ng	gram, milligram, microgram, nanogram
GFP	green fluorescent protein
GH	glycoside hydrolases
HRP	horseradish peroxidase
Hz	hertz
kb	kilobase-pairs
kD	kilo Dalton
L, ml, µl	liter, milliliter, microliter
LPMO	lytic polysaccharide monooxygenase
M, mM, µM	molar, millimolar, micromolar
MAP	mitogen-activated protein
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PPI	protein- protein interaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rpm	rotations per minute
RT-PCR	reverse transcription polymerase chain reaction
T. reesei	*Trichoderma reesei*
Y2H	yeast two-hybrid system
V	volt

Table of contents:

1. Introduction

1.1 *Trichoderma reesei*

T. reesei is a filamentous ascomycete, which was firstly isolated from the Solomon Islands during World War II due to its ability to degrade different cotton based fabrics of the US army. In a US army research program at Natick the cause for this degradation was investigated and the organism was identified as *T. viride* (Reese E. T. 1976). Later it was found out to be a new species and named after Elwyn T. Reese, the Natick laboratory researcher which was the first investigator of this fungus (Simmons E.G. 1977). Still today, all industrial *T. reesei* strains used for enzyme production are derived from this single isolate from the Solomon Islands with the strain name QM6a. *T. reesei* was described as the anamorph (asexual stage) of the fungus *Hypocrea jecorina.* It was found, based on different phylogenetic markers that the asexual fungus *T. reesei* was not distinguishable from the sexual reproducing fungus *Hypocrea jecorina*, which indicates that they belong to the same species (Kuhls et al. 1996). Further research showed that *T. reesei* is in fact female sterile (Seidl et al. 2009). This is the consequence of a non-functional *ham5* gene encoding a MAP kinase scaffold and consequently introduction of a wild-type *ham5* in QM6a can restore sexual reproduction (Linke et al. 2015). Nowadays different mutant strains from the original QM6a strain are widely used in the industrial production of cellulases and other enzymes (Seidl et al. 2009). The cellulolytic and hemicellulolytic enzymes produced by *T. reesei* have received considerable industrial importance over the past years for example in pulp-, paper-, food- or textile- industry. Today new applications for these enzymes are found in the biofuel and biorefinery industry (Kubicek 2013). Hence the emphasis of research in the past decades was put on strain improvement towards cellulase overproduction (Eveleigh et al. 1979). Improved *T. reesei* strains with increased cellulase production were mainly achieved by random mutagenesis, which led to strains capable of producing 20 times as much cellulase as the QM6a isolate (Seiboth et al. 2011) and it is reported that at least 120 g/L can be produced (Cherry et Fidantsef 2003). The original strain QM6a has a genome size of 33 Mb and the whole genome was sequenced and published in 2008 (Martinez et al. 2008). From QM6a a number of hypercellulolytic strains were derived including RUT-C30. At present RUT-C30 is still the most used strain in laboratory in terms of cellulase hyperproduction and serves as a model industrial strain (Le Crom et al. 2009). As it can be seen in Fig. 1, through several steps of mutagenesis introduced by UV-light, nitrosoguanidine (NTG) or linear acceleration (LA) several mutant strains are derived from the origin QM6a for the purpose of improved production of the requested proteins (Seidl et al. 2008). Beside that *T. reesei* is nowadays used for commercial large scale production of enzymes, it is also used as model organism for the regulation of the cellulase genes and for the biochemistry of

cellulose and hemicellulose degradation (Martinez et al. 2008, Kubicek 2013, Schuster et Schmoll 2010, Horn et al. 2012).

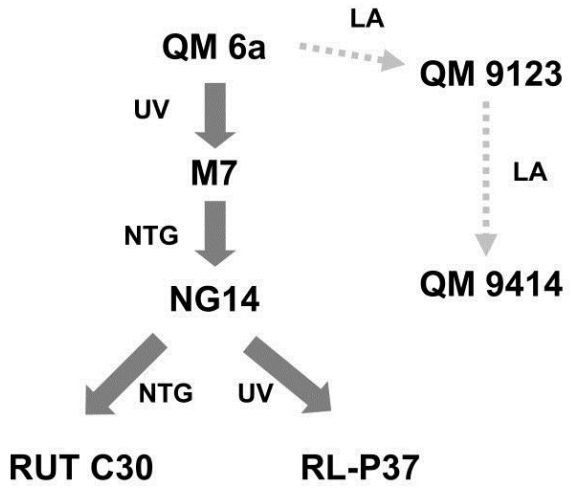

Figure 1: Lineage of mutants from original *T. reesei* isolate QM6a. Mutations were introduced by UV-light (UV), nitrosoguanidine (NTG) or linear acceleration (LA) and subjected to different screening procedures for cellulose production (Seidl et al. 2008).

1.2 Industrial importance of the cellulase-producer *T. reesei*

Lignocellulosic biomass is mainly composed of lignin, cellulose and hemicellulose that form the structural framework of the plant cell wall. This complex polymer is the most abundant renewable biomass, produced by plants through photosynthesis (Himmel et al. 2007). Cellulose is a homopolymer consisting of β-1,4-linked D-glucose units. Cellulose microfibrils (bundles) are composed of crystalline and amorphous regions. Crystalline regions are tightly packed and hard to separate in comparison to amorphous regions which are slightly packed and easier to separate. Cellulose fibrils are embedded into a matrix of lignin and hemicellulose. These hemicelluloses are polysaccharides composed of several monosaccharides, e.g. D-xylose, L-arabinose, D-mannose, D-galactose or D-glucose. Hemicelluloses have usually shorter chains, which are branched and are not crystalline and therefore better water-soluble and biodegradable by enzymes. Lignin is an amorphous highly branched polymer complex of phenylpropan units (Gibson 2012). Approximately up to 40 % of plant dry biomass consists of lignin and it is responsible for the stability of plant tissue (Pandey et al. 2011).

The cellulolytic system of *T. reesei* can be divided into three main types: endoglucanases, exoglucanases and β-glucosidases. These three types work together to degrade cellulase to glucose (Glass et al. 2013). Endoglucanases cleave internal bonds of the cellulose chain, while exonucleases start from the chain ends and cleave cellobiose (therefore called cellobiohydrolases CBHs) and at last

β-glucosidases release D-glucose from soluble oligomeric breakdown products including cellobiose (Turner et al. 2007, Seiboth et al. 2011).

Enzymes which are originally designated as glycoside hydrolase 61 (GH 61) and carbohydrate-binding module 33 (CBM 33) are now re-classified as lytic polysaccharide monooxygenases (LPMO), which belong to the auxiliary activity family 10 in the CAZy database. Copper depending LPMOs are important enzymes for the degradation of cell wall components such as cellulose, hemicellulose and chitin. The enzymatic degradation is enhanced by these enzymes and therefore LPMOs play an important role as industrial enzymes for the conversion of lignocellulolytic biomass into simple sugars. The mechanism is based on an oxidative cleavage of glycosidic bonds in polysaccharides (Busk et al. 2015, Forsberg et al. 2014). Cellobiose dehydrogenase donates an electron via its cytochrome domain to the copper ion of PMO, where dioxygen is partially reduced and attacks the pyranose ring of the glucose at the C-1 or C-4 position, which leads to the destabilization of the glycosidic bonds (Kittl et al. 2012).

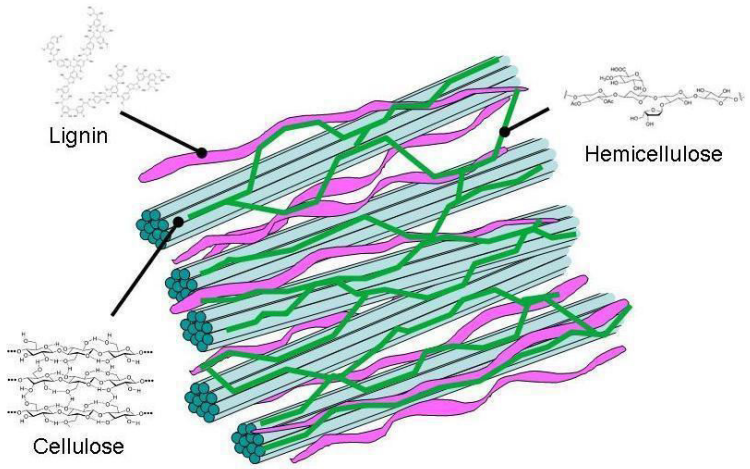

Figure 2: Scheme of lignocellulose composed of cellulose, hemicellulose and lignin. Cellulose and hemicellulose are carbohydrate polymers containing different sugar monomers (pentoses and hexoses) and they are tightly bound to lignin. Cellulose, hemicellulose and lignin form structures called microfibrils, which mediate structural stability in the plant cell wall (Meine et al. 2013).

One of the most interesting applications of lignocellulosic biomass today is its saccharification into simple sugars for the production of bioenergy in the form of biofuels or other biorefinery products (Kubicek et al. 2009). An alternative to the previously used petroleum-based fuels, which lead to different environmental problems, is the usage of bio-ethanol. Ethanol is a high-octane fuel and can be used as additive to gasoline. The current problems, such as the global warming effect caused by the continued anthropogenic enrichment of the atmosphere with greenhouse gases like carbon

dioxide (CO_2), nitrous oxide (NO_2) and methane as well as fossil fuel limitation and increasing prices in the next years, led to the search for alternative renewable energy sources. The release of CO_2 is particularly caused by burning fossil fuels through transport, heating, electricity or industry. Therefore the aim of reducing CO_2 emissions can be achieved by bioethanol-based biofuels (Seidl et al. 2010). Ethanol production is currently based on grain crops (especially corn and wheat) or sugar crops (sugarcane, sugar beets or molasses). Approximately 60 % of world ethanol production use sugar crops, the remaining 40 % are using grain crops as the primary feedstock. The bioethanol production process underlies following steps: biomass is converted to sugars by hydrolysis and then fermented for the production of ethanol (Shapouri et al. 2006). Depending on the carbon source used as feedstock it can be distinguished between first and second generation biofuels. First generation biofuels are already well established, where ethanol is produced by fermentation of either sugar or starch based material. For example sugar based ethanol is produced primarily from sugarcanes in Brazil, and starch based ethanol from corn and grains in the US. There are a few problems involved in the process of first generation biofuels. The usage of raw material is the main problem, because most of the used materials are food staples and the use of biofuels has increased food prices. Therefore first generation biofuels can be seen as a temporary solution. In comparison to first generation biofuels second generation utilizes different types of lignocellulosic material, agricultural residues or waste. The goal of second generation biofuels is the usage of non-food parts (leafs, stalks, straw) which are by-products of the agriculture and therefore do not compete with staple food (Lennartsson et al. 2014). There are also a few limitations in the second generation biofuels process. The major problem is the recalcitrance of the lignocellulose material, because they are less susceptible to chemical and enzymatic degradation and therefore different pretreatment methods are used to separate the different constituent parts and to increase thereby the accessible surface for the enzymes.

Although *T. reesei* industrially employed strains are able to secrete more than 100 g/l cellulases, it is still necessary to reduce the costs and maximize the efficiency of enzyme production. At the present the bioethanol production process is not cost effective enough. Several approaches, including improvements in increased and optimized enzyme production, the plant biomass pretreatment and fermentation process and the optimization of the enzyme mixture, were followed to make industrial scale production economically feasible. However, although the results are promising, improvements are still necessary (Seidl et al. 2010).

1.3 Regulation of cellulases and xylanases in *T. reesei*

T. reesei is the main industrial source of cellulases and hemicellulases and its genome encodes 10 cellulases and 16 hemicellulases (Martinez et al. 2008). *T. reesei* cellulases are usually not formed during growth on monosaccharides such as glucose and require the presence of an inducer for their full expression. A commonly used inducing carbon source for cellulase gene expression can be cellulose itself. But cellulose is insoluble and often other soluble inducing carbon sources are used, for example lactose, which also initiates cellulase expression. The advantage of lactose in comparison to cellulose is the solubility and in comparison to the other inducing disaccharide sophorose its low price. Glucose, the degradation product of cellulose, serves as repressor of the cellulase gene expression (Kubicek et al. 2009).

Cellulase encoding genes can be regulated by several transcription factors including positive ones such as XYR1, ACE2, HAP2/3/5 and negative ones including ACE1 or CRE1 (Kubicek et al. 2009). Another transcription factor was found to be detrimental for cellulase production and the expression of several cellulase genes, namely ACE3. Deletion of the gene decreases the production of cellulases and xylanases, in contrast, overexpression of *ace3 r*esults in a significantly increase of the production. Thus, *ace3* was identified as a novel master regulator of lignocellulose degradation (Häkkinen et al. 2014).

XYR1 (xylanase regulator 1), a zinc binuclear cluster protein, which binds to a GGCTAA motif, is the main transcriptional activator of cellulases and xylanases gene expression in *T. reesei.* Their induction by all known inducing carbon sources (cellulose, lactose, sophorose, xylan and xylose) is switched-off in the absence of XYR1 (Stricker et al. 2008). The *Aspergillus niger* XlnR is the orthologue of XYR1 and is the main regulator protein of more than 10 genes involved in the degradation of xylan and cellulose and regulates also the D-xylose metabolism (Mach-Aigner et al. 2008). The functional domains include N-terminal zinc binuclear cluster domain, a central coiled-coil domain and a C-terminal activation domain (Stricker et al. 2008). The transcripton of *xyr1* under non-inducing conditions occurs at a low basal level, but it is clearly induced by lactose and cellulose. Beside XYR1 other transcription factors are known which modulate cellulase and xylanase transcription.

XYR1 and ACE2 are both able to bind to the same promoter motif [$GGC(T/A)_4$] (Furukawa et al. 2009). While the HAP2/3/5 complex binds to CCAAT motif and is believed to be necessary for the generation of an open chromatin structure and therefore necessary for the complete transcriptional activation (Zeilinger et al. 2003). Deletion of *ace1* leads to an increase of the cellulases and hemicellulases expression, which demonstrates the role of ACE1 as repressor. ACE1 also represses *xyr1* expression during growth on D-xylose (Mach-Aigner et al. 2008). The carbon catabolite repressor CRE1 is a

member of the C_2H_2 type of DNA-binding proteins and acts as the major suppressor of cellulases and hemicellulases gene expression by recognizing a 'SYGGRG-3' consensus sequence (Felenbok et al. 2001). CRE1 can inhibit the basal as well as the inducible cellulase expression and also prevents positive expression of *xyr1.* The CRE1 protein has sequence similarity with other proteins mediating glucose repression, like *Aspergillus nidulans* CreA and *Saccharomyces cerevisiae* MIG1 (Nakari-Setälä et al. 2009, Mach-Aigner et al. 2008, Lichius et al. 2014). Transcription of *xyr1* was increased in *ace3* overexpressing strains and decreased in deletion strains. Therefore, the absence of XYR1 is not an explanation for the total lack of cellulase activity and gene expression exhibited by the deletion strain (Häkkinen et al. 2014).

The transcriptional activator XYR1 regulates all xylanases by a xylose-arabinose and xylan-depending induction (Herold et al. 2013). The cellobiohydrolases CBHI and CBHII are abundantly secreted and play an important role for the degradation of cellulases. For the expression of both major cellulase genes *cbh1* and *cbh2*, sophorose seems to be the most potent inducer. The induction by sophorose results in elevated *xyr1* transcription levels. It was found out that a single point mutation in XYR1 (A824V) is responsible for the high basal level of *cbh1* and *cbh2* expression and the deregulation of *xyn1* and *xyn2* gene expression in an industrial strain background (Derntl et al. 2013). In *A. niger* a V756F mutation for XlnR showed a similar effect resulted in a constant xylanase activity under repressing conditions (Hasper et al. 2004). Lichius et al. (2014) showed that only a N-terminal GFP tagging of XYR1 results in a total functionality of the transcription factor. GFP-XYR1 showed higher gene expression levels and nuclear recruitment compared to the C-terminal tagged XYR1 strain. XYR1 showed a significant increase of nuclear import under cellulase-inducing conditions, for example with carbon sources such as sophorose, lactose and cellulose. Whereas nuclear export occurred quicker the stronger the non-inducing condition was. Furthermore it was shown that cytoplasmic fluorescence intensity remained constant, while the nuclear signal increased constantly during induction, which indicates that newly produced XYR1 became imported into the nuclei upon its biosynthesis. Nuclear loss of XYR1 triggered by export to non-inducing conditions occurred very slowly and during this period the cytoplasmic pool of XYR1 did not increase, which indicates the degradation of exported XYR1. In summary, cellulase gene expression depends on *de novo* biosynthesis and immediate nuclear import of XYR1 (Lichius et al. 2014).

