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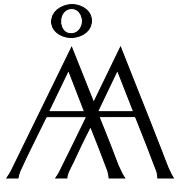

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Supervised by

Dr. Annika Brandt
Biochemistry, Faculty of Science and Engineering
Åbo Akademi University, Finland

Docent Jussi Meriluoto
Biochemistry, Faculty of Science and Engineering
Åbo Akademi University, Finland

Docent Juhani Soini
Faculty of Life Sciences and Business
Turku University of Applied Sciences, Finland

Reviewed by

Docent Taina Lundell
Department of Microbiology,
Faculty of Agriculture and Forestry
University of Helsinki, Finland

Docent Stephen Rudd
University of Turku, Finland

Opponent

Dr. Thierry Tron
CNRS Research Director
Institut des Sciences Moléculaires de Marseille
Aix-Marseille Université, France

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Dedicated to

Maija, Pekka, Heikki and
Henrik

"Yesterday is history, tomorrow is a mystery,
today is a gift of God, which is why we call it the present."

– anonymous –

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Elina Palonen

Abstract

An industrially and pharmaceutically important filamentous fungus, *Aspergillus terreus*, is known to biosynthesise plentiful natural products including lovastatin, a serum cholesterol lowering agent. *A. terreus* has also been observed to cause invasive aspergillosis in immunocompromised patients and to show resistance to one of the antifungal drugs, amphotericin B. In some of the *Aspergillus* spp. the pathogenic features have been observed to include diverse properties of asexual conidia as well.

In bacteria, a phenomenon quorum sensing has been observed to induce a joint activity with the surrounding prokaryotes in response to a specific, cell density reflecting and self-inductive (i.e. autoinductive) molecule of the same species origin. In filamentous bacteria, e.g. *Streptomyces* spp., small γ-butyrolactones have been observed to act as autoinducers with diverse responses to the achieved cell density including secondary metabolism and cellular development. Quorum sensing in fungi has been less studied. In *A. terreus* a secondary metabolite — butyrolactone I — has been observed to increase the production of two secondary metabolites, lovastatin and sulochrin, and to affect the fungal morphology as well thus giving indications of a similar role as observed in *Streptomyces* spp.

The central aim of this thesis was to further elucidate the role of butyrolactone I in the fungus *A. terreus* on the metabolite and especially on the transcriptomic level. To this end, both chromatographic methods with HPLC and MS/MS as well as genome-wide gene expression and transcriptome sequencing were applied.

The positive role of butyrolactone I in lovastatin production was confirmed and its autoinducing feature was revealed together with a potential signalling profile, reminiscent of the quorum sensing phenomenon of bacteria. The suggested positive role of butyrolactone I in secondary metabolism and sporogenesis was further supported by the gene expression profiles of the global regulator, *laeA*, and the orthologs of the key regulators of asexual conidiation (*brlA, abaA, wetA*). An in-depth transcriptome analysis revealed also an unknown gene cluster to be regulated in pace with these conidiation regulators and, furthermore, the key enzyme of the cluster showed similarity to a known pigment-producing key enzyme of another fungus of *Ascomycota*. Moreover, the transcriptome sequencing revealed also interesting splice variants within velvet gene family with numerous regulative functions in the formation of asexual conidia and sexual ascospores of *Aspergillus* spp. All this rises several hypotheses awaiting further studies.

Sammanfattning (Abstract in Swedish)

Aspergillus terreus är en industriellt och medicinskt sett viktig mycelsvamp som producerar flera sekundära metaboliter, bl.a. lovastatin som används till att sänka kolesterolnivån i blodserum. *A. terreus* har även hittats i allt flera fall bland de organismer som orsakar en patologisk svampinfektion, invasiv aspergillos hos patientar med immunbristsyndrom, där läkemedelsresistens mot amphotericin B har observerats. Bland släktet *Aspergillus*, de kända patogena faktorerna omfattar också asexuella svampspor med sina mångsidiga egenskaper.

Ett speciellt bakteriefenomen känt som quorum sensing är en utforskad aktivitet bland prokaryoter. Fenomenet är förknippad med celldensiteten av bakteriefloran av samma art genom att reagera på en viss självinduktiv signaleringsmolekyl, vars produktion har överstigit en tröskelkoncentration som reflekterar den uppnådda celldensiteten. Myceliumformande bakterier, t.ex. av släktet *Streptomyces*, har observerats producera små själv-inducerande molekyler med mångsidig inverkan, bl.a. reglering av sekundär metabolism samt morfologiska förändringar. Däremot har quorum sensing bland mycelsvamparter undersökts i mindre utsträckning. En sekundär metabolit — butyrolakton I — av *A. terreus* har noterats att upphöja produktionen av två andra sekundära metaboliter, lovastatin och sulokrin, samt påverka svampens morfologi, vilket antyder en liknande funktion som har uppvisats för *Streptomyces* bakterier.

Den centrala avsikten med denna avhandling var att klarlägga butyrolakton I och dess roll i mycelsvampen *A. terreus* på molekylärbiologisk och genetisk nivå. För detta ändamål utnyttjades både kromatografiska (vätskekromatografi och MS/MS masspektrometri) samt transkriptomkartläggande (genuttryck och transkriptomsekvensering) metoder.

De uppnådda resultaten bekräftade att butyrolakton I ökar lovastatinproduktionen samt tyder på potentiella själv-inducerande och regulativa signaleringsroller. Dessa liknar det kända quorum sensing-fenomenet i bakterier. Den föreslagna positiva rollen av butyrolakton I i den sekundära metabolismen samt i sporbildningen stöddes ytterligare av genuttrycksprofiler av vissa regulatorgener. Därtill uppdagades ett okänt genkluster, vars centrala gen uppvisade en hög likhet med ett känt pigment-producerande enzym hos en annan svampart av *Ascomycota*, och vars genkluster visade en likartad genuttrycksprofil med regulatorgenerna. Transkriptomanalyset avslöjade också olika genvarianter bland ett annat svampspecifikt genkluster med rikliga regulativa funktioner inom sporbildning. De uppväckta hypoteserna ger anledning till fortsatta undersökningar.

Original Publications

This doctoral thesis is based on the following original publications, which are referred to in the text by Roman numerals (I–IV).

I Raina, S, de Vizio, D, Palonen, EK, Odell, M, Brandt, AM, Soini, JT, and Keshavarz, T. Is quorum sensing involved in lovastatin production in the filamentous fungus *Aspergillus terreus*? *Process Biochemistry*, 47(5):843–852, 2012

II Palonen, EK, Neffling, MR, Raina, S, Brandt, A, Keshavarz, T, Meriluoto, J, and Soini, J. Butyrolactone I quantification from lovastatin producing *Aspergillus terreus* using tandem mass spectrometry — evidence of signalling functions. *Microorganisms*, 2(2):111–127, 2014

III Palonen, EK, Raina, S, Brandt, A, Meriluoto, J, Keshavarz, T, and Soini, JT. Transcriptomic complexity of *Aspergillus terreus* velvet gene family under the influence of butyrolactone I. *Microorganisms*, 5(1):12, 2017

IV Palonen, EK, Raina, S, Brandt, A, Meriluoto, J, Keshavarz, T, and Soini, JT. Melanisation of *Aspergillus terreus* — is butyrolactone I involved in the regulation of both DOPA and DHN types of pigments in submerged culture? *Microorganisms*, 5(2):22, 2017

Contribution of the author in the publications

The author wrote the genome-wide microarray part of Publication I in collaboration with the co-author S. Raina and under supervision of A. Brandt and J. Soini. The author wrote the manuscript of Publication II in collaboration with the co-authors M.-R. Neffling and S. Raina and under supervision of A. Brandt, J. Meriluoto and J. Soini. The manuscripts of Publications III and IV were written by the author in collaboration with the co-author S. Raina and under the supervision of A. Brandt, J. Meriluoto and J. Soini.

The author is responsible for, and the primary and corresponding author of the Publications II, III and IV.

Publication I. The author participated to a lesser extent in the design of the genome-wide microarray experiment in collaboration with the supervisors A. Brandt and J. Soini, and the EU project members S. Raina and Prof. T. Keshavarz. The author conducted RNA extraction, labelling and hybridisation processes, the use of microarray instrumentation and the analysis of the obtained gene expression data under the supervision of A. Brandt and J. Soini.

Publication II. The author conducted the cultivation and HPLC experiment in collaboration with S. Raina and the LC-ESI-MS/MS experiment in collaboration with M.-R. Neffling, and under the supervision of A. Brandt, J. Meriluoto and J. Soini. The further data analysis was performed by the author under the supervision of A. Brandt, J. Meriluoto and J. Soini.

Publication III. The author participated in the planning to utilise an additional transcriptional experiment in collaboration with the EU project partners together with the supervisors A. Brandt and J. Soini. The author prepared and utilised the modified methodological combination of transcriptome sequencing and the already performed microarray experiment under the supervision of A. Brandt and J. Soini. The author performed the RNA extraction and preparation for the subsequent supervised usage of the sequencing instrumentation, and the combined transcriptome assembly and analysis under the supervision of A. Brandt and J. Soini.

Publication IV. The author conducted the further computational analysis of the obtained gene expression and transcriptome data combination under supervision of A. Brandt and J. Soini.

Contents

Abbreviations

ACP acyl carrier domain
AHL acyl homoserine lactone
AT acyl transferase domain
BI butyrolactone I
bp base pair
CAZy carbohydrate active enzyme
cDNA complementary deoxyribonucleic acid
CDW cell dry weight
CMeT C-methyltransferase domain
cRNA complementary ribonucleic acid
DHN 1,8-dihydroxynaphthalene
dUTP deoxyuridine triphosphate
ETP epipolythiodioxopiperazine
FAD flavin adenine dinucleotide
FC fold change
FMN flavin mononucleotide
FPKM fragments per kilobase of exon per million reads mapped
GPY-L glucose, peptonised milk, yeast extract, lactose
HPLC high performance liquid chromatography
HR-PKS highly reducing polyketide synthase
IGV Integrative Genomics Viewer
KS beta-ketoacyl synthase domain
LC-ESI-MS/MS liquid chromatography electrospray ionisation tandem mass spectrometry
***L*-DOPA** 3,4-dihydroxyphenylalanine
MFS major facilitator superfamily
mRNA message ribonucleic acid
NA not available
NR-PKS nonreducing polyketide synthase
NRPS non-ribosomal peptide synthase
ORF open reading frame
PCR polymerase chain reaction
p.i. post-inoculation
PKS polyketide synthase
PT polyketide product template domain
R thioester reductase domain
SAM S-adenosyl-*L*-methionine
SAT starter unit: ACP transacylase domain

SM secondary metabolism
TE thioesterase domain
TE/CLC thioesterase/Claisen cyclase domain
THN tetrahydroxynaphthalene
UTR untranslated region
YME yeast and malt extracts, glucose

Review of the literature

1.1 Filamentous fungi of genus *Aspergillus*

The genus *Aspergillus* is classified to the phylum *Ascomycota* of *Dikarya* subkingdom together with other fungal species of *Candida* and *Fusarium*, for instance. The filamentous species of *Aspergillus* and *Fusarium* belong to the subphylum *Pezizomycotina* of which the genus *Aspergillus* is included in the class *Eurotiomycetes*. The filamentous fungi, including *Aspergillus* spp. contain an abundant reservoir of secondary metabolites including mycotoxins, pigments, and pharmaceutically and industrially important compounds. Numerous metabolites remain still unidentified.

Several of these fungi are also opportunistic pathogens to a range of hosts from plants [1] to humans [2]; for example *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger* and *Aspergillus terreus* have been observed to cause severe fungal infections in immunocompromised patients, a disease known as invasive aspergillosis. Approximately 4 % of these infections have been reported to be caused by *A. terreus* and the mortality is higher for *A. terreus* than for other *Aspergillus* spp.; varying between 38 to 94 % depending on the underlying disease and the global location of the patients. *A. terreus* and *A. flavus* have been reported to show resistance to the commonly used antifungal drug amphotericin B, in contrast to the common pathogen *A. fumigatus*. The reason behind the resistance is currently not known but potential new drugs have been found [reviewed in 3–5].

The diverse natural growth environments where filamentous fungi have been isolated range from moist soil to dry and hot desert, from ripening crop to decomposing matter. *A. terreus* is commonly isolated from soil rhizospheres along with decaying organic matter and produces industrially useful enzymes including plant biomass decomposing CAZy enzymes (carbohydrate active enzymes, CAZy database) [6–15]. The industrially important metabolites of *A. terreus* include for example itaconic acid that has advantageous polymerisation features, and pharmaceutically significant lovastatin. Itaconic acid is utilised in the form of polymerised esters in plastics, adhesives, elastomers and coatings; and as a copolymer of acrylic and itaconic acid in the clinical dentistry, for instance [16–18] [reviewed in 19]. The pharmaceutical property of the secondary metabolite lovastatin is the lowering effect on the cholesterol level of blood serum [20] and is thus used in the treatment of cardiovascular diseases (atherosclerosis and coronary artery disease) as the active ingredient in

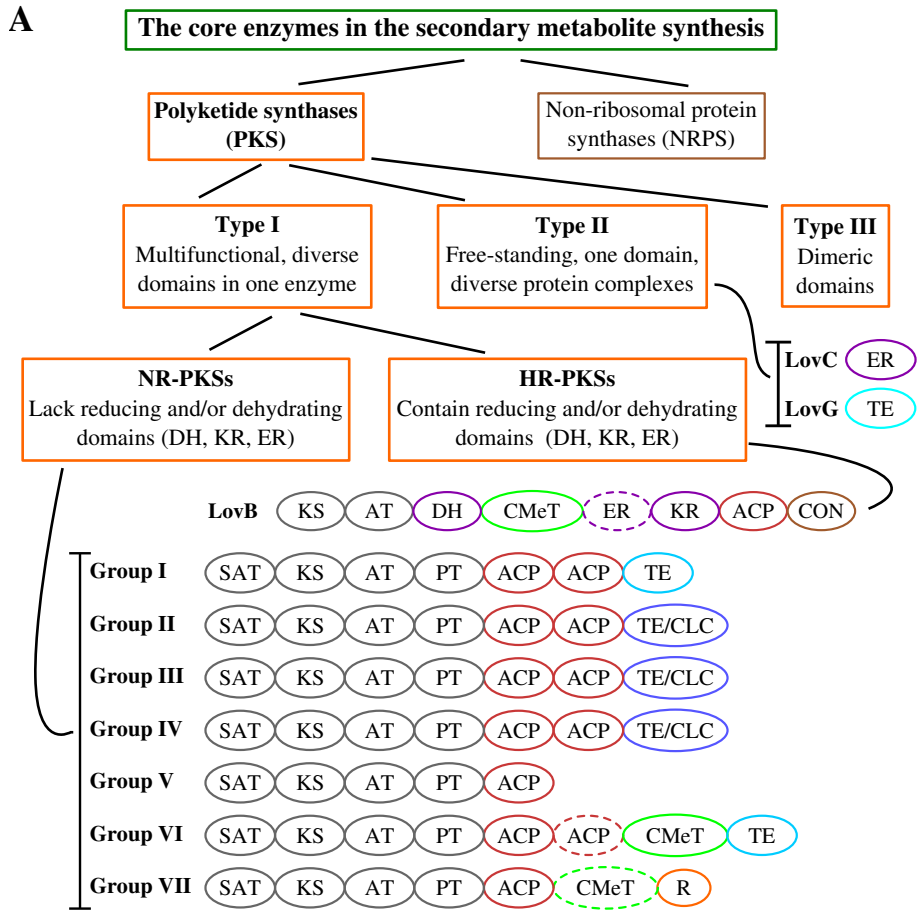
the commercial drugs. Several patents have been established concerning the improved methods for the fungal production of this metabolite and the subsequent lovastatin derivatives (e.g. simvastatin) [21].

In addition to these beneficial compounds, *A. terreus* produces also toxins, among them citrinin [22] and acetylaranotin (belongs to the epipolythiodioxopiperazine (ETP) class of gliotoxins) [23–25]. Gliotoxins have been detected in the clinical samples of patients that suffer from invasive aspergillosis, of which one sample contained *A. terreus* as the causative pathogen [26]. The specific ETP toxin acetylaranotin of *A. terreus* has also been observed to induce colon cancer cell apoptosis, inhibit plant seedling growth, and to have antiviral activities [23, 27, 28]. The other metabolites produced by *A. terreus* include e.g. aspulvinones [29–31], asterriquinones [32, 33], butyrolactones [34–37] and (+)-geodin with the pathway intermediates emodin and sulochrin [38–41].