Another regulator of cellulases and xylanases in *T. reesei* is LAE1 (Seiboth et al. 2012). This putative protein methyltransferase is the orthologue of LaeA which was originally identified as a regulator of secondary metabolite biosynthesis, such as aflatoxin in Aspergillus ssp. (Bok et al. 2004, Sarikaya-Bayram et al. 2015). A *lae1* deletion strain leads to a complete loss of expression of all cellulases as well as the loss of the expression of β-glucosidases and xylanases. Furthermore also auxiliary factors

for cellulose degradation are no longer expressed in a strain with a *lae1* deletion. Deletion of *lae1* also affects asexual sporulation. In contrast, overexpression of *lae1* led to increased expression of cellulases and xylanases, which shows that LAE1 is an attractive target for (hemi)cellulase overproduction and strain improvement (Seiboth et al. 2012). It was shown that the cellulase gene expression modulated by *lae1* is directly dependent on XYR1, and vice versa. The precise mechanism of *lae1*- modulated cellulase gene expression still need to be identified. It is supposed that LAE1 controls the function of genes, which are involved in increasing the fitness of the fungus in its environment. LAE1 further regulates the expression of polyketide or non-ribosomal peptide synthases, ankyrins, iron uptake, heterokaryon incompatibility, PTH11-receptors and different oxidases/monoxygenases (Aghcheh et al. 2014). Additionally, an effect on the expression of GCN5-N-acetyltransferases, amino acid permeases and flavin monooxygenases were identified recently (Fekete et al. 2014). *T. reesei* Δ*lae1* strains and *lae1*-overexpressing strain were cultivated in chemostats on glucose at two different growth rates (0.075 and 0.020 h^{-1}) which resemble repressing and derepressing conditions. Deletion of *lae1* gene affects the regulation of gene expression by growth rate, whereas overexpression of *lae1* has only a little effect (Fekete et al. 2014). *A. nidulans* LaeA is known as a member of the VELVET complex, consisting of LaeA, VeA and VelB, and regulates secondary metabolites as well as sexual and asexual reproduction, such as conidia and fruiting body formation. The VeA orthologue VEL1 in *T. reesei* regulates developmental processes independently of light and is essential for cellulase gene expression. Deletion of *vel1* leads to a complete loss of conidiation, alters hyphal morphology which consequently reduces growth rates and impairs the expression of cellulases and xylanases. In contrast to *A. nidulans*, the transcript of *vel1* is strongly downregulated in a Δ*lae1* strain and not affected in a *lae1*-overexpression strain. It was also shown that the transcription level of XYR1 is strongly reduced, which fits to the results of Δ*lae1*, in which also a reduced *xyr1* transcription level was observed. In conclusion, the cellulase expression in *T. reesei* is also dependent on a functional *vel1* gene and LAE1 regulation occurs by the VELVET complex (Aghcheh et al. 2014).

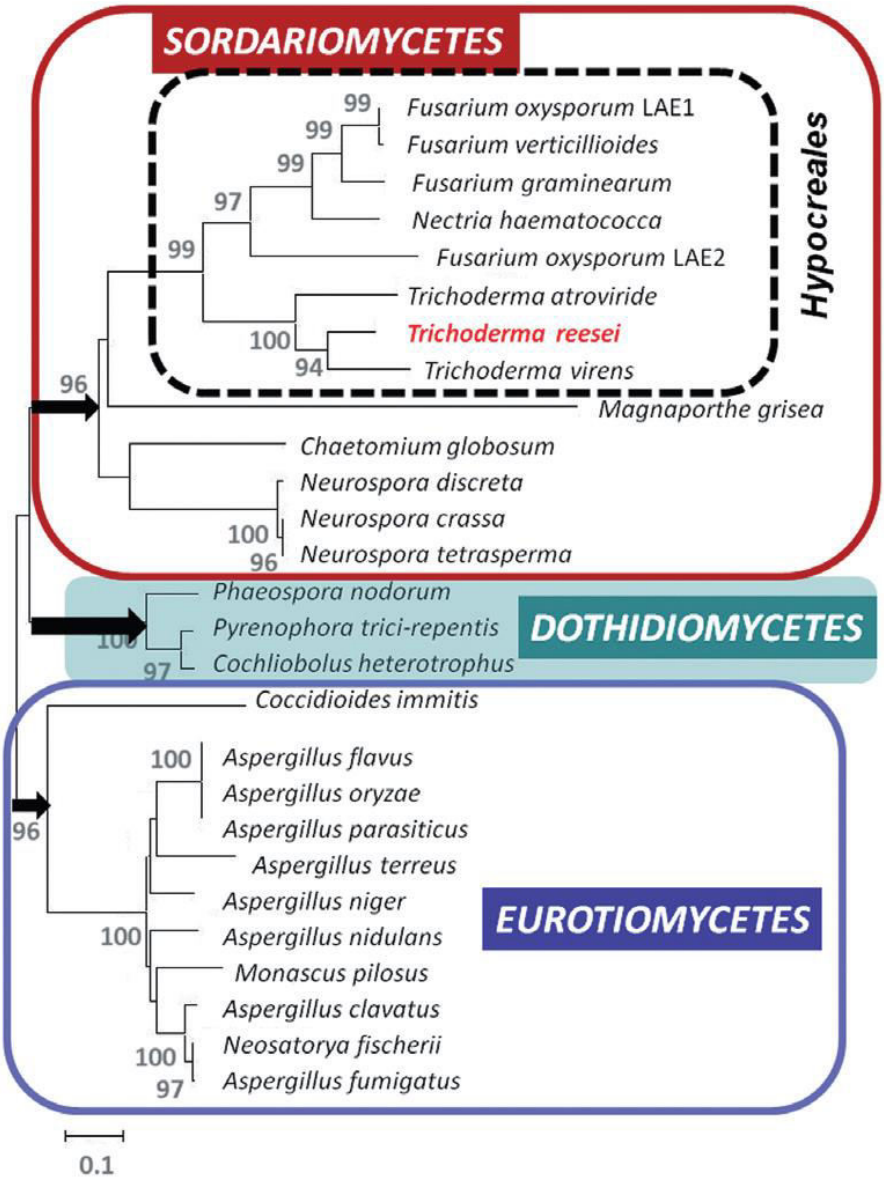

Figure 3: Phylogenetic analysis of LaeA/LAE1 proteins in Eurotiomycetes, Dothidiomycetes and Sordariomycetes (Seiboth et al. 2012).

1.4 Protein-protein interactions (PPIs)

A protein-protein interaction (PPI) delineates an interaction between two or more proteins as a result of biochemical events or electrostatic forces. It relies predominantly on non-covalent interactions, such as Van der Waals forces or hydrogen bonds (Westermarck et al. 2013). PPIs play a key role in virtually all biological processes, for example, DNA replication, transcription, translation, splicing, signal transduction, secretion are only a short list of major research topics where PPIs are involved. PPIs form a network of over 650 000 interactions in the human organism and therefore PPIs are the center of research in many areas of life science (Adams et al. 2005).

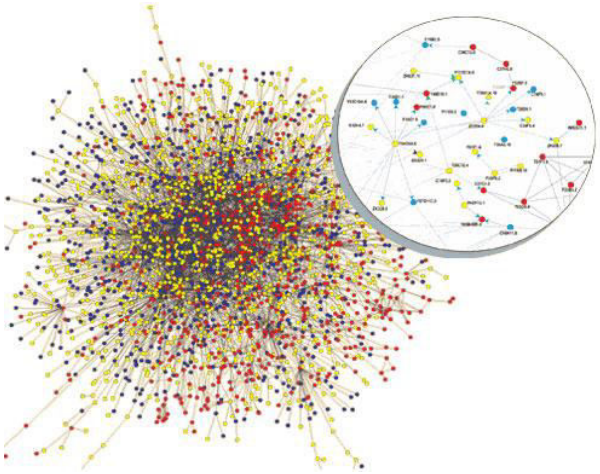

Figure 4: Overview of a protein-protein interaction network map, which links 2,898 proteins by 5,460 interactions to represent a whole cell with its elaborate network of interlocking interactions (Perkel et al. 2004).

Many in vitro and in vivo applications were applied to explore the mechanism of these ubiquitous interactions. Nowadays there are a multitude of methods how to investigate PPIs, but many limitations and disadvantages exist depending on the methods used (Caj et al. 2012).

Maybe the two most conventional and widely used techniques to investigate PPIs are the yeast two-hybrid system and the affinity purification coupled to mass-spectrometry.

Yeast two-hybrid system (Y2H) is based on two functional domains of a transcription factor (usually the *Saccharomyces cerevisiae* Gal4):

- DNA binding domain (bait)
- Activating domain (prey)

The DNA binding domain binds to the particular DNA sequence upstream of the reporter gene, while the activating domain activates reporter gene expression. Both domains are functionally and structurally independent and can be fused to two separate proteins. The protein that is fused to the DNA binding domain is called bait, while the one fused to the activating domain is referred to the prey. In the absence of bait-prey interaction, the activating domain is unable to localize to the reporter gene to drive gene expression. However, by bait-prey interaction the DNA binding domain binds to the DNA localizing the activating domain upstream of the reporter gene, leading to the expression of the reporter gene (Brückner et al. 2009).

In affinity purification coupled to mass-spectrometry (AP-MS) a single protein of interest is affinity captured in a matrix as bait. A protein mixture is passed through the matrix and interacting partners (prey) are retained by interaction with the bait. Proteins that do not interact pass through the matrix and will be discarded. There are multiple variations in the affinity purification step, for example the

protein of interest fused to an epitope-tag is either immune-precipitated by a specific AB or purified by affinity columns recognizing the tag. Affinity purification can make use of an individual tag for single step purification. Subsequently proteins can be processed for direct analysis and identified by MS (Brückner et al. 2009).

Through the present limitations and disadvantages of these two methods, such as false-positive or false-negative results, difficulties in the detection of transient or weak interactions and *S. cerevisiae* as a main host system, researchers look for novel methods to investigate PPIs. One of them is the BioID (Westermarck et al. 2013).

1.5 BioID

Proximity-dependent biotin identification, called BioID, is a recently developed *in vivo* method for identifying protein-protein interactions in a natural cellular environment. It is based on a fusion of a promiscuous *Escherichia coli* biotin protein ligase to the protein of interest. Thus, it is possible to identify proteins and their proximate and/or interacting protein binding partners (Roux et al. 2012). BioID uses a modified form of the 35 kDa bacterial biotin ligase BirA, with a R118G mutation which is called BirA*. BirA catalyzes a two-step reaction: First biotin and ATP is used to form a highly reactive biotinoyl-5´-AMP (bioAMP), afterwards an attachment of bioAMP to a specific lysine results into the biotinylation. BirA* shows an extremely low affinity for the reactive bioAMP intermediate and consequently results in promiscuous protein biotinylation (Morriswood et al. 2012). The isolation of the BioID fusion protein and its biotinylated proximate binding partners is based on a streptavidin-biotin bond. Streptavidin has an extraordinarily high affinity for biotin with a dissociation constant of K_d = 2.5* 10^{13} M^{-1}. It is one of the strongest known non-covalent biological bonds in nature and therefore extensively used in biotechnology and molecular biology applications (Chilkoti et al. 1995).

The addition of biotin to the media is necessary for a strong biotinylation, because of the low affinity of the mutant ligase BirA* to biotin. Due to the supplemented biotin a temporally regulation of the biotinylation process is possible. Through prior studies it was shown that biotinylation is detectable between 1 h and 24 h after the addition of biotin. After biotinylation of proximate proteins, these proteins can be detected on a western blot by using a horseradish peroxidase (HRP)-conjugate streptavidin AB and later identified by mass spectrometry (Morriswood et al. 2012).

Until now BioID was successfully used in the screening of mammalian nuclear lamina, trypanosome bilobe (protist), cell junction complexes and centrosomes (Roux et al. 2012, Morriswood et al 2012, Firat-Karalar et al. 2014, Schweingruber et al. 2016, Mehus et al. 2016). BioID is a screening method

with its own advantages and limitations. One significant advantage is that it enables a selective isolation and identification of proteins including their biotinylated neighbors. Another advantage is that potential interactions are detected in their normal cellular context and natural environment. Additionally, in comparison to other screening methods, BioID can identify weak or transient interactions. BioID can also be applied to insoluble proteins and as mentioned before it is amenable to temporal regulation. The only requirement for BioID is the expression of a single fusion protein in the host cell (Roux et al. 2013). As with any other method, there are also limitations that need to be mentioned. The fusion of the biotin ligase BirA*can affect the function or stability of the protein. The method can also lead to false-negative results, if there is a lack of primary amines for biotinylation (Roux et al. 2013).

In summary BioID is a useful alternative for protein-protein interaction screening methods. It has many advantages in comparison to other present methods. BioID is not without drawbacks and limitations, but it is a relatively new method and needs further research to improve and expand it to other organisms.

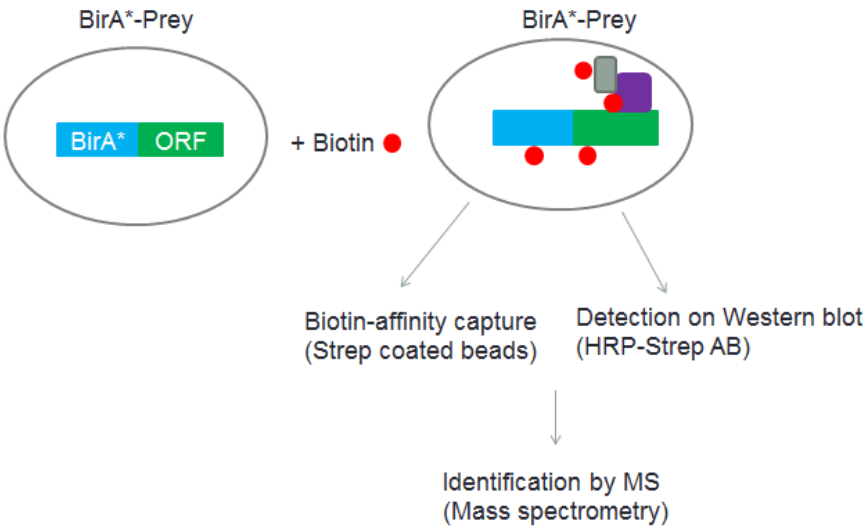

Figure 5: Experimental setup for BioID. It shows the biotinylation of proximity binding partners of the biotin ligase fusion protein (BirA*-prey) with the addition of biotin. Afterwards these protein-protein interactions can be detected through western blot by using a HRP-strep AB or through biotin-affinity capture using streptavidin coated beats. The captured proteins can then be identified by MS.

1.6 Aim of the thesis

The aim of this thesis was to test if the BioID system can be used in fungi such as *T. reesei* to detect PPIs and thereby identifying new interaction partners of the cellulase regulator proteins XYR1 and LAE1.

Different tasks were carried out to achieve this:

- Generation of BirA* tagged LAE1 or XYR1 expressing strains and testing if the fusion proteins perform their physiological function
- Gene expression studies of *xyr1* and *lae1* on lactose
- Testing different cellulase-inducing carbon sources and biotin-concentrations for western blotting
- Western blotting of intracellular proteins to detect biotinylated proteins with the HRP-strep AB
- Testing the BirA* presence, containing the c-Myc epitope with an anti-Myc-HRP AB

2. Material and methods

2.1 Strains and cultivation conditions

T. reesei transformants expressing XYR1- or LAE1- BirA* fusion proteins were created in QM9414Δ*tku70* (Ivanova et al. unpublished data). QM9414 (ATCC 26921) is an early cellulase overproducing mutant isolated from the wild-type strain QM6a, in which the *tku70* gene was deleted resulting in QM9414Δ*tku70* with an improved homologous recombination efficiency (Guangtao et al. 2009).

All transformants were generated before starting this thesis (Ramoni et al. unpublished data). The two cellulase regulators LAE1 and XYR1 were tagged with a mutated biotin ligase (BirA*). LAE1 was tagged N- or C-terminally with BirA* giving rise to the strains BirA*-Lae1 or Lae1-BirA* and XYR1 was tagged only N-terminally with BirA* giving rise to BirA*-Xyr1. BirA*-Xyr1 was only tagged N-terminally because Lichius et al. (2014) showed that a C-terminal tagging of XYR1 by GFP resulted in a reduced *xyr1* expression level and phenotypical differences in comparison to the N-terminally tagged GFP (Lichius et al. 2014). All gene fusion constructs were integrated at the *xyr1* and *lae1* locus respectively and the integration was confirmed by PCR and sequencing of the coding region of the fusion gene. All strains were kept on PDA plates and grown on 28°C. To compare the phenotype of the different transformants with the reference strain QM9414Δ*tku70* and the Δ*xyr1* and Δ*lae1* deletion strains, BirA*-Xyr1 strains were grown on MA-xylan or MA-D-xylose media at 28°C and imaged after 48 and 96 hours. BirA*-Lae1 and Lae1-BirA* strains were grown on PDA at 28°C and imaged after 48, 96 and 192 hours.