1.2 Secondary metabolism

The biosynthesis of secondary metabolites generally involves enzymatic pathways derived from genomic gene clusters which are under complicated transcriptional control. For instance the lovastatin producing cluster in fungi was investigated by Kennedy et al. [42] and other *A. terreus* gene clusters have been described in Guo and Wang [43], Yin et al. [44]. The control of secondary metabolite production includes transcription factors with specific protein domains (e.g. Zn(2)-C6 fungal-type DNA-binding domain as in the lovastatin gene cluster [42]) that bind to specific genomic locations inside the gene cluster in order to regulate the gene transcription. The majority of the key enzymes in these biogenesis pathways can be divided into two classes: polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS). Within these two enzyme classes there are also diverse combinations of different protein domains with specific functions providing the broad scale of intermediates to be further progressed into divergent secondary metabolites by the surrounding cluster enzymes. The PKSs are further divided into three different types, from which the type I is common to fungal PKSs with multidomain and -functional structures. These PKSs include also two subclasses, highly reducing (HR) or non-reducing (NR), determined by the presence or absence of reducing and/or dehydrating domains, respectively. In addition, fungal PKSs are commonly iterative, i.e. the substrate is used in several rounds during the polyketide chain elongation, thereby increasing the diversity of the biosynthesised secondary metabolite intermedi-

ates [reviewed in 45, 46]. The varying domain compositions of the PKS enzymes have been further categorised into various groups; recently non-reducing polyketide synthases (NR-PKS) were suggested to contain seven various groups in *Aspergillus* spp. and *Ascomycota* [47]. In *A. nidulans* (except for the group II) these NR-PKS groups biosynthesise the precursors for the main downstream compounds which include cathepsin K inhibitors F9775A and B (Group I) [48], pigment elsinochrome of *Elsinoë fawcettii* (Group II) [49], YWA1 and melanin (Group III) [50], mycotoxin sterigmatocystin (Group IV) [51], secondary metabolites emodin (Group V) [52], cichorine (Group VI) [47] and asperfuranone (Group VII) [53]. The characteristic domain compositions for the NR-PKSs of the seven suggested groups and the precursors for the above mentioned compounds are illuminated in Figure 1.



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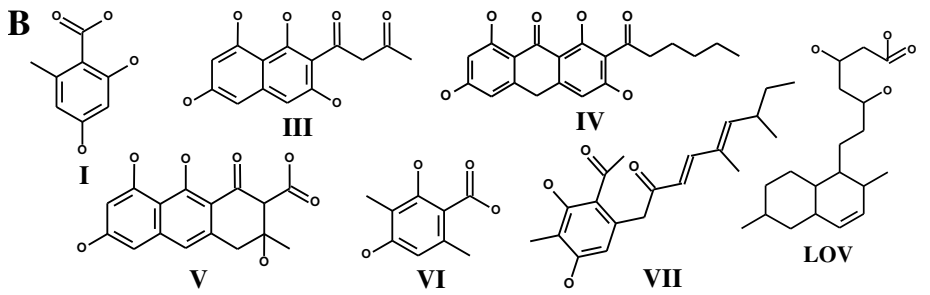

Figure 1: (**A**) A scheme of the core enzyme classes involved in the biogenesis of diverse secondary metabolites [reviewed in 46] and examples of their domain compositions [42, 47, 54]. The domains encircled with a dotted line indicate the absence of the domain in some, but not all, of the members of the corresponding group. Domain abbreviations: SAT; starter unit:ACP transacylase, KS; beta-ketoacyl synthase, AT; acyl transferase, PT; polyketide product template, ACP; acyl carrier, TE; thioesterase, TE/CLC; thioesterase/Claisen cyclase, CMeT; C-methyltransferase, R; thioester reductase. (**B**) Examples of the intermediates produced by the NR-PKSs of the groups I and III-VII that are involved in the secondary metabolite biosynthesis pathways of *A. nidulans* [47] and the produced and released lovastatin intermediate (LOV) of an enzyme complex of the subclass of HR-PKS (LovB) and two PKSs of type II (LovC and LovG) of *A. terreus* [42, 43, 54]. The intermediates and the accession numbers of the example enzymes are: I; orsellinic acid (ANID_07909.1), III; 2-acetoacetyl 1,3,6,8-tetrahydroxynaphthalene (ANID_08209.1), IV; norsolorinic acid anthrone (ANID_07825.1), V; atrochrysone carboxylic acid (ANID_00150.1), VI; 3-methylorsellinic acid (ANID_06448.1) and VII; 6-[(3*E*,5*E*)-5,7-dimethyl-2-oxonona-3,5-dienyl]-2,4-dihydroxy-3-methylbenzaldehyde (ANID_01034.1) and LOV; dihydromonacolin L acid (precursor of lovastatin) (LovB; ATEG_09961, LovC; ATEG_09963, LovG; ATEG_09962).

The regulation of secondary metabolism is dependent on the growth conditions, e.g. the intensity of light and/or aeration levels, with a diversity of known as well as thus far unknown regulatory factors and cascades. One of the factors involved is a global transcription regulator, putative DNA methyltransferase LaeA which has been shown to play a positive role in the biogenesis of sterigmatocystin and penicillin in *A. nidulans*, lovastatin in *A. terreus*, aflatoxin in *A. flavus* and CAZy enzymes in *Trichoderma reesei* [55–57]. In *A. nidulans* LaeA has been demonstrated to form a trimeric complex with two members — VeA and VelB — of a unique fungal regulatory protein family, named as Velvet [58]. The regulation of secondary metabolism is also closely connected to mycelial

development via Velvet proteins (described in the next section 1.3).

1.2.1 Industrial benefits

The profitable industrial usage of *Aspergillus* species has a long history: one of the oldest known processes utilises specific strains of *Aspergillus oryzae* in soy bean fermentation to obtain traditional soya sauce. More recently, filamentous fungi have been studied with the aim to increase the yield and production period of the commercially advantageous compounds and enzymes. Typically, the industrial processes have been applied in submerged cultivation conditions. The studies have paid attention to micro- and macroscopic morphology, culture media contents and more efficient strains [59, 60].

The industrial submerged cultivation processes are usually performed in bioreactors of various sizes and types (batch, continuous and fed-batch) that provide several methods to modify the cultivation conditions used. These methods are described in detail in a review by Papagianni [61]. Typical batch cultivations contain the following fungal growth phases: (i) asexual conidia germination followed by rapid increase in hyphal growth; (ii) stationary phase during which the production of secondary metabolites, including the beneficial ones, initiates; and (iii) pre-autolysis and autolysis which begin through hyphal fragmentation (these phases are further described in the next section 1.3). As represented by Papagianni [61] the continuous cultivation is initiated by supplementing fresh growth medium to the batch culture when the growth enters the stationary phase. The bioreactor vessel is then replenished with fresh medium in order to sustain the optimum growth rate, i.e. there is a continuous in- and outflow of fresh and used media, respectively. The fed-batch cultivation is also based on the batch culture: a fresh, concentrated nutrient of the original medium is supplemented to the batch cultivation when a specific component of the substrate has been fully utilised [61]. The shared aim of these diverse culture types is to improve the industrial cultivation conditions and to enable the modelling of growth and production rates in order to increase the production efficiency of the commercially beneficial products.

Morphologically the significant differences between the industrial cultivation and the natural fungal growth are the hyphal and mycelial growth and development patterns which are influenced by the externally controlled cultivation conditions and medium properties (nutrients, pH). Growth in agitated submerged liquid conditions was visualised and modelled by Bizukojc and Ledakowicz [59], while *A. terreus* has been iso-

lated from terrestrial environment [6]. The industrially formed morphological structures have been reviewed by e.g. Papagianni [61], Bizukojc and Ledakowicz [62] and Krull et al. [63]. Cultivation in industrial bioreactors with the controlled submerged conditions leads to the formation of either free hyphae followed by dispersed mycelial filaments, or aggregated hyphae and mycelia that form pellets. The formed pellets have been observed to consist of at least two types: non-coagulative and coagulative. The non-coagulative type of pellets are formed by hyphae and mycelia derived from asexual conidia that were aggregated after germination whereas the coagulative type of pellets consist of aggregated asexual conidia of which only a fraction were germinated and developed into hyphae and mycelia. The physical properties of these pellets, including their density and diameters, affect the production rates and kinetics of the desired compounds [61–63]. In addition, the formation of either dispersed, non-pelleted mycelia or mycelial pellets has been observed to have different impacts on the production efficiencies of certain commercially beneficial compounds or enzymes in question [61]. Therefore the control of mycelial morphology is important. The factors that affect fungal morphology and thereby the industrial submerged cultivation include: the initial conidia concentration, cultivation pH, medium composition and mechanical stress (e.g. agitation and aeration) [59, 62]. These factors have been studied in *A. terreus* along with pellet density, size and age that were observed to influence the proportion of active mycelia [59].

Although the industrial cultivation conditions differ from the natural growth conditions the enzymes and production pathways for the desired compounds are the same, and therefore the knowledge of the natural processes is of high importance as well.

Lovastatin

One of the known, beneficial secondary metabolites of *A. terreus* is lovastatin (also known as mevinolin or monacolin K, Figure 2A). It is a polyketide with inhibitory activity against HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase which is the rate-limiting enzyme in cholesterol biosynthesis [20] and is therefore used as a pharmaceutical product to lower the serum cholesterol level.

The biosynthesis pathway of lovastatin contains two HR-PKSs, iterative type (LovB) and in fungi uncommon non-iterative type (LovF), together with two free-standing enzymes, LovC (enoyl reductase, ER) and LovD (acyl transferase, AT) [42, 43, 54]. After the discovery of its clini-

cal utility [20] the industrial production of lovastatin has been studied and the factors improving the production rates examined. Schimmel et al. [60] revealed that exogenous addition of butyrolactone I, another secondary metabolite of *A. terreus*, results in a threefold increase in the production of lovastatin and in an approximate twofold increase in the amount of produced sulochrin (an intermediate of (+)-geodin metabolite biosynthesis (Figure 2C, D [41, 64]) while addition of lovastatin caused insignificant decrease in sulochrin production under submerged and shaken cultivation conditions.

The kinetic effects of various nutrition, aeration and pH combinations as well as morphological development on the production of lovastatin by *A. terreus*, both in submerged shaken flask and large-scale bioreactor cultivation conditions, have been thorougly investigated. Bizukojc and Ledakowicz [62] comprehensively reviewed and concluded the preferable nutrient composition to include: (i) lactose, starch or glycerol as carbon source, ideally in fed-batch culture; (ii) amino acids as a complex organic nitrogen source, and to avoid ammonium and nitrates as well as higher nitrogen levels; (iii) exogenous beneficial compounds such as B-group vitamins, methionine as well as butyrolactone I. The optimal aeration level appears ambiguous due to the reported contradictory effects: both higher and lower oxygen concentrations in the used cultivation broths have been observed to increase the lovastatin production, whilst the appropriate pH value varies due to the impact of the nutrition contents. However, higher aeration was found to increase while slightly alkalic pH to decrease the co-biosynthesised but undesired (+)-geodin metabolite production. Obtaining more optimal lovastatin-producing cultivation conditions is further challenged due to the high carbon-dependency and the mutually beneficial nitrogen levels of both lovastatin and (+)-geodin production, thus complicating the fed-batch cultivations. The morphological association of lovastatin production is also more restricting: dependency on the amount of growing hyphae (i.e. smaller and active hyphal pellets) has more influence on lovastatin production than on (+)-geodin production; therefore lovastatin production is more growth phase-dependent. It initiates prior to the stationary phase while the continuation relies on the nutrient availability, in contrast to (+)-geodin biosynthesis, which occurs during the stationary phase with no correlation to the pellet development as concluded by Bizukojc and Ledakowicz [62].

Kumar et al. [65] reported that a fed-batch cultivation of an *A. terreus* strain DRCC 122 (a mutant of strain ATCC 20541) produced lovastatin at

2200 mg/l. The cultivation was performed in a bioreactor of 1000 litres containing 775 litres of production medium with glucose, maltodextrin, starch and lactose (peptonised milk) as carbon sources, and corn steep liquor, yeast extract and peptonised milk as nitrogen sources while corn steep liquor and maltodextrin were also added as the feeding substrate [65]. The production strategy was slightly controversial in comparison to the summarised contents of the beneficial nutrients as was remarked in the review by Bizukojc and Ledakowicz [62]. In a simplified approximation a volume of 0.5 l of the final fed-batch culture would be required to produce 1 g of lovastatin in the cultivation conditions applied by Kumar et al. [65]. Since the recommended dose of lovastatin in the treatment of cardiovascular diseases ranges from 20 mg up to 80 mg per day (as stated by the ratiopharm GmbH and STADA Arzneimittel AG in Germany) it would thus require approximately 10–40 ml of the final fed-batch culture to obtain a daily dose of a patient [65].

Butyrolactone I

Butyrolactone I, another valuable secondary metabolite of *A. terreus*, was discovered in 1977 by Kiriyama et al. [34] while in 2013 its lactone core with two aromatic benzene rings (Figure 2B) was revealed to be biosynthesised by a NRPS-like enzyme complex, BtyA [66]. The enzymes involved in the subsequent modification with attached methyl and prenyl groups have not yet been identified. Butyrolactone I has been reported to inhibit cyclin dependent kinases CDK1 and CDK2 [67, 68], and was subsequently observed as an antiproliferative compound against colon carcinoma cell lines [69].

In addition to the shown increase in secondary metabolism (lovastatin and sulochrin production), the supplementation of butyrolactone I was observed to cause morphological changes as well; enhanced hyphal branching and conidiation [60] in a similar manner as has been demonstrated for filamentous actinobacteria. In *Streptomyces* spp., small γ-butyrolactone-containing autoregulative signalling molecules have been observed to control secondary metabolite production as well as morphological differentiation, especially by the known regulator A-factor in *Streptomyces griseus* (Figure 2E) [70, 71], and is deeply reviewed by Horinouchi and Beppu [72]. Consequently, Schimmel et al. [60] raised a hypothesis of an analogous role for butyrolactone I in *A. terreus*.

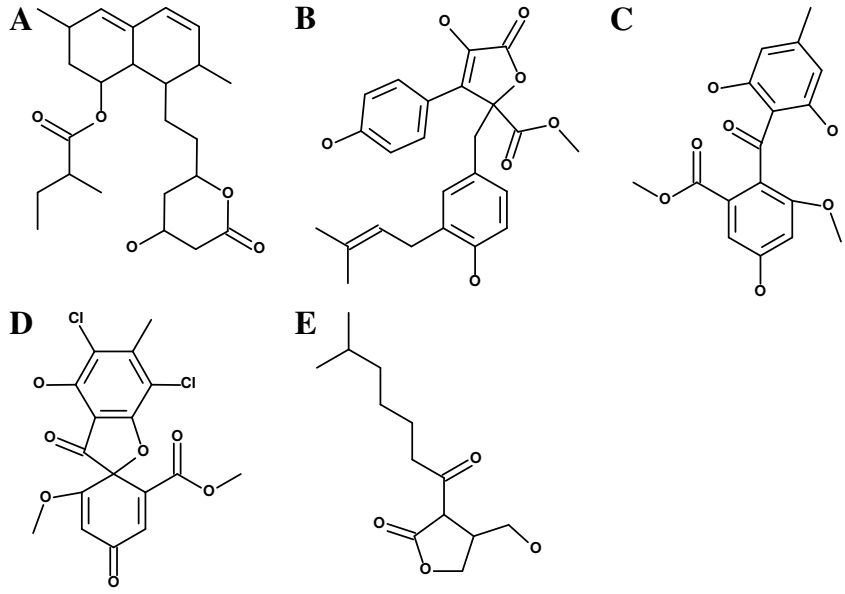

Figure 2: *A. terreus* secondary metabolites (**A**) lovastatin, (**B**) butyrolactone I, (**C**) sulochrin, (**D**) (+)-geodin and (**E**) the autoinducer A-factor of *S. griseus*.