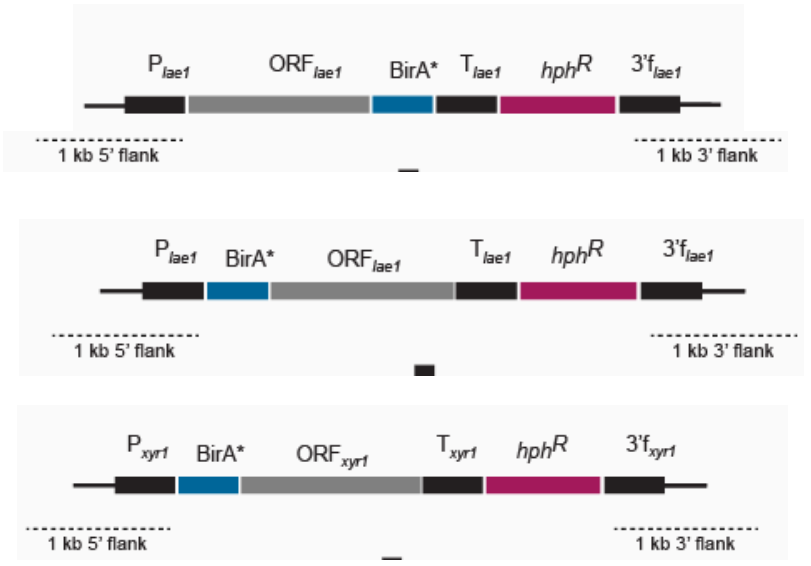

Figure 6: Schematic representation of the *lae1* and *xyr1* replacement cassettes to express Lae1-BirA*, BirA*-Lae1 and BirA*-Xyr1. They consist of the promotor, open reading frame, terminator, hygromycin resistance cassette and the N- or C- terminally tagged BirA* fusion genes and were transformed into *T. reesei* QM9414Δ*tku70.*

For shake flask cultivation conidia were harvested from PDA plates with 10 ml NaCl-Tween 80 solution by using a sterile Drigalski spatula. The spores were filtered through glass-wool tubes and the spore concentration was determined at 600 nm with a photometer. Afterwards $2*10^6$ spores per ml were inoculated in 250 ml MA-medium and 1 % carbon source and 0.4 g/l $CaCl_2$ were additionally added into 1 L Erlenmeyer flasks. The flasks were incubated in a shaker for 24 h at 28°C and 250 rpm. Following, the given amount of biotin for 10 µM, 50 µM or 100 µM was added. After the addition of biotin, samples were drawn at defined time points. For gene expression experiments the mycelium was filtered through a Whatman filter by using a vacuum pump, put into aluminum foil, immediately frozen in liquid nitrogen and stored at -80°C.

$$\frac{\text{conidia}}{\text{mL}} = \frac{\text{conidia}}{\text{square}} * 10^3 * \frac{\text{dilution}}{0.004}$$

Formula 1: Amount of spores per ml medium using the Thoma cell counting chamber.

Additionally, two replacement experiments a sophorose replacement for the recombinant *xyr1* strains and a D-xylose replacement for the recombinant *lae1* strains, were performed.

For the sophorose replacement experiments, the harvested spores were inoculated in 170 ml MA-medium with 1 % glucose and 0.4 g/l $CaCl_2$ and incubated for 18 h at 28 °C and 220 rpm (Lichius et al. 2014). The mycelia were then washed with sterile tap water and put into flasks, containing 105 ml MA-replacement-medium followed by a 30 min starvation period (consumption of remnant glucose from pre-culture). Afterwards 1.75 mM sophorose and 50 µM biotin were added. Samples were taken within the first 3 h at defined time points.

For the D-xylose replacement spores were inoculated in 250 ml MA-medium supplemented with 1 % glucose and 0.4 g/l $CaCl_2$ and incubated for 24 h at 28°C and 250 rpm. Thereafter the mycelium was filtered, washed with sterile tap water and put into flasks containing 200 ml MA-replacement-medium. The mycelia were incubated for 30 min for starvation before 1 mM D-xylose and 50 µM biotin were added. Samples were taken within the first 5 h at defined time points.

2.2 Molecular biology techniques

2.2.1 DNA extraction

The mycelium was removed from agar plates with a sterile needle and mixed in 500 µl lysis buffer with a vortex. Additionally, 150 µl P_3 (K-acetate) buffer, cooled to 4°C, was added and incubated for 5 min on ice. Afterwards the suspension was vortexed again and centrifuged for 5 min at 15 000 g at room temperature. 300 µl of the supernatant was transferred into new microtubes containing 300 µl ice cold isopropanol. After vortexing, the DNA was precipitated for 20 min at -20°C.

Subsequently the solution was centrifuged for 10 min at 13 000 g and 4°C. The supernatant was discarded and the DNA pellet was washed with 300 µl ice cold 70 % ethanol. The suspension was centrifuged again at the same conditions and the supernatant was discarded again. At last the pellet was dried at room temperature and the DNA was resuspended in 50 µl sterile water.

2.2.2 PCR

The correct length of fragments inserted in the genome of the recombinant *xyr1* and *lae1* strains, containing the biotin ligase BirA*, was checked through PCR using Phusion™ Polymerase.

PCR consists of typically 3 cycle steps:

- Denaturation: This step causes DNA melting and separates the DNA double helix into two single strands. It is performed at 98°C for about 20-30 seconds.
- Annealing: Allows specific primers to bind the target DNA. The temperature depends on the melting temperature of the primers and is performed at 55- 60°C for 30 seconds.
- Elongation: The DNA polymerase synthesizes the complement DNA strand from 5' to 3' end and starts with the 3' end of the annealed primer. Temperature and time depends on the DNA polymerase optimum. In this work PHIRE® and PHUSION® polymerase were used, which require an elongation temperature of 72°C

Usually the thermocycler cools down the reaction to 4°C to allow storage overnight.

Table 1: PCR thermal cycling program of the *BirA fusion genes**

STEP	TEMP	TIME
Initial denaturation	98°C	30 sec
30 cycles	98°C	10 sec
	touch (63x3/60x3/57x24)	20 sec
	72°C	30 sec/kb
Final extension	72°C	490 sec
Hold	15°C	-

To check whether the amplified fragment with the correct size was generated, agarose gel electrophoresis was employed.

2.2.3 Agarose gel electrophoresis

First 0.7- 2 % (w/v) agarose was suspended in 1x TAE buffer, heated in the microwave and cooled down to 50°C. Then 1.5- 3 µl SYBR®Safe, a common DNA gel stain, was added. The solution was poured into a gel tray and a comb was used to create pockets in the gel. After solidification, the comb was removed and the gel was put into an electrophoresis chamber. The chamber was filled with 1x TAE buffer. For loading, the samples were mixed with DNA loading dye and the samples were pipetted into the respective chamber. For DNA size comparison, additionally 5 µl of a molecular weight standard (GeneRuler 1 kb DNA Ladder) was pipetted into one chamber. The gels were run at 95 V, 400 mA, until bands were visible.

2.2.4 Sequencing

To test if the integrated fragment in the transformants has the correct sequence, they were sent to Microsynth AG for sequencing.

After purification of the DNA, performed according to the protocol "QIAquick PCR purification kit (using a microcentrifuge)", the concentrations of all samples were determined using Nanodrop ND-1000. A volume of 15 µl containing 1200 ng DNA and 30 pmol primer was sent to Microsynth AG.

An optimal primer design with preferred values enables specific amplification with high yield. A primer length of 18-22 bp, a melting temperature of 52- 58°C and a percentage of GC bases in the primer of 40- 60 % were used for an optimal result. These values were checked on the primer3plus homepage and listed below.

Table 2: Primers for sequencing the whole coding region of BirA*Lae1 25A

Number	Name	5'-3' sequence	Nucleotides	% GC	Tm (°C)
P130	Fw_Seq_Bira*	gccatgagaagagtggagga	20	55	60
P131	Seq_Plae1_BirA*	cggcttgggattaacaaact	20	45	59
P132	Seq_Olae_Bira*	gagccaagaaggagatgtgc	20	55	60
P154	P154_MRSuperP1	tcttggaagcctgggaaata	20	45	60
P155	P154_MRSuperP2	ttcgttcggttttctggc	18	50	60

Table 3: Primers for sequencing the whole coding region of Lae1-BirA* 5A

Number	Name	5'-3' sequence	Nucleotides	% GC	Tm (°C)
P130F	Fw_Seq_Bira*	gccatgagaagagtggagga	20	55	60
P131	Seq_Plae1_BirA*	cggcttgggattaacaaact	20	45	59
P132	Seq_Olae_Bira*	gagccaagaaggagatgtgc	20	55	60
P142	Seq2_pLAE1_birA*	tacagcctgcctgagcctat	20	55	60
P155	P154_MRSuperP2	ttcgttcggttttctggc	18	50	60

Table 4: Primers for sequencing the whole coding region of BirA*-Xyr1 26

Number	Name	5'-3' sequence	Nucleotides	% GC	Tm (°C)
P110	P3F_BirA*start	atggaacaaaaactcatctcag	22	36.4	56
P130	Fw_Seq_Bira*	gccatgagaagagtggagga	20	55	60
P143	Seq1_bira_xyr1	taacaacccccagtctctgc	20	55	60
P144	Seq2_bira_xyr1	ccatgtgcccattgtatagg	20	50	59
P145	Seq3_bira_xyr1	tgcgatatcctgtgcttgag	20	50	60
P1R	P1R_xyr1	cttatccgcaaggaggatgtgg	22	55	65
P3R	P3R_xyr1	agtcgctcatgatcctaccag	21	53	59

2.2.5 Protein extraction and determination of protein concentration

All steps were performed on ice or in liquid nitrogen as indicated:

In 2 ml microtubes 1 g glass beads (0.5 mm acid-washed) and 900 µl protein extraction buffer was prepared. At least 100 mg frozen mycelia were grounded in liquid nitrogen, added to the microtubes and vortexed. In the precooled mixer mill the mycelia suspension was homogenized for 1 min at 30 Hz and immediately cooled down for 1 min in an ice bath, containing NaCl for a better cooling effect. This step was repeated three times. Afterwards the cell debris was spun down in the precooled centrifuge for 5 min at 16 000 g and 4°C. The supernatant was transferred into a new microtube and again centrifuged under the same conditions.

Subsequently the protein concentration was determined using the Bradford method.

First the protein extraction sample was diluted 1:1 with distilled water. Then 795 µl distilled water was mixed with 200 µl of the Bradford reagent and 5 µl of the protein extraction sample. The solution was vortexed and incubated for 10 min at room temperature. The absorbance was measured at 595 nm. Measurement was performed in duplicates and the mean value was used to calculate the protein concentration in µg/ml through a BSA standard curve.

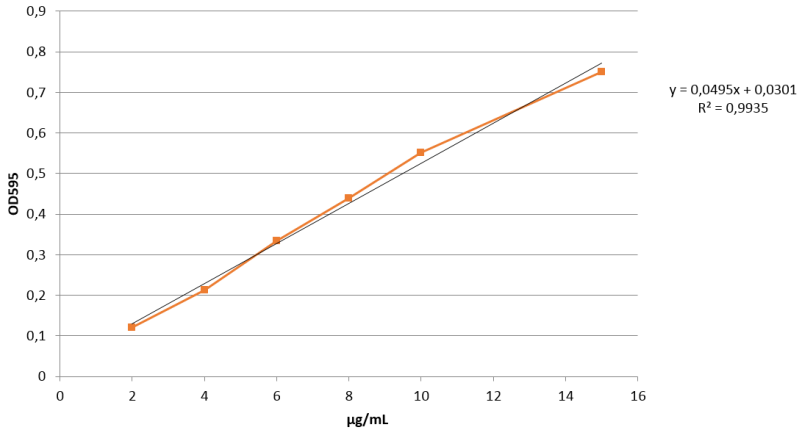

Figure 7: Bradford BSA standard curve

2.2.6 SDS PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis describes a commonly used technique to separate proteins. The procedure contains the preparing of the acrylamide gels, the sample preparation and the electrophoresis step.

- Preparation of acrylamide gels:

For the separating gels an acrylamide concentration of 14 % was used.

Table 5: Components of SDS PAGE separating gels

Separating gel	for 2 gels	for 4 gels
dH_2O (ml)	3.4	6.8
Separating buffer (ml)	3	6
Acrylamide (ml)	5.6	11.2
10 % APS (µl)	60	120

TEMED (µl)	6	12

Table 6: Components of SDS PAGE stacking gels

Stacking gel	**for 2 gels**	**for 4 gels**
dH_2O (ml)	3	6
Stacking buffer (ml)	1.25	2.5
Acrylamide (ml)	0.75	1.5
10 % APS (µl)	25	50
TEMED (µl)	5	10

First the separating gel was prepared and mixed, avoiding air bubbles. The suspension was pipetted into the gel rack and overlaid with dH_20 to ensure a straight level of the gel. After 20 min the gel was polymerized, the water was poured off and the remaining water was removed with filter paper. Meanwhile the stacking gel was prepared and pipetted on top of the separating gel. Additionally, a comb was placed in the still liquid stacking gel, to create pockets in the gel. After 30 min the stacking gel also polymerized and the comb was removed.

- Sample preparation:

The sample concentration was determined through Bradford. Usually 20 µg protein per lane was loaded. The calculated amount of sample was mixed with loading dye (1x FSB) and heated for 7 min at 97°C.

- Electrophoresis:

After loading the samples, the rack was put into the electrophoresis chamber and filled up to 2/3 with 1x running buffer. The running conditions were 120 V, 400 mA.

Thereafter the gel was stained with colloidal Coomassie staining solution overnight and washed with dH_20.

2.2.7 Western blot

Western blot is a widely used analytic technique to identify specific proteins. A mixture of proteins is separated through gel electrophoresis and then transferred to a membrane and incubated with

specific antibodies to detect a protein of interest. Western blotting includes two steps: blotting of proteins from SDS gel to membrane followed by a detection and visualization step.

The proteins were either blotted onto a nitrocellulose membrane (for chemiluminescent detection with X-ray photofilms) or PVDF membrane (for fluorescent detection with Typhoon FLA9000). For blotting, a correct assembling in the blotting chamber is necessary. Proteins move from the cathode (SDS gel) to the anode (membrane) due to their negative charge by SDS. Two Whatman papers, soaked in electroblotting buffer, formed the bottom level. Thereupon the membrane was placed followed by the SDS gel and again two Whatman papers, soaked in electroblotting buffer. The gel was blotted overnight with 15 mA in the blotting chamber.

During the detection step it is essential that the membrane does not dry out. The membrane was blocked for 1 h in 25 ml PBS-T plus 1 % BSA by shaking. Thereafter the membrane was wrapped into a Falcon tube and incubated with one of the following solutions (3 ml):

- To detect biotinylated proteins the streptavidin-horseradish peroxidase antibody (streptavidin HRP- strep AB, Life Technologies, Cat. No 43-4323) was used. Streptavidin HRP-strep AB is based on a specific bond between streptavidin and biotin with extremely high affinity (K_d= 10^{-15}M). Streptavidin is a tetrameric protein, containing four identical subunits binding biotin molecules. A dilution of 1:1000 was used in western blotting for an optimum result.
- To detect the c-Myc epitope on the recombinant proteins the c-Myc epitope tag antibody, HRP conjugate (anti-Myc-HRP AB, Life Technologies, Cat. No R951-25) was used. It is a monoclonal mouse AB that allows detection of recombinant proteins containing the c-Myc epitope, with the following sequence: Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu. Anti-Myc-. HRP AB is a crosslink of the anti-Myc AB with horseradish peroxidase. A dilution of 1:5000 was used for the optimum result in western blotting.

These solutions were incubated for about one hour while rotating at room temperature. Next the membrane was washed three times with PBS-T for 5 min while shaking. For visualization of the western blot, two different methods were used.

For chemiluminescence detection, the membrane was basted with the Super-signal™ West Pico Chemiluminescent Substrate (Thermo Scientific), which provides intensive signal and picogram sensitivity for western blotting with horseradish peroxidase enzyme conjugates. In a dark room the membrane was placed on an X-ray photofilm and incubated for a few seconds. Then the photofilm

was put into the development solution (Sigma Aldrich) for 1 min, into the fixing solution (Sigma Aldrich) for further 1 min and afterwards the reaction was immediately stopped with dH_20.

For visualization through Typhoon FLA 9000 the membrane was covered with Pierce ™ECL 2 Western blotting Substrate (Thermo Scientific) and incubated for 5 min. The membrane was put into the Typhoon FLA 9000 upside down and scanned at 200-500 V with a pixel size of 100 µm.

2.2.8 RNA extraction and cDNA synthesis

In 2 ml microtubes a mixture of 700 µl Chirgwin reagents and 6 µl 2-mercaptoethanol was prepared. Frozen mycelia were grounded with a pestle and mortar in liquid nitrogen. Then the biomass powder was added to the previous mixture and vortexed for 10-15 sec until a homogenous suspension has formed. Stepwise, 70 µl 2 M NaAc (pH 4.0), 700 µl phenol (pH 4.0) and 400 µl chloroform: isoamylalcohol was added and well vortexed after every addition. Afterwards the mixture was incubated for at least 15 min on ice. The tubes were centrifuged for 20 min at 13 000 g and 4°C.

From here RNAse free materials were required (DEPC treated tips, tubes and water).

The aqueous phase (upper phase, maximum 700 µl) was transferred to a 1.5 ml microtubes containing 700 µl isopropanol (4°C) and mixed gently. RNA was precipitated by incubating for 1 h at -20°C. The next step was to pellet the RNA by centrifugation for 20 min at 13 000 g and 4°C. Afterwards the supernatant was removed and the pellet was washed two times with each 1 ml 70 % ethanol. The pellet was dried at room temperature for 10 min and dependent on size and solubility, it was dissolved in 50-200 µl DEPC treated water (for better solubility the tube can be incubated for further 10 min in the thermoblock at 55°C). The RNA concentration was determined through Nanodrop ND-1000. If RNA was not immediately processed, it was stored at -80°C.

The cDNA synthesis was performed by using the RevertAid^{TM} H Minus 1st Strand Synthesis Kit according to the manufactures protocol, by Fermentas.

The first step was the DNA digest: 5 µg RNA, 1 µl DNAse reaction buffer (10x) and 1 µl DNAse are filled up to a volume of 10 µl with DEPC water and incubated for 30 min at 37°C in the thermomixer. Thereafter the reaction was stopped by adding 1 µl 25 mM EDTA. To inactivate DNAse it was further incubated for 10 min at 65°C.

Secondly, for the cDNA synthesis (1st strand synthesis) 11 µl DNAse digested RNA mix, 0.5 µl random hexamer primer and 0.5 µl oligo-(dT) primer were incubated for 5 min at 65°C. Afterwards 4 µl RT reaction buffer (5x), 1 µl RiboLock^{TM} RNAse inhibitor (20 u/µl), 2 µl 10 mM dNTPs and 1 µl reverse transcriptase were added and incubated for 60 min at 42°C, then 5 min at 70°C. At last the cDNA was cooled down on ice and stored at -20°C.

2.2.9 qPCR analysis

In order to amplify and quantify the amount of specific gene transcripts, quantitative real time PCR (qPCR) was used. The analysis was carried out in a BioRad® IQ™ thermal cycler. The PCR reaction mix consists of 12.5 µl SYBR® Green Supermix, 8.5 µl ultrapure water, 2 µl primer (forward+ reverse) and 2 µl diluted cDNA (1:100), which leads to a total reaction volume of 25 µl for all samples. The reaction mix was pipetted into a 96-well plate and covered with optical foil. The housekeeping reference gene *tef1* (translation elongation factor 1) was used for reference calculation and data normalization. All reactions were performed in technical triplicates. Used primers are listed in Table 7. Data was analyzed through REST® (Relative Expression Software Tool V2.013) (Pfaffl et al. 2002).

Table 7: Primers used for qPCR

Gene	Name	5'-3' sequence	Efficiency (%)
tef1	qPCR-**tef1-F**	CCACATTGCCTGCAAGTTCGC	94
	qPCR-**tef1-R**	GTCGGTGAAAGCCTCAACGCA	94
lae1	qPCR-**lae1-F**	ACTGGAGATTGACTGGATGC	100
	qPCR-**lae1-R**	TTCTGCGTCTGGTAGCCTC	100
xyr1	qPCR-**xyr1-F**	CCATCAACCTTCTAGACGAC	100
	qPCR-**xyr1-R**	AACCCTGCAGGAGATAGAC	100
cbh1	qPCR-**cbh1-F**	CCGAGCTTGGTAGTTACTCTG	98
	qPCR-**cbh1-R**	GGTAGCCTTCTTGAACTGAGT	98

2.2.10 RT-PCR

Reverse transcription polymerase chain reaction is a combination of a common PCR and the use of reverse transcriptase to detect and quantify gene expression. The principle is based on RNA which is converted into cDNA by using a reverse transcriptase. cDNA is then used as template for the amplification by PCR. The stronger the intensity of the amplicon on the agarose gel, the higher are the mRNA abundancy and therefore gene expression for the gene of interest. RT-PCR is often used for relative quantification.

Tef1 (translation elongation factor 1) was used as housekeeping gene to quantify the total mRNA in the respective samples. Oligonucleotides were designed, which bind in the BirA* fusion protein

encoding gene as well as in the open reading frame of *xyr1* and *lae1* (see figure 8). Following PCR, samples were loaded on an agarose gel to analyze if they are expressed.

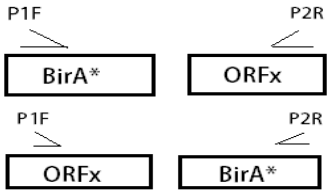

Figure 8: Schematic presentation of the binding sites of the different oligonucleotides in the *BirA fusion genes.**

Table 8: Reaction mix for the setup of RT-PCR in a total volume of 12.5 µl

Components	reaction volume (µl)
Water	6.95
GoTaq® 5x Green buffer (Promega)	2.5
$MgCl_2$	1.25
dNTP (10 mM)	0.25
Forward and reverse primer (diluted 1:10)	0.5
GoTaq Flexi DNA Polymerase (Promega)	0.05
cDNA (diluted 1:10)	1

Table 9: PCR thermal cycling program for amplification of *tef1*

Step 1	95°C	2min	
Step 2	95°C	1min	Step 2-4: 30x
Step 3	59°C	1min	
Step 4	72°C	1min	
Step 5	72°C	10min	
Step 6	15°C	-	

Table 10: PCR thermal cycling program for RT-PCR of the *BirA fusion genes**

Step 1	95°C	2min	
Step 2	95°C	1min	Step 2-4: 3x
Step 3	63°C	1min	
Step 4	72°C	2.50min	
Step 5	95°C	1min	Step 5-7: 3x
Step 6	60°C	1min	
Step 7	72°C	2.50min	
Step 8	95°C	1min	Step 8-10: 34x
Step 9	57°C	1min	
Step 10	72°C	2.50min	
Step 11	72°C	7min	
Step 12	15°C	-	

Table 11: Primers used in RT-PCR

Primer name (forward+ reversed)	**Analyzed (fusion) gene**	**Sequence 5'-3'**	**Amplicon length** (bp)
Tr_tef1_Fw Tr_tef1_Rev	*tef1*	GTACTGGTGAGTTCGAGGCTG GGGCTCGATGGAGTCGATG	350
P137 P158	*lae1-birA**	TTGGAAGCCTGGGAAATAC TGCTCAGGTAAGGAGCCAGT	2082
P157 P142	*birA*-lae1*	TTCTGCGTCTGGTAGCCTC TACAGCCTGCCTGAGCCTAT	1821
P130 P1R	*birA*-xyr1*	GCCATGAGAAGAGTGGAGGA CTTATCCGCAAGGAGGATGTGG	2817

2.3 Instruments & materials

Autoclave	Certoclav^{R} EL18L
Vortex	Scientific Industries Vortex Genie^{R} 2
Incubator	Sanyo, MIR-153
Nanodrop	Nanodrop Technologies, ND-1000
Geldocumentation	Biorad
Photometer	Thermoscientific
Thermoblock	Eppendorf
Centrifuges	Eppendorf centrifuge 1-15PK, Sigma 1-16K, Sigma 330K
PCR cycler	Biometra Thermocycler T3000
qPCR	Eppendorf Realplex Mastercycler
pH meter	Radiometer Copenhagen, PHM82 standard pH meter
Gel-electrophoresis+ Power supply	Biorad
SDS-PAGE equipment	Biorad
Waterbath	Fisher scientific Isotemp 220
Biorad IQ thermal cycler	Biorad
Exhaust hood	Fischer & Co
Ice machine	Scotsman^{R} AF80
Typhoon FLA 9000	GE Healthcare Life Science
-80°C freezer	New Brunswick Science
Magnetic stirrer	Heidolph Instruments, Hei-standard
Cuvettes	Plastikbrand^{R} Einmal-Küvetten
Petri dishes	Greiner Bio One Petri dish
Filtration material	Calbiochem. Miracloth
Filters	Whatman^{TM}
PCR tubes	Starlab StarPCR^{R} Tube
96well plate	Biorad PCR plates 96well
X-ray Film	Thermo Scientific
PVDF membrane	BioRad
Nitrocellulose membrane	BioRad
Filter paper	Whatman
Filter tips (10µl, 100µl, 1ml)	Greiner Bio One

Pipette tips (5ml, 1ml, 200µl, 20µl)	Greiner Bio One
Pipetman Neo (P10N, P20N, P200N, P1000N)	Gilson
Centrifuge tubes (25ml, 50ml)	Greiner Bio One
Developer solution (Carestream® Kodak® autoradiography GBX developer/replenisher)	Sigma Aldrich
Fixing solution (Carestream® Kodak® autoradiography GBX fixer/replenisher)	Sigma Aldrich
Mixer mill	Retsch

2.4 Chemicals and suppliers

Acrylamid	Merck
Agar Agar	Merck
Agarose	Starlab
Ammoniumpersulfat (APS)	Sigma Aldrich
Beechwood xylan	Roth GmbH
Bradford reagents	Biorad protein assay
Bovine serum albumin (BSA)	Pierce
Calciumchloride ($CaCl_2$)	Sigma Aldrich
Cellulose	Merck
Chloroform	VWR
D-xylose	Merck
D-biotin	Life Technologies
DNA Ladder (1kb)	Gene Ruler
DNA loading dye (6x)	Thermoscientific
Ethylenediaminetetraacetic acid (EDTA)	Roth GmbH
Ethanol	Merck
Glucose	Roth GmbH
GoTaq 5x green buffer	Promega
GoTaq Flexi DNA Polymerase	Promega
Phosphoric acid (H_3PO_4)	Merck
Isoamylalkohol	Roth GmbH
Isopropanol	Merck
Potassium chloride (KCl)	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Lactose	Merck

Methanol	Merck
2-mercaptoethanol	Merck
Magnesium chloride ($MgCl_2$)	Roth GmbH
Magnesium sulfate heptahydrate ($MgSO_4*7H_2O$)	Roth GmbH
Disodiumhydrogenphosphate ($Na_2HPO_4* 2H_2O$)	Merck
Sodium chloride (NaCl)	Roth GmbH
Ammoniumsulfate ($(NH4)_2SO4$)	Roth GmbH
Nuclease free water	Roth GmbH
Phosphate buffered saline (PBS)	Sigma Aldrich
Peptone	Merck
Phenol	Applichem
Potato dextrose agar (PDA)	Difco
Potassium acetate	Merck
50x TAE buffer	Roth GmbH
TEMED	Sigma Aldrich
Tris	Roth GmbH
Tween 80	Merck
Sodium dodecyl sulfate (SDS)	Roth GmbH
Sophorose	Merck
Sybr Green Supermix	Promega
SYBR® Safe	Invitrogen
Urea	Merck
Water for analysis	Merck
Xylan from beechwood	Sigma Aldrich
Xylose	Sigma-Aldrich

2.5 Media and solutions

For all solutions and media distilled water was used unless otherwise indicated. All solutions were autoclaved under following conditions: 125°C for 25 min.

2.5.1 Potato dextrose agar (PDA)

PDA (1 L):

potato dextrose agar	39 g/l

2.5.2 Mandels-Andreotti (MA)-medium

MA-medium (1 L):

$(NH_4)_2SO_4$	1.4 g/l
KH_2PO_4	2 g/l
$MgSO_4.7H_2O$	0.3 g/l
Urea (5 mM)	0.3 g/l
Pepton	1 g/l
50 x trace elements	20 ml
Tween 80	0.5 ml
dH_2O	930 ml

- Adjust pH to 5
- Add 0.4 g/l $CaCl_2$ and 10 g/l C-source of a sterile stock solution after autoclaving

2.5.3 MA-xylan/ MA-xylose

MA-xylan/MA-xylose (1 L):

$(NH_4)_2SO_4$	1.4 g/l
KH_2PO_4	2 g/l
$MgSO_4.7H_2O$	0.3 g/l
AgarAgar	15 g/l
50 x trace elements	20 ml
dH_2O	930 ml

- For xylan (unsoluble carbon source): add 10 g xylan before autoclaving
- For D-xylose (soluble carbon source): add sterile 50 ml of a 20 % D-xylose-stock solution after autoclaving
- Add 0.4 g/l $CaCl_2$ of a sterile stock solution after autoclaving

2.5.4 Replacement media

Replacement media (1 L):

$(NH_4)_2SO_4$	1.4 g/l
KH_2PO_4	2 g/l
$MgSO_4.7H_2O$	0.3 g/l
50 x trace elements	20 ml

dH_2O	930 ml

- Adjust pH to 5
- Add 0.4 g/l $CaCl_2$ and 10 g/l C-source of sterile stock solutions after autoclaving

Trace elements (1 L):

$FeSO_4$* $7H_2O$	250 mg
MnSO4*H_2O	80 mg
$ZnSO_4$*H_2O	70 mg
CaCl2*$2H_2O$	100 mg

2.5.5 10x phosphate buffered saline (PBS)/ PBST

10x PBS (1 L):

NaCl	80 g/l
KCl	2 g/l
Na_2HPO_4* $2H_2O$	18.1 g/l
KH_2PO_4	2.4 g/l
dH_2O	800 ml

- Adjust pH to 7.4 with HCl

PBST (1 L):

10x PBS	100 ml
Tween 80	3 ml
dH_2O	900 ml

2.5.6 Protein extraction buffer

Protein extraction buffer (1 L):

10x PBS	100 ml
500 mM 100x EDTA	10 ml

- Fill up to 1 L with analysis water
- Add 10 protein extraction tablets per liter (cOmplete ULTRA Tablets, Mini, EDTA-free from ROCHE)

2.5.7 SDS PAGE

Acrylamide stock solution (1 L):

Acrylamide	300 g/l
Bisacrylamide	8 g/l

- Fill up to 1 L with ddH_20 (double distilled water)
- Store dark at 4°C

5x running buffer (1 L):

Tris	15 g/l
Glycine	72 g/l
SDS	5 g/l

Separating gel buffer:

Tris	180 g/l

- Adjust pH to 8.8 with HCl

Stacking gel buffer:

Tris	60 g/l

- Adjust pH to 6.8 with HCl

Colloidal Coomassie staining solution (100 ml)

$(NH_4)_2SO_4$	8 g
ddH_2O	78.1 ml
85 % H_3PO_4	1.9 ml
5 % Coomassie Blue G250	1.6 ml

MeOH	20 ml

2.5.8 Western blot

Electroblotting buffer (500 ml):

Tris	1.2 g
Glycin	5.64 g
MeOH	10 ml

- Adjust pH to 8.9 with NaOH
- Store at 4°C

2.5.9 DNA/RNA extraction

Chirgwin reagents:

Guanidinium thiocyanate	250 g
H_20 sterile	293 ml
Na_3citrat 0.75 M, pH7	17.6 ml
Sarcosyl solution	26.4 ml

- Dissolve at 65°C

Potassium acetate buffer (P_3 buffer) pH 4.8 (100 ml):

K-acetate 5 M	60 ml
Glacial acetic acid	11.5 ml
dH_20	28.5 ml

Lysis buffer (1 L):

Tris-HCl	48.5 g/l
EDTA	17.5 g/l
NaCl	8.8 g/l

- Adjust pH to 8 with HCl
- Afterwards add 10 g SDS

2.5.10 Biotin stock (1L)

The maximum solubility of Biotin is 220 mg/l. For preparation of stock concentrations, 220 mg of biotin were dissolved in 1000 mL MA media, which was subsequently used to add to the cultures after precultivation on different carbon sources.

3 Results

3.1 Phenotype analysis of BirA* fusion protein strains

QM9414Δ*tku70* was transformed with different fusion constructs in which the *birA** ligase gene was either attached at the 5' end of the coding region (*xyr1*, *lae1*) or at the 3' end of the coding region (*lae1*). Following transformation and purification of the transformants they were tested for their growth behavior and sporulation pattern. Photos were taken after incubation of 2 d, 4 d and 8 d of growth on PDA plates for *lae1* strains and 2 d and 4 d on xylan or D-xylose plates for xyr1 strains. As further control the deletion strains QM9414Δ*xyr1* and QM9414Δ*lae1 w*ere also grown under the same conditions.

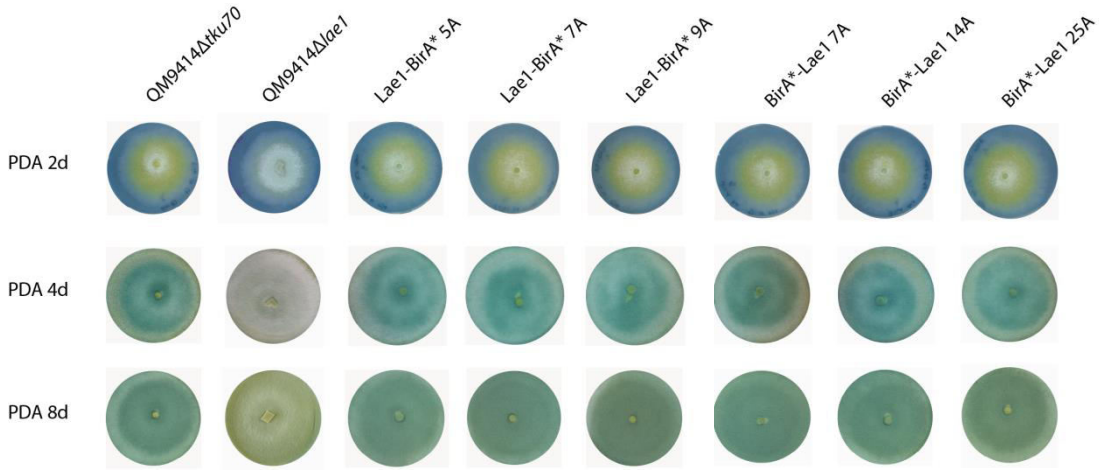

Figure 9: Phenotypic analysis of QM9414Δ*tku70* (reference strain), QM9414Δ*lae1* and the C-and N-terminally tagged LAE1 expressing transformants. The growth and sporulation phenotypes of C- and N-terminal tagged LAE1 strains are comparable to the reference strain, but not to the *lae1* deletion strain QM9414Δ*lae1.*

As shown in Fig. 9, strains in which the fusion gene had integrated at the respective locus (*lae1*) showed a similar growth and sporulation phenotype as the reference strain (QM9414Δ*tku70*). All transformants containing the fusion of *birA** to *lae1* displayed similar growth behavior and the characteristic yellow pigmentation and green conidiospores like the reference strain QM9414Δ*tku70*. Sporulation defects which were observed in QM9414Δ*lae1* (Seiboth et al. 2012) were not detected in any of the transformants, indicating that the fusion of *birA** to *lae1* does not affect its physiological function as LAE1 loss of function in QM9414Δ*lae1* results in the disappearance of the yellow pigmentation and reduced sporulation.