1.3 Fungal growth phases

In contrast to the unicellular budding yeasts, e.g. *Saccharomyces cerevisiae*, numerous filamentous fungi have an elaborate growth cycle that begins with spore or conidia germination followed by subsequent polarised hyphal tip growth and frequent branching, whereby substantial mycelial growth is enabled. Under specific environmental conditions, such as intensity of light, nutrition and temperature, the growth and extension of hyphae may cease leading to mycelial differentiation including formation of conidia together with pigmentation [73]. From an industrial point of view, the duration of the stationary growth phase is of high importance. This phase begins through transition from the active hyphal growth and branching state to the stabilised state where the increase and decrease of the hyphal mass are in balance, and continues until the pre-autolysis begins during which the cultivation gradually reaches the autolysis phase. The pre-autolysis can be caused by nutritional depletion or internal toxic accumulation, for instance, and leads to an increase in hyphal differentiation, including fragmentation under submerged cultivation as comprehensively reviewed by Papagianni [61]. Submerged and shaken, or fed-batch bioreactor cultivation conditions with elongated stationary phase are widely applied in the production of useful secondary metabolites and

are thus optimised continuously. In *A. terreus* the industrial submerged growth has been observed to begin with an early hyphal pellet formation, indicating simultaneous conidia aggregation and germination leading to increase of pellet size with hyphal development from active apical to mature mycelial aggregates. When the pre-autolysis phase is reached, fungal secondary metabolism is altered with a decrease in lovastatin production [59]. In another industrially important fungus, *A. niger*, the autolysis phase of the submerged, carbon-deficient cultivation has been observed to induce conidiation and pigmentation [74, 75].

In addition to the optimisation of the submerged, industrially beneficial cultivation conditions by preventing or delaying the final steps of morphological development, investigating the role of conidiation in *A. terreus* is important for pathological reasons as well.

1.3.1 Fungal spore formation

In addition to conidia (also known as asexual mitotic spores), *A. terreus* is known to produce ascospores (sexual meiotic spores) [76] as well as unique, hyphal accessory conidia (aleurioconidia) which are often found in relation to *A. terreus* infections [77–79]. Furthermore, the role of asexual conidia of *A. terreus* in the invasive aspergillosis has been reported to differ from the role of asexual conidia produced by *A. fumigatus*. The *A. terreus* conidia were observed to retain their vitality, in contrast to germination, in the macrophages after phagocytosis even in immunocompetent hosts thus awaiting potential immunosuppression [80].

The pathways for formation of both asexual conidiation structures (conidiophores) and sexual fruiting bodies (cleistothecia) are scarcely studied in *A. terreus* while in the model filamentous fungus, *A. nidulans*, both asexual conidia and sexual ascospore formation has been studied more thoroughly. Under aerobic cultivation conditions and in the presence of light, limiting nutrional factors or environmental stress are generally the main factors that initiate asexual conidia formation with mycelial growth cessation. Subsequently, foot cell and stalk formation begins followed by stalk elongation. When the appropriate stalk length is reached, a vesicle is formed on the tip of the stalk enabling budding of numerous metulae with unique nuclei. Each of the metulae gives rise to specific phialides that produce chains of conidiophores through mitosis followed by conidia maturation (Figure 3) [reviewed in 73, 81].

On the genetic level, three known core transcription factor proteins of asexual conidia formation — BrlA, AbaA and WetA — control these

central steps in chronological order [82–87]. BrlA is required in the initiation of conidiation, i.e. in the emergence of vesicles and metulae on the tips of the formed stalks [84]; AbaA controls the formation of phialides [85]; WetA is involved in the maturation of conidia and in the sustainment of their viability [86, 87] together with a trehalose biosynthesis enzyme, TpsA [88].

Velvet family of proteins

In *Ascomycota* the coordination of conidiation and sexual ascospore formation together with secondary metabolism has been observed to involve a unique group of regulative proteins and transcription factors of the *velvet* gene family. In *Aspergillus* species this family appears to consist of four proteins — VeA, VelB, VelC and VosA — with specific Velvet domains and a global regulator LaeA with a DNA methyltransferase domain. In *A. nidulans* these family members have been more thoroughly investigated and have been found to form diverse homo- and heterodimeric complexes as well as one heterotrimer, at least, with specific functional roles in the regulation of mycelial development and secondary metabolism. Under solid cultivation conditions, hyphae has been observed to contain multimeric complexes, such as: VelB–VeA, VelB–VeA–LaeA, VelB–VelB, VosA–VosA and a putative VelC–VosA complex. In phialides and conidia, a VosA–VelB complex has been found [55, 58, 89–92].

The Velvet domain in the corresponding regulatory proteins is conserved in filamentous fungi of *Ascomycota* and *Basidiomycota* phyla and appears to have a unique amino acid sequence found only in the proteins of the Velvet complex. The suggested transcription factor-like functions of these Velvet proteins were confirmed by Ahmed et al. [93]. In *A. nidulans*, the Velvet domain of VosA (one of the Velvet complex proteins) was shown to bind directly to the target DNA, specifically to the promoter regions of the target genes. The crystal structures of VosA and the VosA–VelB dimer have been resolved and the Velvet domain was reported to be reminiscent of a DNA-binding domain (RHD) of a known mammalian transcription factor (NFκB) although no significant sequence similarity was observed. The Velvet domains were also observed to be involved in the dimerisation of VosA and VelB proteins [93].

The changes in the cultivation conditions, including light and dark environment, have been observed to affect both the formation of these different Velvet complexes and their mycelial locations. These variances were shown to either induce or inhibit secondary metabolism and/or mycelial

differentiation, thereby enabling the balance within the occurrence of secondary metabolism, mycelial differentiation ranging from hyphae to sexual ascospore or asexual conidia formation, as well as pigmentation [55, 58, 89–92, 94, 95]. These regulative functions are visualised in the Figure 3 with solid arrow lines representing the reported steps in regulation of conidiation whereas the dotted arrow lines indicate further deductions based on the reported observations by Park et al. [91] and Sarikaya Bayram et al. [90].

In *A. terreus* only LaeA of this *velvet* gene family has been studied through heterologous expression of the *A. nidulans* LaeA orthologue and was observed to increase lovastatin production [55], while the predicted genes encoding the orthologues of the Velvet complex members as well as the endogenous LaeA have not been in focus. Intriguingly, Schimmel et al. [60] reported exogenous butyrolactone I to enhance conidiation of *A. terreus* under submerged cultivation conditions while neither LaeA nor Velvet complex members were included in the study.

1.3.2 Melanins and pigments in *Ascomycota*

One of the antagonistic responses of filamentous fungi of the phylum *Ascomycota* to hostile habitat conditions, such as UV irradiation or unfavourable pH, is formation of melanins and other pigments on the conidia and ascospores. Several fungi of *Ascomycota* phylum produce pigments with polyketide synthase (PKS) enzyme complexes followed by enzymatic tailoring and potential polymerisation of the compounds. Diverse melanin types omitting PKS enzymes in their biogenesis pathways have been observed as well [96].

Thus far numerous pigments and their specific morphological locations together with their biogenesis and polymerisation pathways amongst filamentous fungi remain still undiscovered. The *Fusarium* genus of *Ascomycota* has been reported to contain several pigments generated by NR-PKS enzyme complexes: red pigments (i) bikaverin and (ii) perithecial fusarubin (located in perithecia, one form of sexual fruiting bodies) in *Fusarium fujikuroi* [97, 98], and (iii) a red pigment aurofusarin and (iv) a black perithecial pigment (PGL1) in *Fusarium graminearum* [99–101], for instance. Intriguingly, the two pigments of *F. fujikuroi* have been observed to be produced in contrasting cultivation conditions, being either alkalic or acidic [98].