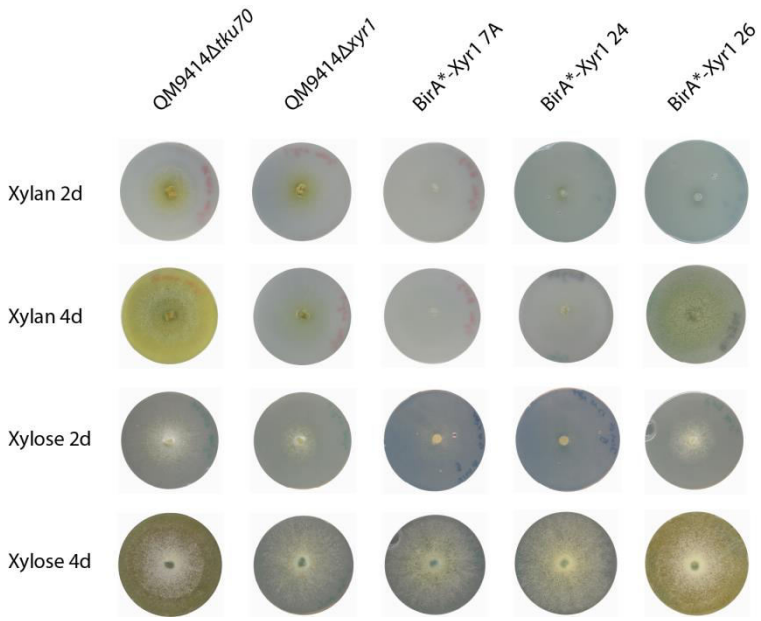

Figure 10: Phenotypic analysis of QM9414Δ*tku70* (reference strain), QM9414Δ*xyr1* and *birA tagged *xyr1* transformants. The phenotypes of the BirA*-Xyr1 26 strain is comparable to the reference strain, but differs from the *xyr1* deletion strain. All other transformants show growth defects similar to QM9414Δ*xyr1.***

The growth behavior of *birA*-xyr1* fusion transformants was analyzed by use of xylan and D-xylose plates. Although the *birA*-xyr1* fusion gene could be detected in all transformants at the *xyr1* locus, growth defects were recognized in all transformants except BirA*-Xyr1 26, the strain which was used for further experiments. No growth defects were observed on control D-glucose plates (data not shown). Hence the growth of BirA*-Xyr1 26 is not impaired on these carbon sources as found in a *xyr1* deleted strain (Mach-Aigner et al. 2008). Thus the physiological function of *xyr1* is exerted in strain BirA*-Xyr1 26. The deletion strain QM9414Δ*xyr1* as well as the strains BirA*-Xyr1 7A and BirA*-Xyr1 24 did not show the same sporulation pattern as the reference strain QM9414Δ*tku70* during growth on D-xylose and only a weak sporulation can be recognized in the negative strains due to their strong impaired ability to use D-xylose as carbon source.

3.2 Sequencing

Strains which did not show any growth defects were further tested by PCR and sequencing of the amplicon to reveal if the correct fusion gene sequence was present. The following strains did not reveal any mutation within the open reading frame of the fusion gene:

- Lae1-BirA* 5A
- BirA*-Lae1 25A
- BirA*-Xyr1 26

The whole coding region was sequenced and the result is shown for each strain in the appendix (7.1). Those three positive tested strains were used for further experiments.

3.3 BioID: optimization of the biotin concentration

Different biotin concentrations were tested to obtain the optimum concentration for the detection of BirA* mediated biotinylation. High concentrations of biotin results in the inhibition of cellulase expression (Bischof R. and Seiboth B., data not shown), and therefore only concentrations up to 50 µM biotin were tested. Therefore the strains BirA*-Lae1 25A, Lae1-BirA* 5A and QM9414Δ*tku70* as control were cultivated in a liquid culture under cellulase inducing conditions using lactose as carbon source. After 24 h of cultivation on lactose 10 µM, 50 µM or 100 µM biotin was added to the medium. Samples were taken before biotin addition and further 4 h and 12 h after biotin addition. Intracellular protein extracts of all samples were then separated by SDS PAGE and analyzed either by western blotting or with Coomassie Blue staining as a loading control. The Coomassie Blue stained SDS gels can be found in the appendix (7.2). HRP-strep AB was used to detect biotinylated proteins and the western blot was visualized using X-ray photofilm.

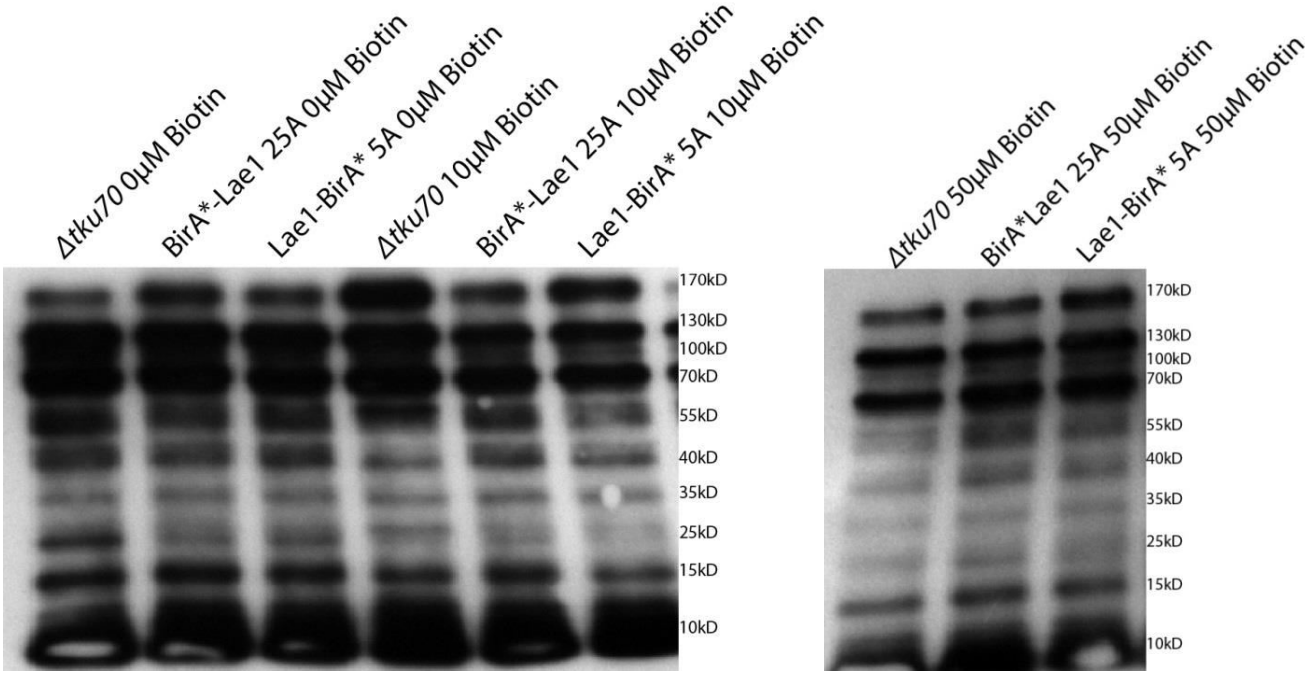

Figure 11: Western blot of cell free protein extracts (20 µg per lane) 4 h after biotin addition to lactose grown QM9414Δ*tku70* and strains expressing the N-terminal (BirA*-Lae1 25A) and C-terminal (Lae1-BirA* 5A) fusion proteins. Putatively biotinylated proteins were detected using the HRP-strep AB. The bands in QM9414Δ*tku70* represent background signals. No obvious additional bands or stronger bands which would indicate additional biotinylated proteins are present in the transformants compared to the control.

It is expected that the BirA* ligase not only biotinylates the target proteins but also the fusion protein, which should result in a biotinylated fusion protein in the Lae1-BirA* and BirA*-Lae1 strains

of 79.5 kD. Despite the dark blurred bands, no difference between the protein extracts of the control strain QM9414Δ*tku70* and the Lae1-BirA* and BirA*-Lae1 strains are visible. There is also no influence of the different biotin concentrations seen on the protein pattern or signal strength in the western blot. Samples without addition of biotin show the same pattern as samples treated with biotin, suggesting that a strong background signal is present. Thus no additional protein band can be seen and no obvious additional or stronger biotinylation is found after 4 h of incubation with different concentrations of biotin using lactose as carbon source.

Similar results were observed for the strains which were incubated for 12 h on lactose following the addition of biotin (see Fig. 12). In this picture the exposure time was shortened which results in weaker background signals, but again similar results as shown in Fig. 11 were obtained.

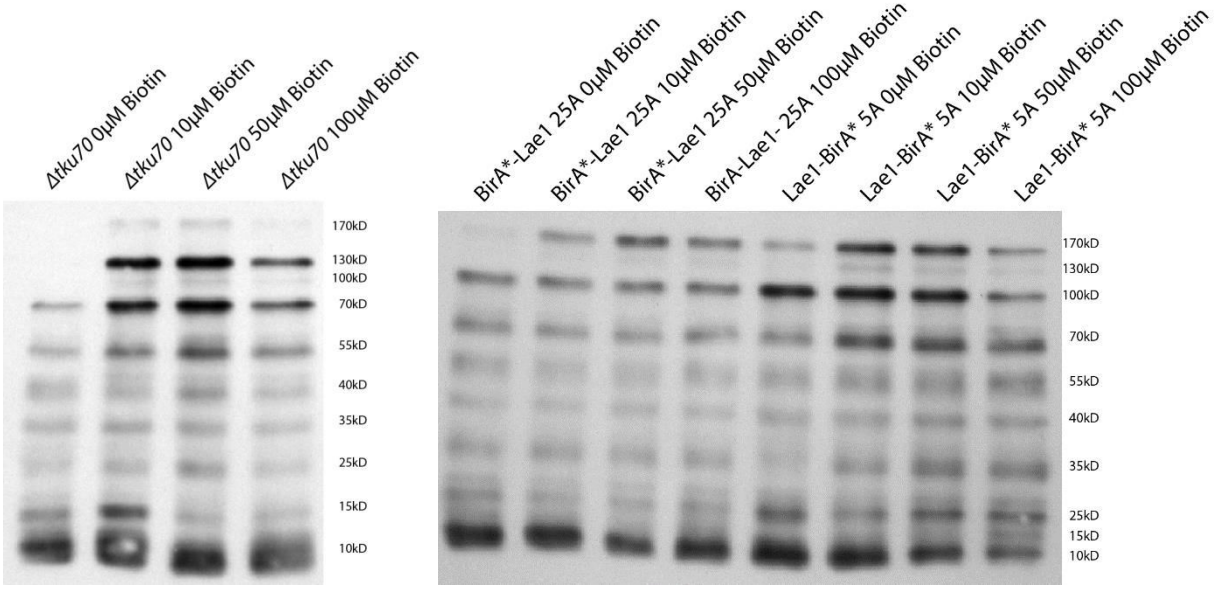

Figure 12: Western blot of cell free protein extracts 12 h after addition of different biotin concentrations to different lactose grown QM9414*Δtku70* and strains expressing the N-terminal (BirA*-Lae1 25A) and C-terminal (Lae1-BirA* 5A) fusion proteins. Putatively biotinylated proteins were detected using the HRP-strep AB. The bands in QM9414*Δtku70* represent background signals. No obvious bands which would indicate newly biotinylated proteins are present in the protein extracts of the different transformants treated with different biotin concentrations.

The western blot shows a distinctive and clear protein band pattern. However, again there are no differences between the transformants and the reference strain in respect of the biotin concentration and C- or N-terminal tagged versions of LAE1. The visible protein bands all correspond to background signals found in untreated strains or the control strain. However, the QM9414Δ*tku70* 0 µM biotin lane seems to show a different protein pattern, but in accurate comparison only the signal is weaker and no outcome can be concluded about this difference.

To summarize, 4 h or 12 h following addition of biotin to lactose grown cultures did not lead to obvious additional biotinylated proteins. Comparison of 10 µM, 50 µM or 100 µM biotin addition also did not show any reproducible difference in the protein pattern, so we further used a concentration of 50 µM biotin as it was used in a previous study, where 50 µM was chosen to achieve an optimum biotinylation pattern (Morriswood et al. 2012).

3.4 BioID: influence of different carbon sources upon fusion protein expression

3.4.1 Lactose and cellulose

In comparison to the previous experiment the BirA*-Xyr1 26 sample was tested for biotinylation and a second carbon source cellulose was used, because it is a significantly stronger inducer in comparison to lactose. The lactose fermentation was carried out as described before. The fermentation of the precultures with the strains QM9414Δ*tku70,* BirA*-Lae1 25A, Lae1-BirA* 5A and BirA*Xyr1 26 were performed for 24 h. Afterwards biotin concentrations of 10 µM or 50 µM were added to the shake flasks containing all strains. In the cellulose fermentation all four strains were cultivated for 38 h on cellulose and 50 µM biotin were added. In this experiment a sampling time point of 8 h was chosen. Again all samples were protein extracted, loaded on a SDS gel with each 10 µg and blotted on a western blot membrane. The western blot was now visualized via Typhoon FLA 9000 in order to achieve an easier handling and to obtain better comparable results. For detection of the biotinylated proteins again the HRP-strep AB was used, but additionally the anti-Myc-HRP AB was used to detect the c-Myc epitope which is fused to the BirA*-ligase. The expected size of BirA*-Xyr1 is 138 kD, Lae1-BirA* and BirA*-Lae1 is 79.5 kD.

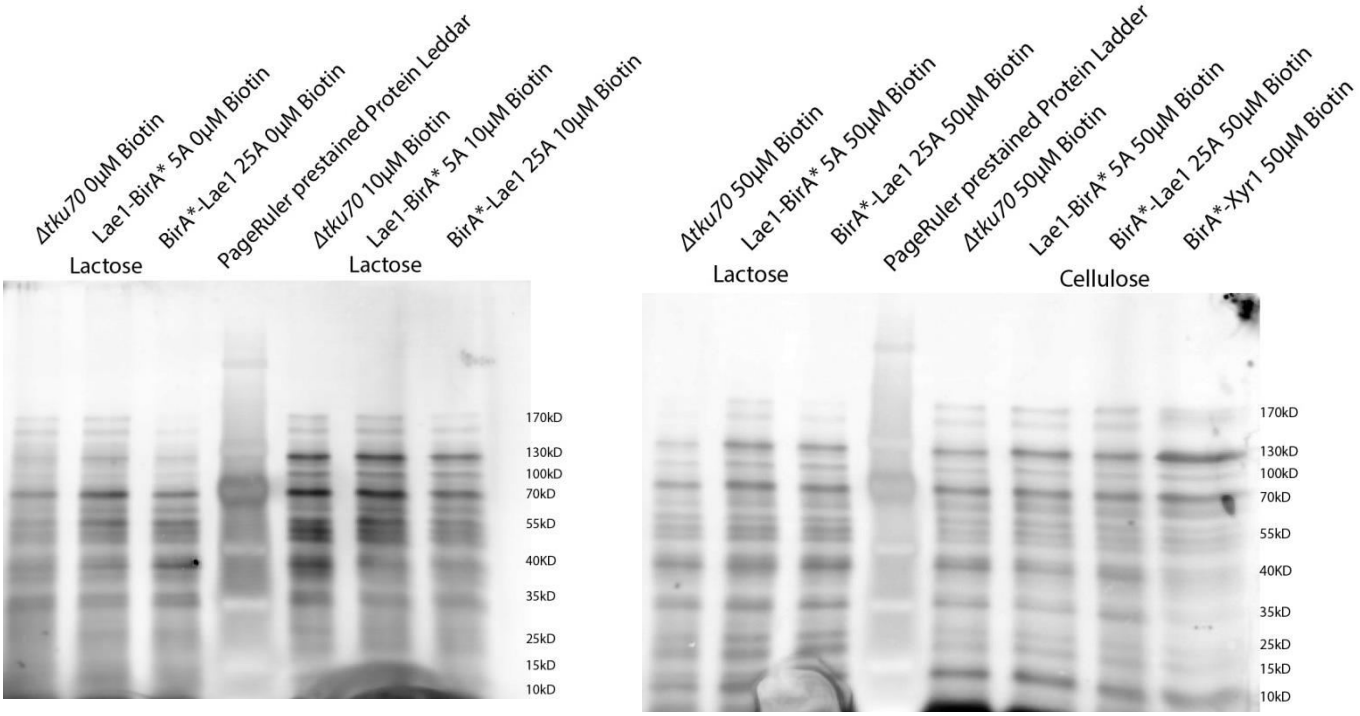

Figure 13: Western blot of cell free protein extracts of QM9414*Δtku70* and strains containing the N-terminal (BirA*-Lae1 25A, BirA*-Xyr1 26) and C-terminal (Lae1-BirA* 5A) fusion proteins 8 h after addition of biotin to lactose or cellulose grown cultures. Putatively biotinylated proteins were detected using a HRP-strep AB. The bands in QM9414*Δtku70* represent background signals. No obvious additional or stronger bands which would indicate additional biotinylated proteins are present in the different transformants.

In Fig. 13, the protein extracts of QM9414Δ*tku70,* BirA*-Lae1 25A and Lae1-BirA* 5A 8 h after addition of biotin (0 µM, 10 µM and 50 µM) to a lactose grown culture and of QM9414Δ*tku70,* BirA*-Lae1 25A, Lae1-BirA* 5A and BirA*Xyr1 26 after addition of 50 µM biotin to a cellulose grown culture are compared. Again all strains grown on lactose have a similar protein pattern. A strong background is recognized in the control samples, but no additional band indicating biotinylated proteins were detected. The control strain QM9414Δ*tku70* shows similar protein bands as the N- or C-terminally tagged LAE1 version. Also for the cellulose samples no difference between the control strain and the *lae1* or *xyr1* tagged strains can be recognized. The addition of different biotin concentrations does not lead to differences in the protein pattern or band strength. Again a strong background signal is present.

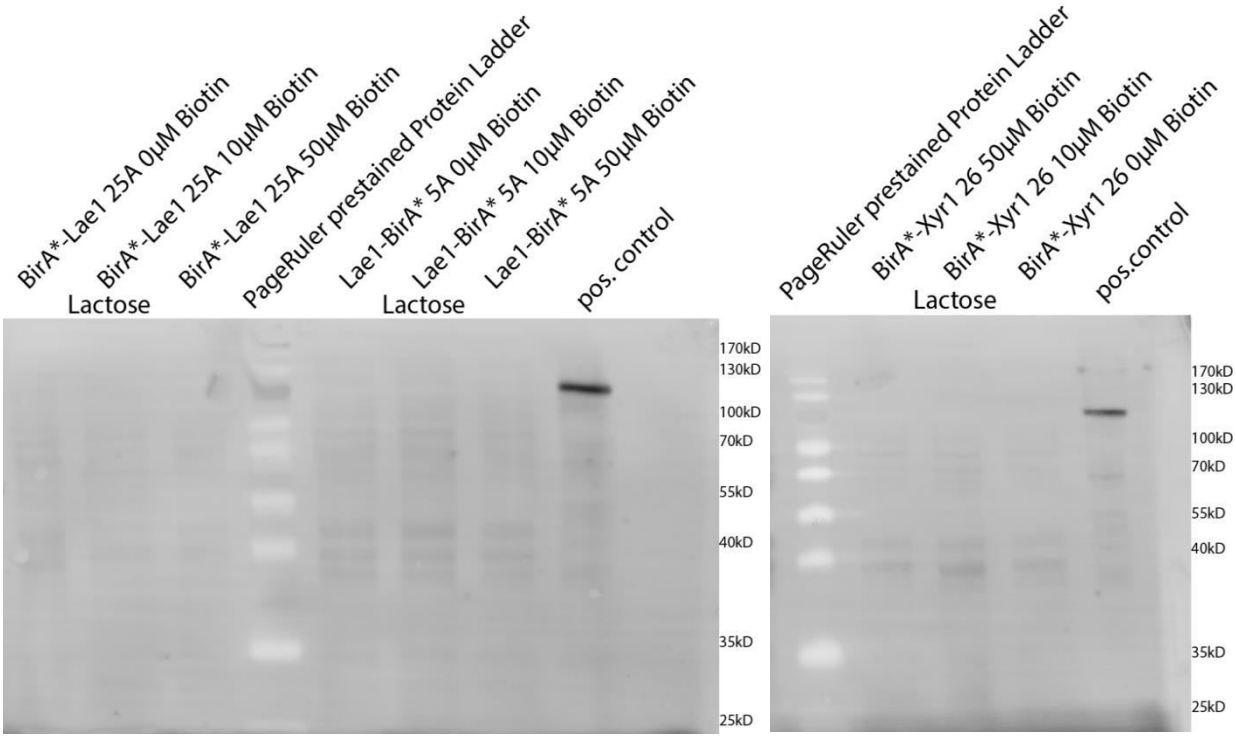

Figure 14: Western blot of cell free protein extracts of QM9414*Δtku70* and strains expressing the N-terminal (BirA*-Lae1 25A, BirA*-Xyr1 26) and C-terminal (Lae1-BirA* 5A) fusion proteins 8 h after addition of biotin to lactose or cellulose grown cultures. The c-Myc epitope on the BirA* ligase was detected using an anti-Myc-HRP AB. Only the positive control (cell free extracts with BirA*-labeled bilobe protein (Morriswood et al. 2012)) shows a signal for the c-Myc epitope.

As a c-Myc epitope is present on the N-terminal end of the BirA* biotin ligase, the same cell free extracts were used to detect the fusion protein using an anti-Myc-HRP AB. Lactose samples of BirA*-Lae1 25A, Lae1-BirA* 5A and BirA*Xyr1 26 treated with 0 µM, 10 µM or 50 µM biotin were tested. Furthermore a positive control of cell free extracts with BirA*-labeled bilobe protein (Morriswood et al. 2012), kindly provided by Brooke Morriswood, was added. As it can be seen, only the positive control shows a visible band in the size of the BirA*-labeled bilobe protein. For the tested lactose grown samples only faint bands, most likely background was detected, but no signal for the c-Myc epitope was found.

Furthermore, the control strain QM9414Δ*tku70* on lactose and all four strains on cellulose were tested. In the western blot below (Fig. 15) again only the positive control shows a visible band. The different biotin concentrations have no influence on the detection of the c-Myc epitope on the BirA*-ligase and in none of these samples a positive signal with the anti-Myc-HRP AB was detected, except of the positive control. The present results would indicate that the fusion protein i.e. the BirA* is instable or that the present fusion protein concentration is too low to be detected.

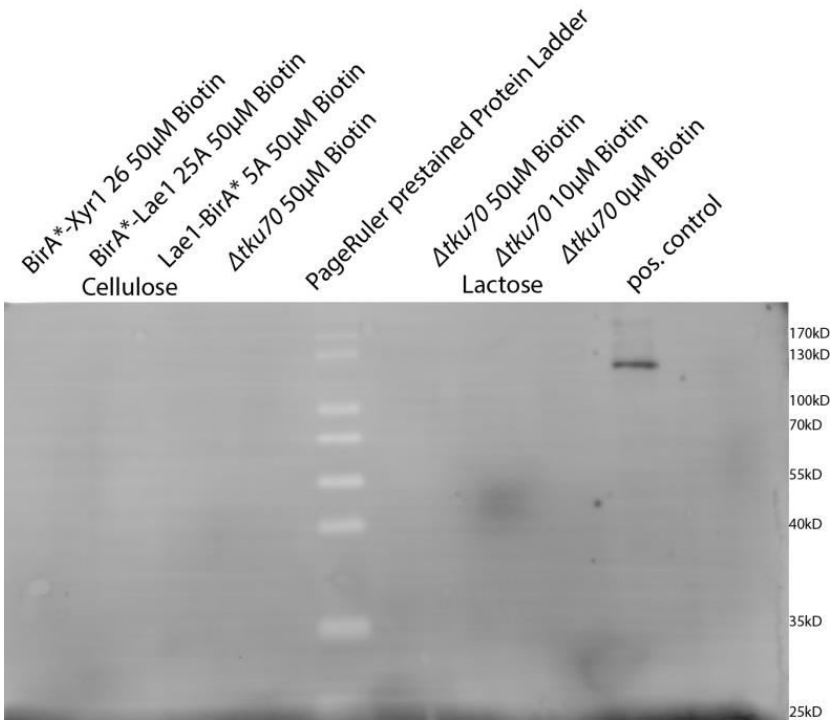

Figure 15: Western blot of cell free protein extracts of QM9414*Δtku70* **and strains expressing the N-terminal (BirA*-Lae1 25A, BirA*-Xyr1 26) and C-terminal (Lae1-BirA* 5A) fusion proteins 8 h after addition of biotin to lactose or cellulose grown cultures. The c-Myc epitope on the BirA* ligase was detected using an anti-Myc-HRP AB. Only the positive control (cell free extracts with BirA*-labeled bilobe protein (Morriswood et al. 2012)) shows a signal for the c-Myc epitope.**

To summarize, the BirA* tagged transformants did not differ in the biotinylation pattern compared to the reference strain under the chosen conditions. Although lactose and cellulose are cellulase inducing carbon sources and LAE1 or XYR1 are essential for cellulase expression, no effect by the addition of biotin can be achieved. Furthermore, the detection of the c-Myc epitope on the BirA*ligase did not yield a positive signal at the expected size for the BirA* tagged LAE1 or XYR1. Only a positive control shows a visible band detected by the AB. Hence, again no indication for a BirA* mediated biotinylation could be detected and the presence of the BirA* tagged fusion proteins could not be confirmed.

3.4.2 D-xylose and sophorose

XYR1 is the primary activator of transcription of cellulases and hemicellulases and when tagged with GFP its full size can only be detected in western blots within the first three hours after induction with sophorose and is then rapidly degraded and smaller sized fragments appear (Lichius et al. 2014). Therefore the next step was a replacement experiment with D-xylose for BirA*-Lae1 25A and Lae1-BirA* 5A strains and sophorose for BirA*-Xyr1 26 strain. Procedure of the replacement experiment is described in 2.1. QM9414Δ*tku70,* BirA*-Lae1 25A and Lae1-BirA* 5A were induced with 1 mM D-xylose and 50 µM biotin, BirA*-Xyr1 26 was induced with 1.75 mM sophorose and 50 µM biotin. Samples were taken after 24 h preculture on glucose (24 h glucose), directly after D-xylose addition

(0 h xylose) as well as 1 h, 3 h and 5 h after addition. For the sophorose replacement samples were taken after 18 h preculture (18 h glucose) as well as 1 h, 2 h and 3 h after addition of sophorose. Protein extracts were prepared and subjected to SDS PAGE and western blotted using the Typhoon FLA 9000 for visualization. Both antibodies, HRP-strep AB to detect biotinylated proteins as well as anti-Myc-HRP AB to detect the presence of the biotin ligase BirA*, were used for blotting. Additionally, the HRP-strep AB was diluted 1:5000, to reduce intensive background signals observed in the prior western blots. Again additional bands at a size of 79.5 kD (BirA*-Lae1 and Lae1-BirA*) and 138 kD (BirA*-Xyr1) are expected.

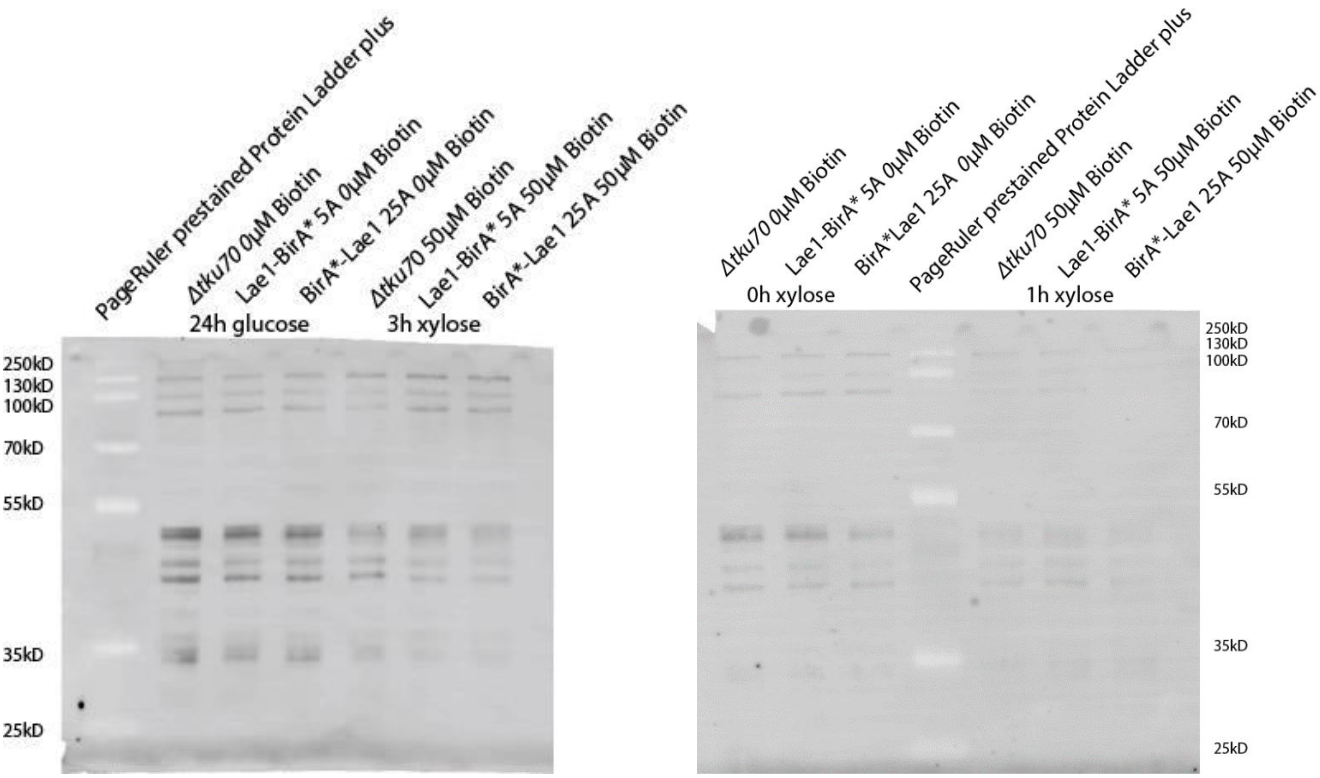

Figure 16: Western blot of cell free protein extracts of QM9414*Δtku70* **and strains expressing the N-terminal (BirA*-Lae1 25A) and C-terminal (Lae1-BirA* 5A) fusion proteins 24 h to glucose and 0 h, 1 h, 3 h to D-xylose grown cultures. Putatively biotinylated proteins were detected using a HRP-strep AB. The bands in QM9414*Δtku70*** **represent background signals. No obvious additional or stronger bands which would indicate additional biotinylated proteins are present in the different transformants.**

The western blot containing the samples of protein extracts after 24 h of growth on glucose, 0 h, 1 h and 3 h after D-xylose addition with the diluted HRP-strep AB showed less background signal. Nevertheless the protein pattern is largely the same in all samples including the control samples. Neither the addition of 50 µM biotin nor the putatively expressed fusion proteins result in a different protein pattern or signal strength. BirA*-Lae1 25A and Lae1-BirA* 5A showed no biotinylation induced by D-xylose and also the different sampling time points have no influence.

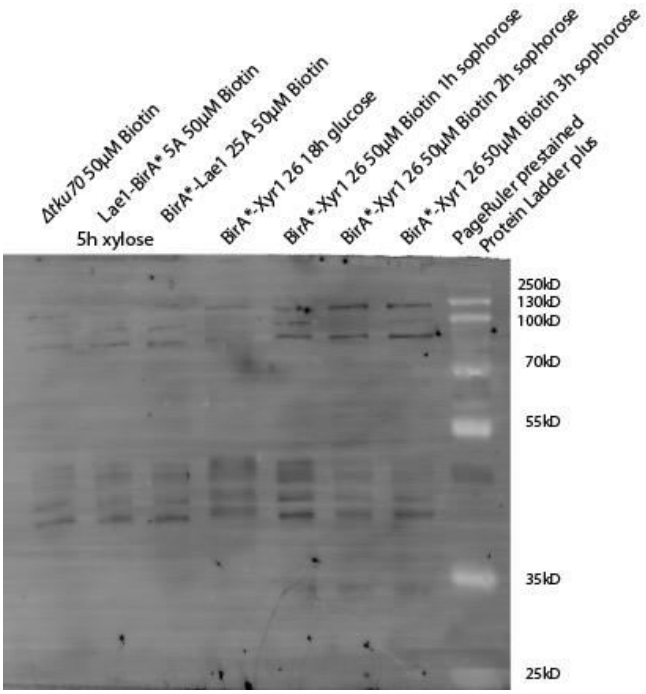

Figure 17: Western blot of cell free protein extracts of QM9414Δ*tku70* and strains expressing the N-terminal (BirA*-Lae1 25A) and C-terminal (Lae1-BirA* 5A) fusion proteins 5 h to D-xylose and 18 h to glucose, 1 h, 2 h, 3 h to sophorose grown cultures. Putatively biotinylated proteins were detected using a HRP-strep AB. The bands in QM9414Δ*tku70* represent background signals. No obvious additional or stronger bands which would indicate additional biotinylated proteins are present in the different transformants.