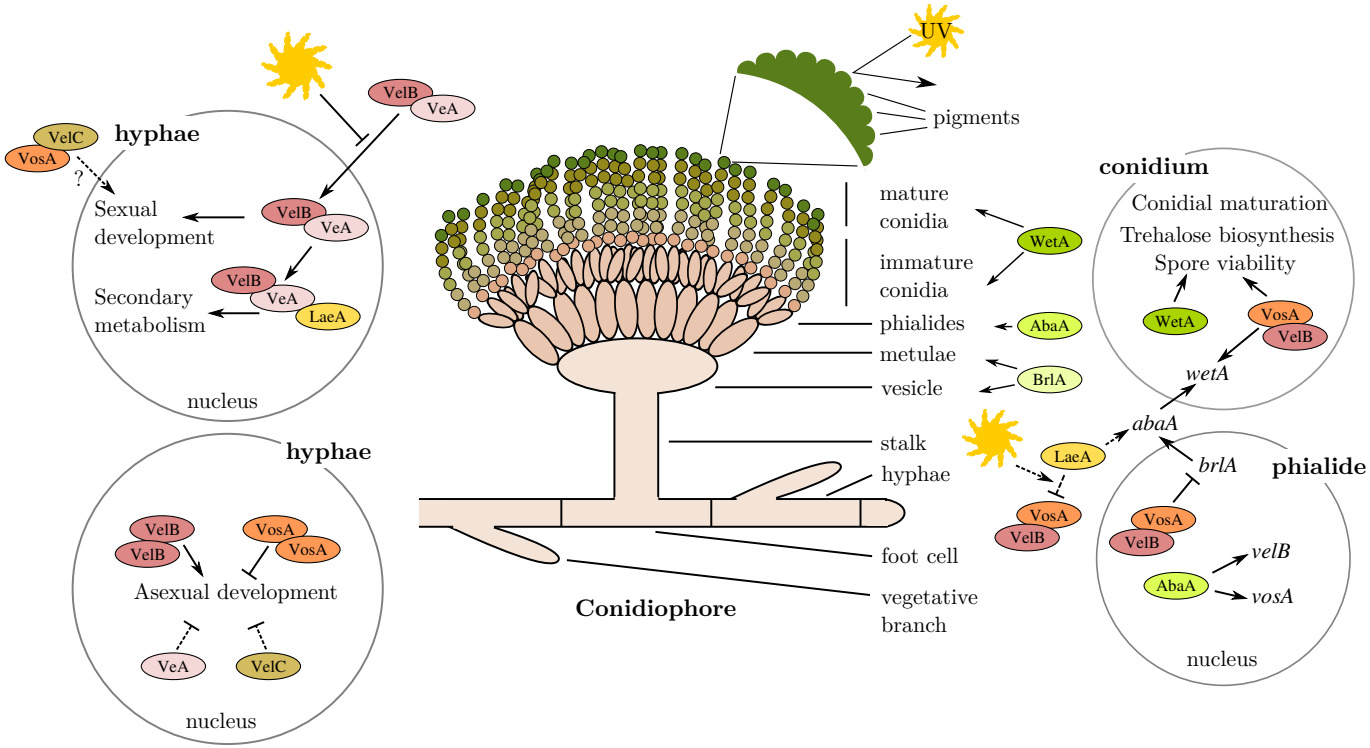

Figure 3: A schematic overview of *A. nidulans* conidiophore structure and the proposed regulative roles of the key factors of the Velvet complex influencing asexual development and conidiation. Adapted from Figure 1 in Publication III and [90, 102].

Melanin synthesis pathways are also divided into two different classes (DHN or *L*-DOPA) depending either on the type of pathway intermediates or on the inhibition of a certain enzyme in the pathway. The biosynthesis pathway of DHN (1,8-dihydroxynaphthalene) class includes hydroxynaphthalene intermediates and hydroxynaphthalene reductase(s), in addition to a PKS [103, 104]. The effective reductase inhibitors used in the classification include tricyclazole, phthalide and pyroquilone [103]. The *L*-DOPA type of melanin biosynthesis includes a tyrosinase enzyme with precursors tyrosine or 3,4-dihydroxyphenylalanine (*L*-DOPA) and is inhibited by kojic acid and tropolone [105, 106], prior to the commonly occurring polymerisation [96, 107].

In the genus *Aspergillus*, the DHN-type of conidial pigmentation in *A. fumigatus* is well studied and contains a typical gene cluster with the common NR-PKS (Alb1) along with two reductase enzymes (Arp1 and Arp2) both of which can be inhibited with tricyclazole [104]. In *A. terreus* an uncommon Asp-melanin, derived from aspulvinone E, together with its biogenesis pathway containing an NRPS-like MelA enzyme and a tyrosinase TyrP, was recently discovered [66, 108]. However, no PKS-involving pigmentation pathway has been found in *A. terreus* while Pal et al. [109] reported indications of potential presence of a DHN-type pigment by using the inhibitors tricyclazole and phthalide.

1.4 Cell–to–cell communication in microbiota

In both gram-negative and -positive bacteria the production of species-specific or interspecies, and selfregulative or autoinducive molecules have been observed to increase until a certain concentration threshold is reached, indicating that the corresponding cell density and required growth phase are reached as well [70, 71, 110]. This is a process known as quorum sensing, a form of cell–to–cell communication. These signalling molecules include acyl-homoserine lactones (AHL), with a lactone ring linked to a methionine moiety which is bound to acyl side chains of diverse length; autoinducer-2 family (AI-2) with e.g. furanosyl borate diesters; small ring-formed oligopeptides; and γ-butyrolactones that are common in filamentous bacteria such as *Streptomyces* species. These γ-butyrolactones are similar to AHLs apart from the methionine module as can be seen in the autoinducer A-factor of *S. griseus*, for instance (Figure 2E on page 2) [reviewed in 111].

One of the features of the quorum sensing phenomenon is the stimulation of the biogenesis of the signalling molecule itself, thereby initiating a

positive feedback loop. This enables the synchronised cell–to–cell communication to initiate both common and species-specific responses corresponding to the achieved cell density and growth phase. These responses include secondary metabolism (e.g. antibiotic carbapenem biogenesis in *Erwinia carotovora* [112, 113]); conjugal transfer (e.g. *Agrobacterium tumefaciens* resulting into tumorigenesis, crown gall disease in plant hosts [114, 115]); morphological development together with wide secondary metabolism (e.g. *S. griseus* [70, 71, 116]); and bioluminescence (symbiotic *Vibrio fischeri* [117]). Additional factors affecting the initiation of quorum sensing response(s) include the availability of receptor(s), while the response timing has been reported to vary independently to the time point of exogenous supplementation of the specific autoinducer to a mutant lacking the autoinducer synthesis [118].

Fungal cell–to–cell communication has also been investigated and some similar processes have been observed in few species. In a dimorphic fungus *Candida albicans* a 15-carbon oxylipin, farnesol, as well as farnesoic acid (the corresponding fatty acid-like molecule) have been shown to suppress the transition from yeast cell budding to hyphal growth. Another cell–to–cell communication compound, tyrosole, was shown to have a contradictory activity, i.e. to enhance hyphal growth. Both of these processes occurred in a quorum sensing manner with relation to the achieved cell density [119–121]. In the filamentous fungus *A. nidulans* certain oxylipins (derived from oleic and linoleic fatty acids) have been reported to mediate the balance between asexual conidia and sexual ascospore formation as well as to affect production of the secondary metabolites sterigmatocystin and penicillin [122–128]. In *A. flavus* the presence or absence of linoleic acid-derived oxylipin affected the switch between conidia to sclerotia formation in a mycelial density dependent manner, in addition to influencing the biogenesis of aflatoxin. Furthermore, oxylipins were suggested to be involved in host–pathogen communication through the oxylipin pools of the host plants [124, 129, 130]. In *A. terreus* the production of the secondary metabolite, lovastatin, has been observed to be increased by exogenous supplementation of linoleic acid at the beginning of the submerged cultivation, presumably at low mycelial density [131]. However, no further mycelial density related studies were performed.

Altogether, oxylipins appear to play a role in the cell–to–cell communication to coordinate morphological development and production of secondary metabolites in a presumably common manner in filamentous fungi as proposed by Tsitsigiannis et al. [127] and Tsitsigiannis and Keller

[128]. Intriguingly, Schimmel et al. [60] revealed a secondary metabolite of *A. terreus*, butyrolactone I, to influence the morphology and production of the secondary metabolites lovastatin and sulochrin in the same species, i.e. *A. terreus*, in a reminiscent manner as has been reported to occur in bacterial species of *Streptomyces*, as reviewed by Horinouchi and Beppu [72]. However, further effects of butyrolactone I on its own biogenesis or on the *A. terreus* transcriptome have not been studied.

The aims of this study

The main purpose of this study was to elucidate the regulative role of the metabolite butyrolactone I in the secondary metabolism and mycelial development of the filamentous fungus *A. terreus*. Chromatographic and in-depth transcriptional studies were applied during the different growth phases in submerged cultures for a nine-day long cultivation in order to characterise the biotechnological usage of this fungus. Originally to fulfil the major aim, the central plan was to conduct a genome-wide microarray gene expression study, utilizing the at-that-time newly sequenced and computationally annotated genome of *A. terreus* (Broad Institute [132]), in parallel with a time-specific butyrolactone I and lovastatin biogenesis study. This original aim unfolded into the following sections during the project maturation:

(i) To build a transcriptional overview of the effects of exogenous butyrolactone I on *A. terreus* submerged cultivation, by a microarray gene expression study containing all the supplemented and control sample "snapshots" [Publication I].