The samples 5 h after D-xylose addition were also tested. Again no biotinylated protein bands at the expected size can be recognized. By testing the BirA*-Xyr1 26 strain on sophorose, the same result occurs. A biotinylation of XYR1 is either not present or detectable. Although the background was reduced, no additional bands were detected in the different transformants.

Furthermore samples of the replacement experiment were also detected with the anti-Myc-HRP AB. The presence of the biotin ligase should be detectable by binding at the c-Myc epitope.

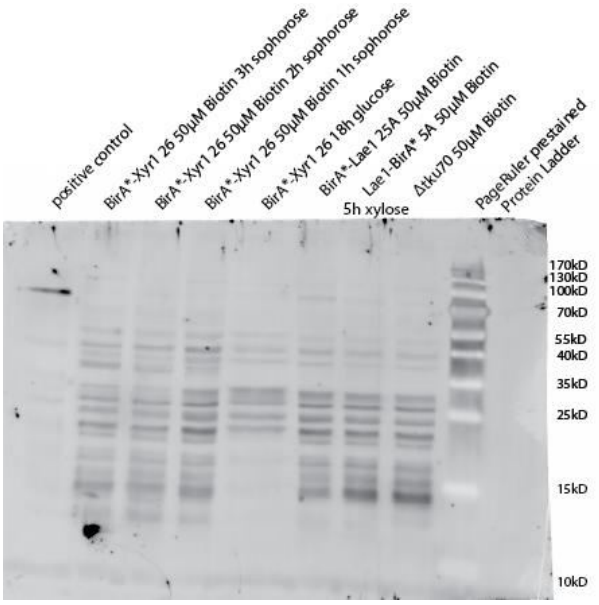

Figure 18: Western blot of cell free protein extracts of QM9414Δtku70 and strains containing the N-terminal (BirA*-Lae1 25A, BirA*-Xyr1 26) and C-terminal (Lae1-BirA* 5A) fusion proteins 5 h to xylose and 18 h to glucose, 1 h, 2 h, 3 h to sophorose grown cultures. The c-Myc epitope on the BirA* ligase was detected using an anti-Myc-HRP AB. Only the positive control (cell free extracts with BirA*-labeled bilobe protein (Morriswood et al. 2012)) shows a signal for the c-Myc epitope.

As it can be seen in the Fig. 18, by detecting the c-Myc epitope with anti-Myc-HRP AB only a strong background signal was visualized with Typhoon FLA 9000. However, a positive signal for the c-Myc epitope is not recognized in any sample, except the positive control.

In summary, also the strong inducing carbon sources D-xylose and sophorose seem to be unable to induce the biotinylation of BirA*-Lae1, Lae1-BirA and BirA*-Xyr1. No additional bands, at the expected sizes, were recognized. Furthermore the c-Myc epitope and thus the presence of the biotin ligase BirA* could not be visualized in the used samples.

In the present BioID experiments following conditions were used:

- different strains (QM9414Δ*tku70,* BirA*-Lae1 25A, Lae1-BirA* 5A, BirA*Xyr1 26)
- different carbon sources (lactose, cellulose, sophorose, D-xylose)
- different sampling time points (lactose: 4 h,8 h,12 h, cellulose: 8 h, sophorose: 1 h,2 h,3 h, D-xylose: 0 h,1 h,3 h,5 h)
- antibodies: HRP-strep AB to detect biotinylated proteins, anti-Myc-HRP AB to detect the presence of BirA*-ligase

In conclusion, all samples show an intense signal which might also prevent the detection of newly biotinylated proteins. BirA*-fusion proteins were not detectable in the used intracellular protein

extracts with the HRP-strep AB. Also BirA* presence was not detectable in the used samples with anti-Myc-HRP AB.

3.5 Transcriptional regulation of *T. reesei lae1* and *xyr1*

Based on the results of the western blots, an analysis whether *lae1* and *xyr1* genes are actually transcribed under the applied conditions was performed. Expression of different cellulase and xylanase activating genes, like *cbh1* (encoding cellobiohydrolase 1), *xyr1* (encoding the cellulase and xylanase regulator 1) and *lae1* (putative protein methyltransferase 1) in the reference strain QM9414Δ*tku70* were analyzed by quantitative real time PCR (qPCR). Samples after 24 h of growth on lactose as carbon source (=0 h lactose) and 4 h after addition of different concentrations of biotin to this culture (=4 h lactose) were analyzed.

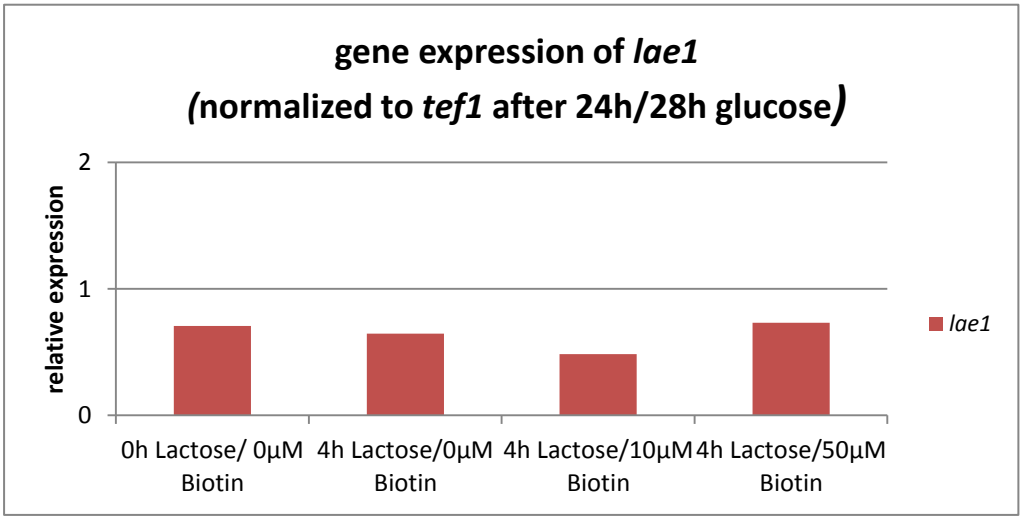

Figure 19: Transcription of *lae1* in QM9414Δ*tku70* in comparison to the repressing carbon source glucose. Different sampling time points and biotin concentrations were used. Biotin was added after 24 h of growth on lactose as carbon source (0 h lactose with 0 µM biotin), and the culture continued for further 4 h after the addition of 0 µM biotin (4 h lactose with 0 µM biotin), 10 µM biotin (4 h lactose with 10 µM biotin) or 50 µM biotin (4 h lactose with 50 µM biotin). The *lae1* transcript levels are related to the expression after 24 h and 28 h of growth on glucose and normalized to *tef1*.

As can be seen in Fig. 19, *lae1 s*hows no upregulation on lactose in comparison to the repressing carbon source glucose. Neither the different sample time point nor the additional biotin shows a distinctive effect on the transcript level of *lae1*.

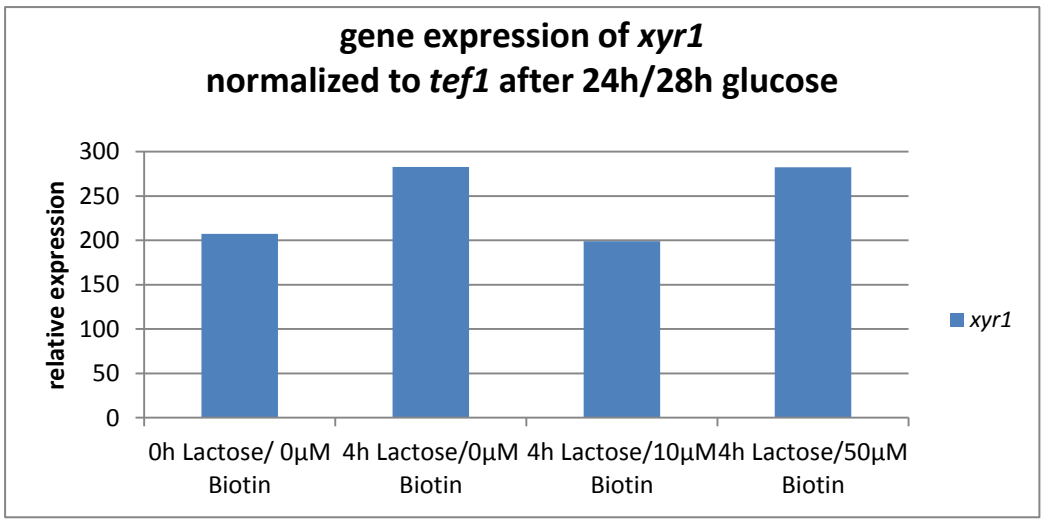

Figure 20: Transcription of *xyr1* in QM9414Δ*tku70* in comparison to the repressing carbon source glucose. Different sampling time points and biotin concentrations were used. Biotin was added after 24 h of growth on lactose as carbon source (0 h lactose with 0 µM biotin), and the culture continued for further 4 h after the addition of 0 µM biotin (4 h lactose with 0 µM biotin), 10 µM biotin (4 h lactose with 10 µM biotin) or 50 µM biotin (4 h lactose with 50 µM biotin). The *xyr1* transcript levels are related to the expression after 24 h and 28 h of growth on glucose and normalized to *tef1*.

In comparison to *lae1, xyr1* is clearly upregulated in QM9414Δ*tku70*. No influence on the expression of *xyr1* is found by an addition of biotin or different sampling time points.

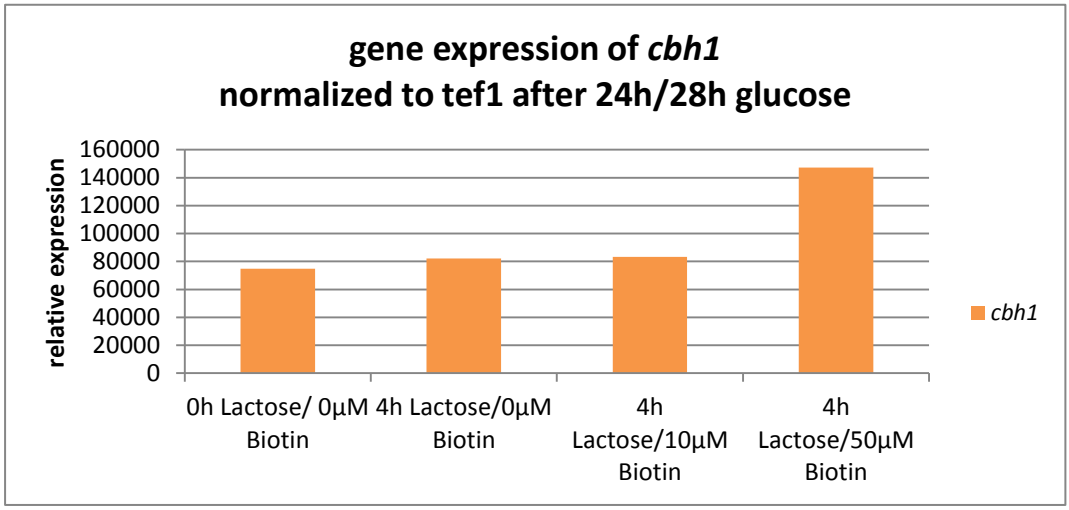

Figure 21: Transcription of *cbh1* in QM9414Δ*tku70* in comparison to the repressing carbon source glucose. Different sampling time points and biotin concentrations were used. Biotin was added after 24 h of growth on lactose as carbon source (0 h lactose with 0 µM biotin), and the culture continued for further 4 h after the addition of 0 µM biotin (4 h lactose with 0 µM biotin), 10 µM biotin (4 h lactose with 10 µM biotin) or 50 µM biotin (4 h lactose with 50 µM biotin). The *cbh1* transcript levels are related to the expression after 24 h and 28 h of growth on glucose and normalized to *tef1*.

Additionally, the expression of the key gene for cellulase production *cbh1* was analyzed. A strong upregulation can be seen. An increase in expression can be seen with a higher biotin concentration of 50 µM.

To summarize, during growth on lactose *cbh1* and *xyr1* are upregulated and the addition of 50 µM biotin seems to further stimulate the *cbh1* expression levels. In contrast, no upregulation for *lae1* during growth on lactose in comparison to glucose can be seen and no effect by adding different concentrations of biotin was achieved. Biotin does therefore not influence *cbh1* transcription negatively. It was also noted that *lae1* transcription occurs at a very low level indicated by a high Ct-value and might therefore be present at very low levels in the cell.

3.6 RT-PCR analysis

In a further experiment we wanted to analyze if the mRNA of the two fusion proteins can be detected during growth of the different transformants. This was performed by RT-PCR. Primers were selected as follows: one primer binds within the BirA* ligase and the other primer binds in the fusion protein partner gene (Table 11).

Following samples were chosen:

- Lae1-BirA* 5A 4 h grown on lactose after addition of 0 µM Biotin
- Lae1-BirA* 5A 4 h grown on cellulose after addition of 50 µM Biotin
- BirA*-Lae1 25A 4 h grown on lactose after addition of 0 µM Biotin
- BirA*-Lae1 25A 4 h grown on cellulose after addition of 50 µM Biotin
- BirA*-Xyr1 26 4 h grown on lactose after addition of 0 µM Biotin
- BirA*-Xyr1 26 4 h grown on cellulose after addition of 50 µM Biotin
- QM9414Δ*tku70* 4 h grown on lactose after addition of 0 µM Biotin
- QM9414Δ*tku70* 4 h grown on cellulose after addition of 50 µM Biotin

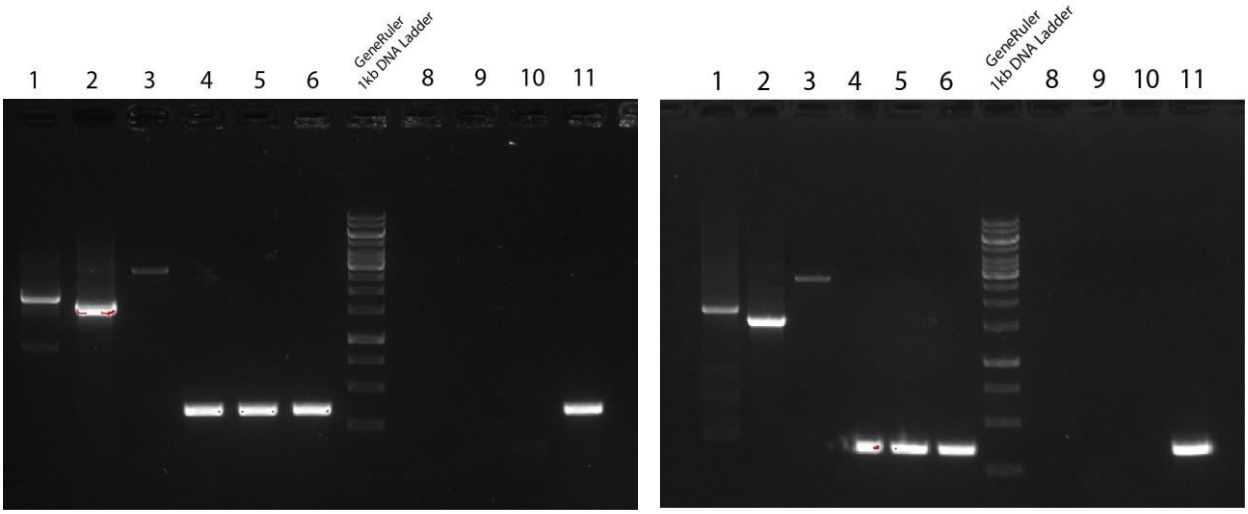

Figure 22: Left: RT-PCR of the lactose induced samples (Lae1-BirA* 5A, BirA*-Lae1 25A, BirA*-Xyr1 26, QM9414Δ*tku70* 4 h grown on lactose + 0 µM Biotin). Right: RT-PCR of the cellulose induced samples (Lae1-BirA* 5A, BirA*-Lae1 25A, BirA*-Xyr1 26, QM9414Δ*tku70* 4 h grown on cellulose + 50 µM Biotin).

Lane 1-3 shows the amplified transcript of fusion proteins including BirA* (BirA*-Xyr1: 2817 bp, Lae1-BirA*: 2082 bp, BirA*-Lae1: 1821 bp). Lane 4-6 shows the same samples to amplify *tef1*, with an expected size of 350 bp. Lane 8-10 shows the control strain QM9414Δ*tku70* with the same primers used for LAE1 or XYR1.

The left picture in Fig. 22 shows the lactose samples. Lane 1-3 shows the amplified transcript of fusion protein including BirA* with the correct sizes of 2817 bp (BirA*-Xyr1), 2082 bp (Lae1-BirA*) and 1821 bp (BirA*-Lae1). In lane 4-6 the same samples were used to amplify *tef1* (control to ensure that the same amounts of DNA were used in the PCR reactions), with an expected size of 350 bp. In lane 8-10 the control strain QM9414Δ*tku70* was tested with the same primers used for LAE1 or XYR1, where no visible band is present, as expected. Lane 11 shows a signal of the control strain with *tef1* primers at an expected size of 350 bp. The same order replies to the cellulose samples in Fig.22 (right).