(ii) To identify and quantify in detail the produced butyrolactone I utilizing high performance liquid chromatography and tandem mass spectrometry in order to re-examine and confirm the previously, in Publication I, observed effects of supplemented exogenous butyrolactone I on its concentration — both on intra- and extracellular levels [Publication II].

(iii) To focus on the potential secondary metabolism- and mycelial development-related effects of the exogenous butyrolactone I on *A. terreus* gene expression during the submerged cultivation, through re-analysing the more reliable microarray results, which were obtained by whole transcriptome sequencing of the cultivated strain (MUCL 38669). This enabled to estimate the reliability of the used microarray probes, whose design was based on annotated genes on the published genome of the *A. terreus* NIH2624 strain (PRJNA15631) [Publications III, IV].

(iv) To examine the structures of the obtained transcripts amongst the secondary metabolism- and mycelial development-related genes, within the sequenced transcriptome and under the influence of exogenous butyrolactone I, thereby providing potential investigation targets involved in the transcriptional regulation [Publication III].

Materials and methods

3.1 Cultivation conditions

A. terreus MUCL 38669 was obtained from CABI Biosciences UK Centre, Surrey, United Kingdom. This strain was maintained on yeast and malt extract (YME) agar slants at 28 °C for 7 days after which it was stored at 4 °C. It was inoculated into a medium of corn steep liquor, tomato paste, oat flour, dextrose and trace element solution, followed by cultivation under agitated conditions in submerged flask cultures using GPY-L medium (consisting of glucose, peptonised milk, yeast extract and lactose) as described in Publication II. These conditions were applied throughout the thesis study and in all Publications I–IV.

3.2 Experimental design

Aspergillus terreus strain MUCL 38669 was cultivated for 216 hours in three experimental sets, to which exogenous butyrolactone I (100 nM, i.e. 42.5 ng/ml, in final concentration) was supplemented at 24 hours (set 1), 96 hours (set 2) and 120 hours (set 3) post-inoculation (p.i.), as well as in one control set where no butyrolactone I was supplemented. Each of the sets (both experimental and control) were cultivated in three biological replicates and sampled at 24 hours, 48 hours, 96 hours, 120 hours, 144 hours and 216 hours p.i. as described in Publication II (Figure 4). This experimental setup was utilised throughout the thesis study and in all Publications I–IV. The approximate growth phases defined in this thesis are partially based on the observed increase in mycelial density (mycelial dry weight, CDW) up to the reached plateau of biomass accumulation at 48 hours p.i. [Publication I, Additional File 2]. The designation is also based on the submerged cultures of *A. terreus* as described by Bizukojc and Ledakowicz [59]. Exponential growth (*a*): rapid increase in mycelial density with active accumulation of mycelial pellets; Transition phase (*b*): proceeding from exponential to the stationary growth, i.e. a metamorphosis (termed by Bizukojc and Ledakowicz [59]) during which the actively growing apical mycelia on the pellet surface develop to form hyphae in the pellet cores as determined by the observed stabilisation of the biomass accumulation; Stationary growth (*c*): the maintenance of the mature pellets with the hyphal cores that were formed during the transition phase as deduced by the continuously stable plateau of the biomass amount [Publication I, Additional File 2]; Pre-autolysis (*d*): preparation to less-favourable conditions for fungal growth. These modelled phases of mycelial development in *A. terreus* are also based on the illustrative

description of pellet development by Bizukojc and Ledakowicz [59]. The approximated lengths of these phases were re-defined according to the results obtained from Publications I–IV. These above assigned phases of pellet development will be used in the forthcoming chapters and sections.

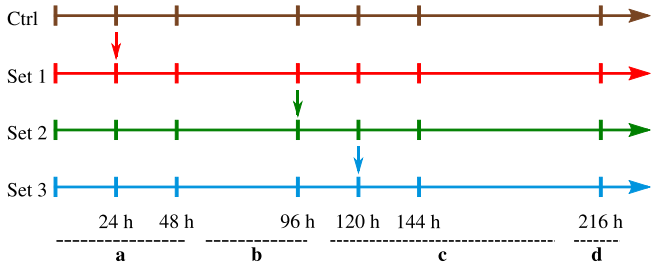

Figure 4: A schematic description of the experimental design of the phases of mycelial growth of *A. terreus* under the cultivation conditions adopted in the thesis study. The time points of the exogenous butyrolactone I supplementation are indicated with arrows. The approximate submerged cultivation growth phases are indicated with dashed lines as follows: (**a**) exponential, (**b**) transition, (**c**) stationary and (**d**) pre-autolysis phase.

3.3 Chromatographic and tandem mass spectrometric analyses

Butyrolactone I was extracted with methanol, in duplicate, from both the mycelial pellets and the culture supernatants of the cultivation sets 1, 2, 3 and control, identified using liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) and quantified using both high performance liquid chromatography (HPLC) and LC-ESI-MS/MS instruments (Table 1). The used chemicals, extraction procedures, details of the used instruments and equipment, as well as detection and quantification parameters are described in Publications I, II.

3.3.1 Quantification

The butyrolactone I quantification with LC-ESI-MS/MS was based on the signal areas for four main transitions (*m/z* 307, 331, 363, 393) and on a concentration curve of external butyrolactone I standard. The quantification analysis with HPLC was based on the obtained external butyrolactone I concentration curve as well. The two sample student's *t*–test by using the language and environment R [133] was applied to evaluate the statistical significance of the time-point specific differences in the butyrolactone I concentration between the control and supplemented sets obtained with HPLC and LC-ESI-MS/MS [further described in Publication II].

Table 1: Specifics of the butyrolactone I detection and quantification procedures using LC-ESI-MS/MS and HPLC.

	LC-ESI-MS/MS [a]	**HPLC** [b]
Instrumentation	Agilent Technologies 1200 Rapid Resolution LC; Bruker Daltonics High Capacity Trap Ultra Ion Trap MS with electrospray (ESI) ion source	Dionex, Ultimate 3000 HPLC
Column	Agilent Zorbax Eclipse XBD-C8; 5.0 µm particles, 4.6 mm × 150 mm	Dionex, Acclaim 120; C18 5.0 µm particles, 4.6 mm × 150 mm
Extraction solvent	CH_3OH	CH_3OH
Mobile phase	CH_3CN with 0.1 % CH_2O_2, and H_2O	55:45 ratio of CH_3CN : H_2O with 0.1 % H_3PO_4 [c]
Gradient	10 % to 90 % CH_3CN	-
Flow rate	1 ml/min	1 ml/min [c]
Injection volume	1 µl	25 µl [c]
Scan range, with auto MS/MS mode	*m/z* 220 to 500	-
Detection wavelength	-	308 nm

[a] further detailed in Publication II
[b] further detailed in Publication I
[c] Same parameters as applied by Sorrentino et al. [131]

3.4 Transcriptomics

Genome-wide differential gene expression analysis and transcriptome sequencing of *A. terreus* strain MUCL 38669 were performed under the same cultivation conditions with butyrolactone I supplementation and sampling time points as displayed in Figure 4 [detailed in Publication III, File S1]. The total RNA used in both of these experiments was extracted from the snap-frozen mycelia samples derived from the six time points of each of the three biological replicates of all cultivation test sets and control [described further in Publication I, Supplementary File 1].

3.4.1 Genome-wide gene expression studies

Custom Agilent 4 $\times$ 44K format microarray slides were designed on the basis of sequenced *A. terreus* strain NIH2624 genome [132] using Agilent eArray application. The resulting arrays contained four 60 bp oligonucleotide probes per gene, two replicate pairs with different 3' biased mRNA locations. The one-colour labelling of the extracted RNA was conducted as per manufacturer Agilent's protocols and resulted into hybridisation of labelled cRNA of one biological replicate per one array, i.e. providing triplicate arrays [detailed in Publication I, Supplementary File 1].

Raw data quality, normalisation and analysis

The statistical computing language and environment R and the linear models for the microarray data (limma package) [133–137] were used in the normalisation and statistical analysis of the obtained microarray data. The gene expression analysis was diverged into two separate pathways (*i* and *ii*) due to the nucleotide-level differences between strains MUCL 38669 (the strain in focus) and NIH2624, genome of which was used in the probe design. The time-point specific and statistically significant differential gene expression was determined through $\log_2$ transformation and subsequent moderated *t*-statistics calculation with adjusted *p*-value for multiple testing in both of these analysis pathways [further details in Publication I, Supplementary File 1].