The RNA encoding the fusion proteins are detectable with the fusion gene-specific primers. BirA*-Xyr1, Lae1-BirA* and BirA*-Lae1 shows a visible signal at the expected size. The control strain QM9414Δ*tku70* shows no signal, because the fusion ligase is not present in this strain and therefore the forward primer cannot bind. Only with *tef1* primers it was shown that the cDNA of QM9414Δ*tku70* is still active.

4. Discussion

Proteins rarely act alone and many physiological processes are carried out by proteins which are organized by their PPIs between two or more proteins. BioID is a relatively new method to investigate protein-protein interactions *in vivo* by detection of potential binding partners including their near neighbors, which do not need to directly interact with each other. In 2012 the system was first tested in mammalian cell lines (Roux et al. 2012) and has several advantages, such as the application to insoluble proteins, the identification of weak or transient interactions and the temporal regulation, which characterize the BioID system (Roux et al. 2013). However, the application of BioID for fungi and in particular *T. reesei* was not known at the beginning of this thesis. Aim of this thesis was therefore to test and establish the BioID technique in *T. reesei*, in which two proteins, XYR1 and LAE1, were chosen to detect known or new interaction partners. The transcriptional factor XYR1 regulates amongst others cellulase and xylanase transcription, D-xylose and L-arabinose catabolism (Akel et al., 2009). LAE1, which is a putative methyltransferase, also regulates cellulase and xylanase transcription (Seiboth et al. 2012) and previously it was shown that it interacts with VEL1 in a yeast two-hybrid assay (Karimi-Aghcheh et al. 2013).

To detect interaction partners, the biotin ligase BirA* is either fused N- or C-terminally to the protein of interest. In the case of XYR1 and LAE1 it was relatively easy to test if the BirA* tagging affects the function of XYR1 or LAE1. A phenotypic comparison of the reference strain (QM9414Δ*tku70*), the different BirA* tagged strains (BirA*-Lae1, Lae1-BirA* and BirA*-Xyr1) to the respective *xyr1* and *lae1* deletion strains (QM9414Δ*xyr1* or QM9414Δ*lae1)* indicated that the main functions of the regulators were not affected by the tagging. All transformants showed the characteristic yellow pigmentation and green sporulation as the reference strain QM9414Δ*tku70* indicating that the fusion proteins still exert their physiological function, while loss of *lae1* led to reduced sporulation and yellow pigment formation. Loss of XYR1 function affects the growth behavior during growth on D-xylose, as it can be seen in the *Δxyr1* deletion strain (Fig. 9, Fig. 10). In the case of XYR1 only a N-terminal fusion of BirA* to XYR1 was performed as a fusion of GFP to XYR1 was only functionally when GFP was found at the N-terminus (Lichius et al.2014). In conclusion the phenotypic experiments showed that *birA** gene fusions with *xyr1* or *lae1* did not affect the growth behavior of the recombinant strains and presented therefore the ideal set-up to screen for interaction partners.

The gene fusion in the positive strains were then also verified by sequencing, to exclude a non-functional BirA* biotin ligase caused by point mutations. Interaction partners for the two proteins (LAE1 and XYR1) were then screened under different cellulase and xylanase inducing conditions and it was tried to detect newly biotinylated proteins in the intracellular protein extracts by western

blotting. Different inducing carbon sources including lactose, cellulose, sophorose (cellulases) and D-xylose, as well as different biotin concentrations and time points were tested for the suitability of the system. A limiting factor for the detection of newly biotinylated proteins was the strong background signal on the western blots, which hindered the detection of newly or additionally biotinylated proteins. Based on these results it is not clear if the BirA* ligase is able to biotinylate proteins at all, as this could not be detected by the used methods.

Another important factor is the supplemented biotin concentration, which is important for the biotinylation by the BioID fusion proteins due to a reduced affinity of the mutant ligase to biotin (Roux et al. 2013). In this thesis a wide range of biotin concentrations (up to 100 µM) were added to the media. Addition of higher biotin concentrations was not tested, because a higher concentration would lead to a repression of cellulase formation and would therefore change the necessary conditions to detect interaction partners (Seiboth B, unpublished data). This fact in comparison with the high background signal observed with the HRP-Strep AB could be a knock-out criterion for the system in *T. reesei*.

Further an anti-Myc-HRP AB was used to detect BirA* fusion proteins. However, also in these experiments no protein could be detected except for the positive control. At present it is not clear if the BirA* ligase is stably expressed at all or if a (partial) degradation of the biotin ligase leads to the failure of its detection and proof of activity.

A gene expression study of *lae1*, *xyr1* and *cbh1* in the reference strain QM9414Δ*tku70* was carried out under the applied conditions for biotyinlation. These experiments showed that *lae1* is not induced when *T. reesei* was cultivated on lactose compared to glucose. On the opposite, *xyr1* and *cbh1* were highly upregulated under the same conditions as expected. Although *xyr1* is induced by lactose and the tagged strain BirA*-Xyr1 retained his phenotypical function, a biotinylation of proximal proteins or its presence detected with antibodies as shown before for a GFP-XYR1 fusion could not be reached (Lichius et al. 2014).

Due to the fact that BioID is a relative new method only a few publications deal with the BioID system. Successfully subcellular locations where BioID were tested include the nucleus, cytoplasm, endoplasmic reticulum and the mitochondrial matrix, as well as an expression in mammalian cells and *Trypanosoma brucei* was tested (Roux et al. 2013). With the results of this diploma thesis it was possible to test the biotin ligase tagged strains phenotypically, which indicate that the fusion protein performs its physiological function and is not blocked or altered by the mutated biotin ligase BirA*. However, on the one hand it is not clear if the BirA*ligase is stably expressed and exhibit biotin activity. On the other hand the biotin turn-over could be too high so that not enough biotin is

present for the biotin ligase. During this study, evidence accumulated that the system also fails in other fungi including *Pichia pastoris* (unpublished data) and no positive results were reported for other fungi to date.

Further studies will be necessary to advance the knowledge about the critical parameters and limitation of the BioID system in fungi and if the system in its present form is applicable at all for fungi.

5. Outlook

The results obtained in this thesis indicate that BioID is not readily transferable to fungi for study protein-protein interactions. To gain insight why the current approach to use BioID for *T. reesei* failed, the stable and active expression of the mutated biotin ligase BirA* needs to be established.

An easy approach would be to overexpress and purify BirA*. The purified protein can then be added together with biotin in an *in vitro* experiment to *T. reesei* cell free extracts, which are then analyzed on western blots for additional biotinylation. If BirA* is active the samples containing the ligase should show distinctively stronger biotinylation than the negative control samples.

Another approach would be the overexpression of the BirA* fused proteins under a strong promoter with and without a fusion partner. When overexpressed, it should be easily possible to detect at least the Myc-tag on the BirA* ligase with the anti-Myc-HRP AB and to test for biotin ligase activity in cell free extracts using different biotin concentrations. From the named *in vitro* experiments, the results will show if the BirA* ligase works in *T. reesei* and if it is possible to detect newly biotinylated proteins. Even if these experiments would indicate that BirA* is functional in *T. reesei* certain limitations cannot be excluded.

The present experiments show that *T. reesei* obviously uses biotinylation of proteins under physiological conditions to a very high extent, which caused high background signals in western blots and it cannot be excluded that faint bands caused by BirA*-biotinylation are difficult to detected. In addition, higher concentration of biotin in the medium leads to down-regulation of major cellulases, which might restrict the range of useful concentrations to detect interaction partners under relevant physiological conditions.

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7. Appendix

7.1 Genotype

Genotype of BirA*-Lae1 25A, Lae1-BirA*5A and BirA*-Xyr1 26. Shown is the open reading frame of the tagged genes BirA*-Lae1, Lae-BirA* and BirA*-Xyr1 without revealing any mutation.

BirA*-Lae1 25A:

atggaacaaaaactcatctcagaagaggatctcgacaaggacaacaccgtgcccctgaagctgatcgccctgctggccaacggcgagttccact
ctggcgagcagctgggagagaccctgggaatgagcagagccgccatcaacaagcacatccagacactgagagactggggagtggacgtgttc
accgtgcctggcaagggctacagcctgcctgagcctatccagctgctgaacgccaagcagatcctgggacagctggatggcggaagcgtggccg
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ggcagaggaggcagaggacggaagtggttcagcccattcggagccaacctgtacctgtccatgttctggagactggagcagggacctgctgctg
ccatcggactgagtctggtgatcggaatcgtgatggccgaggtgctgagaaagctgggagccgacaaggtgagagtgaagtggcctaatgacct
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Lae1-BirA*5A:

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BirA*-Xyr1 26:

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7.2 SDS gels

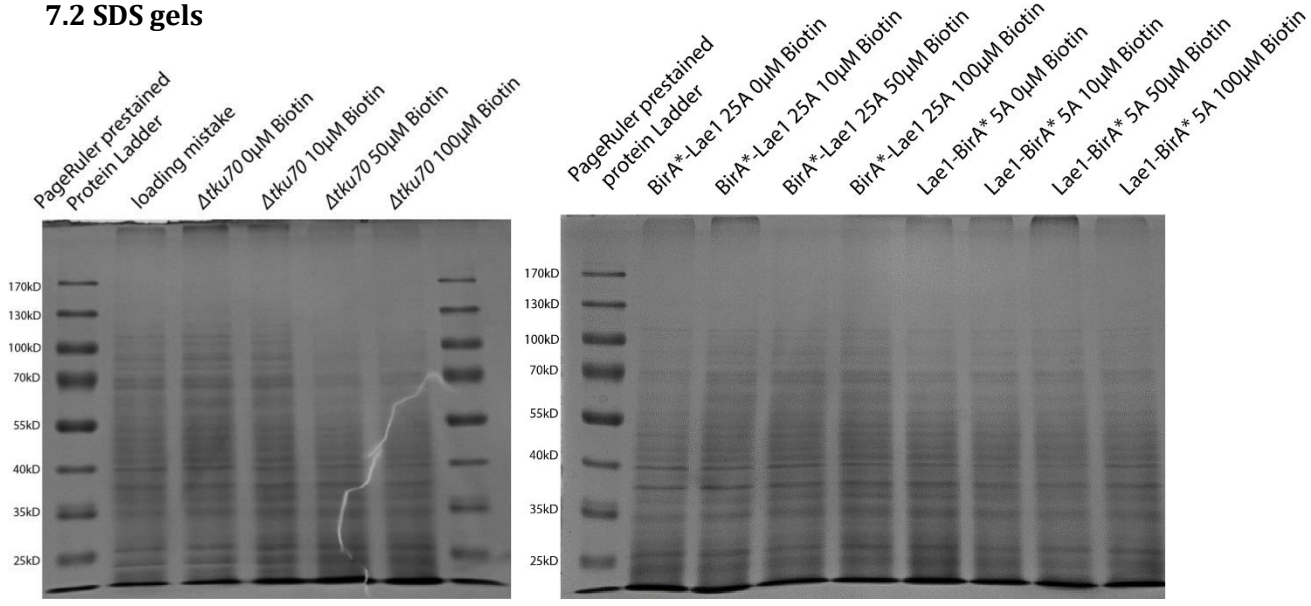

Figure 23: SDS gels of cell free extracts of QM9414Δ*tku70* and strains expressing the N-terminal (BirA*-Lae1 25A, BirA*-Xyr1 26) and C-terminal (Lae1-BirA* 5A) fusion proteins 12 h to lactose grown cultures, with the addition of different biotin concentrations (0 µM, 10 µM, 50µM and 100 µM). The gels were stained overnight with Coomassie Blue staining and washed with dH_20. The visible bands represent background signal

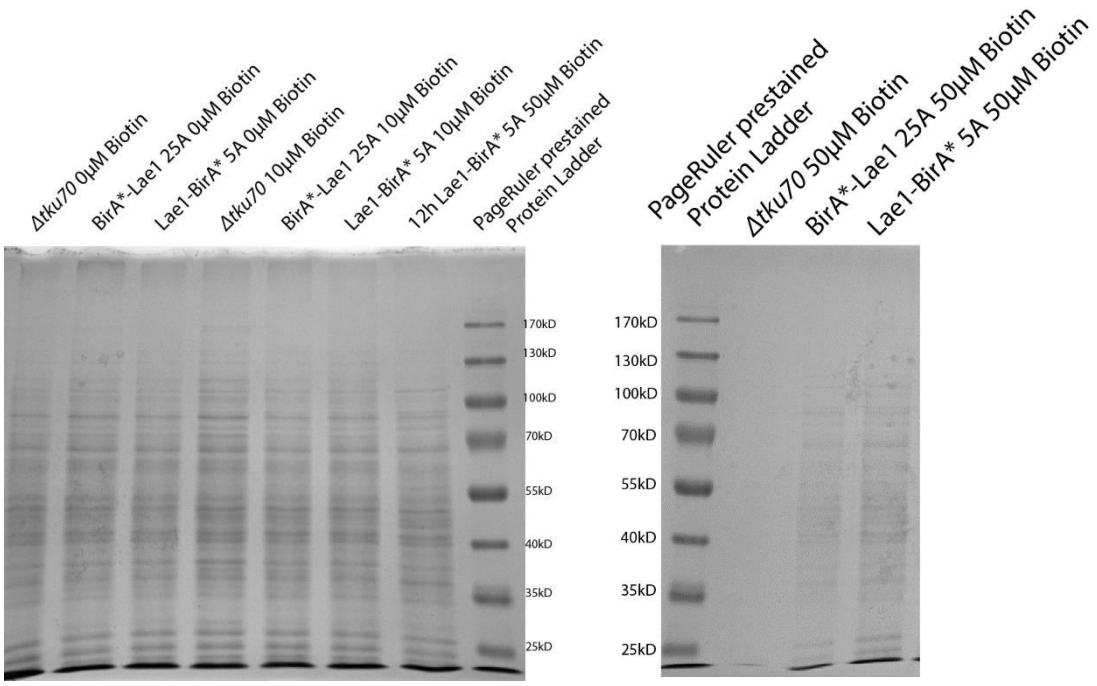

Figure 24: SDS gels of cell free extracts of QM9414Δ*tku70* and strains expressing the N-terminal (BirA*-Lae1 25A, BirA*-Xyr1 26) and C-terminal (Lae1-BirA* 5A) fusion proteins 4 h to lactose grown cultures, with the addition of different biotin concentrations (0 µM, 10 µM and 50 µM). Gels were stained overnight with Coomassie Blue staining and washed with dH_20. The visible bands represent background signal.

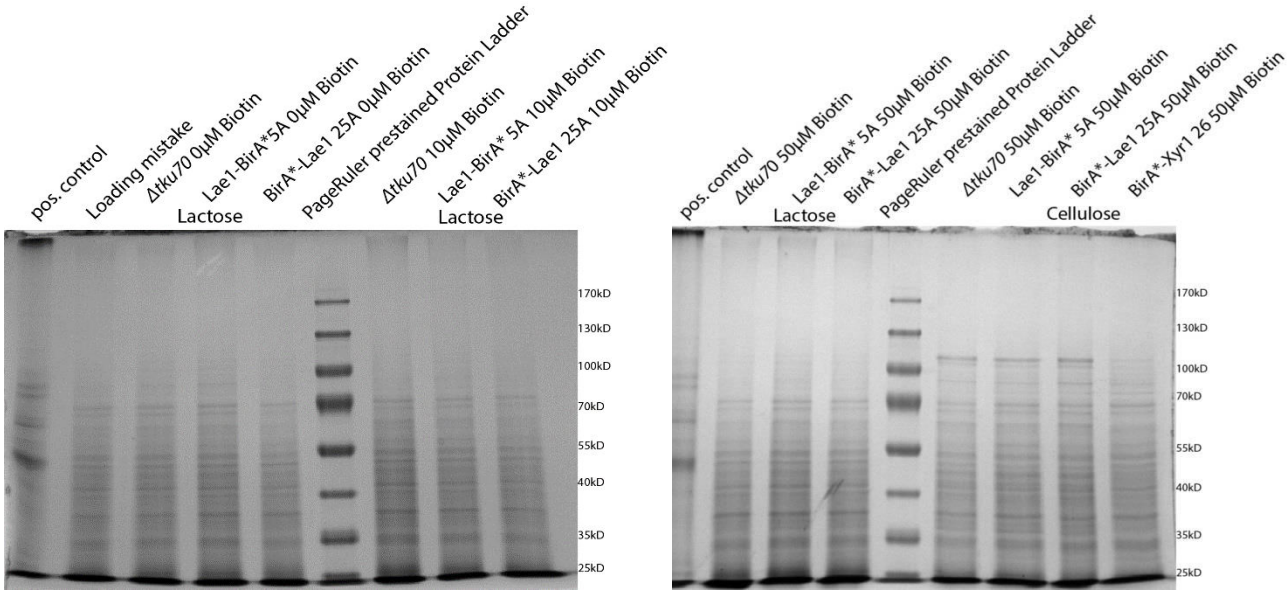

Figure 25: SDS gels of cell free extracts of QM9414Δ*tku70* and strains expressing the N-terminal (BirA*-Lae1 25A, BirA*-Xyr1 26) and C-terminal (Lae1-BirA* 5A) fusion proteins 8 h to lactose and cellulose grown cultures, with the addition of different biotin concentrations (0 µM, 10 µM and 50 µM). Gels were stained overnight with Coomassie Blue staining and washed with dH_20. The visible bands represent background signal.