(*i*) Preliminary normalisation and analysis prior to transcriptome sequencing [Publication I] The normalised microarray probe signal intensities resulted in non-optimal normal distributions of the intensity values (Figure 5A). The lower limits contained inconstant values amongst the sample microarrays and were significantly closer to the second quartiles

(Q2; median) in comparison with the higher limits. In addition, the first quartiles (Q1) were smaller in comparison with the third quartiles (Q3). The standard algorithms of the limma package were applied in the analysis, except for an additional Pearson correlation-based refinement due to the detected inconsistent gene expression profiles of probe duplicates. If the correlation coefficient between the expression patterns of duplicate probes was above 0.7, the corresponding differential gene expression results were included in the further analysis [detailed in Publication I, Supplementary File 1].

(*ii*) Normalisation of the extracted, more reliable raw data and the final analysis [Publication III] The gene expression analysis was improved through performing a series of additional, uncommon bioinformatic steps utilizing BLASTN software [138] together with the sequenced transcriptome data prior to the normalisation, linear modelling and analysis of the gene expression data in order to verify the reliability of the previously designed probes. The probe reliability evaluation was based on specific accuracy criteria for the alignment of the 60-mer probe sequences with the sequenced transcripts of strain MUCL 38669. Only the probes that fulfilled the criteria were considered reliable and were thus included in the further steps [detailed in Publication III, File S1]. The normalisation of the signal intensities of these filtered probes resulted into more optimal normal distributions of the signal intensities (Figure 5B). The second quartiles (Q2; median) of the normalised signal values were more equally distributed between the lower and higher limits as well as the first and third quartiles (Q1 and Q3, respectively), in comparison with the preliminary normalisation quartiles (Figure 5A). Furthermore, the lower limit values remained constantly on the same intensity levels and the number of lower outliers was smaller than in the unfiltered, normalised data. All of this enables a more reliable comparative analysis of the normalised probe signal intensities to obtain more valid differential gene expression results.

Due to the probe filtering the presented differential gene expression values ($\log_2$FC) are the median of either four or two probe replicates. The error bars represent the maxima and minima of these values. The differential gene expression is considered statistically significant if at least one of the probe replicates fulfils the criteria (adjusted p-value ≤ 0.05 and $|\log_2\text{FC}| \geq 0.5$) regarding the three biological replicates.

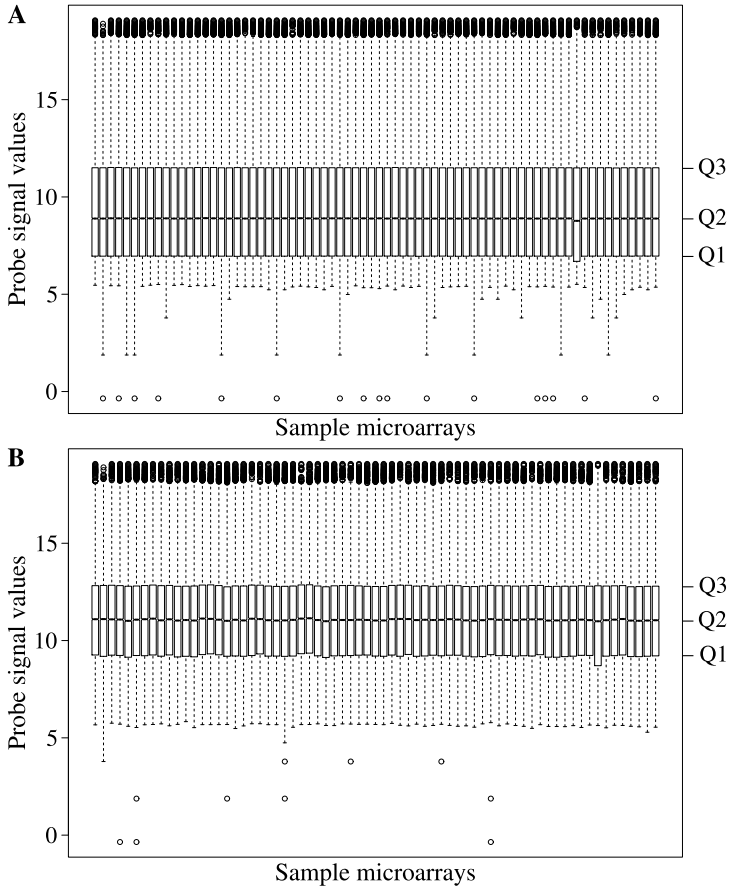

Figure 5: The microarray probe signal intensities represented in quartiles after (**A**) normalisation alone (analysis pathway *i*), (**B**) sequence-based filtering followed by normalisation (analysis pathway *ii*). Q1 indicates the first quartile, i.e. the intensity value of the 25^{th} percentile of the ranked values; Q2 the second quartile, i.e. median (50^{th} percentile); Q3 the third quartile, i.e. the 75^{th} percentile. The ends of dashed lines indicate the lower and higher limit values, while the outliers are indicated with circles [modified Figures S2 and S3 of Publication III].

3.4.2 Strand-specific transcriptome sequencing

The extracted total RNA from all of the six mycelia samples of the cultivation set 3 where butyrolactone I had been supplemented at 120 hours p.i. (Figure 4 on page 20) was pooled prior to the strand-specific transcriptome sequencing in order to obtain a sufficient amount of RNA. The strand-specific double-stranded cDNA (ds cDNA) was synthesised using a combination of three protocols provided by Parkhomchuk et al. [139], Marioni et al. [140] and Levin et al. [141] together with the standard Illumina mRNA preparation protocol in which an essential excep-

tion was included. The exception consisted of the usage of uracil-N-glycosylase to digest the second strand with incorporated dUTPs. The acquired strand-specific RNA was sequenced with Illumina GAII with the paired-end reads protocol [detailed in Publication III, File S1].

Transcriptomic sequence assembly and analysis

The sequenced strand-specific paired-end reads were trimmed to remove multiplex adapters, PCR primers and low quality regions, after which the quality of the obtained reads was evaluated resulting into over 36.4 million trimmed, paired-end reads with Phred score medians above 35, i.e. with an estimated base accuracy above 99.97 % (Table 2).

Table 2: Quality information of the obtained paired-end (PE) read sequences [modified Table S1 of Publication III].

	Number of reads	**Median read length (bp)**	**GC%** [a]	**Phred score median** [b]	**Over-represented reads** [c]
Raw PE1	18752589	101	55	≥ 35.0	0
Raw PE2	18752589	101	55	≥ 33.5	0
Trimmed PE1	18204865	101	55	≥ 36.5	0
Trimmed PE2	18204865	101	55	≥ 35.2	0

[a] GC %: Guanine and Cytosine nucleotide proportion in the read sequences
[b] Per base sequence quality; Phred score: $-10 \times log_{10} P$, where P is a probability of an incorrect base call
[c] A read is overrepresented if the number of its duplicates > 0.1 % of total number of read sequences

The strand-specific transcriptome assembly of the good quality reads was begun with a *de novo* step followed by an alignment of the obtained transcripts to the genome of strain NIH2624, after which the aligned transcripts were assembled and concatenated with the *de novo* transcripts. The resulting transcripts were re-aligned strictly to the NIH2624 genome and re-assembled with a crucially divergent AMOS pipeline (instead of the prevalent pathway) in order to preserve the nucleotide differences between the strains MUCL 38669 and NIH2624. The assembly resulted into an estimated sequence coverage of 88, consisting of over 40000 transcripts with N50 value of 1796 bp (Table 3). See Table 4 for the used software and File S1 in Publication III for all the sequencing and assembly details.

Table 3: Primary details of the assembled transcriptome [modified Table S2 of Publication III].

Total number of transcripts [a]	41695
Total length of the assembly [a]	41.92 Mbp
Transcript minimum length [b]	125 bp
Transcript maximum length [b]	13056 bp
Transcript N50 length [c]	1796 bp
Approximate sequence coverage [d]	88

[a] The assembly contains also putative splice variants and antisense sequences
[b] The length of transcript contigs
[c] The lowest transcript length of the upper half of the nucleotide sum of the length-sorted transcripts
[d] Total number of trimmed reads (36.4×10^6) with median length of 101 bp / assembly length (Mbp)

Further analysis of the transcriptome (pooled cultivation set 3) included (*i*) visualisation of both aligned read sequences and the assembled, re-aligned transcripts to the annotated genome of NIH2624, (*ii*) calculation of an approximate median coverage (represented as counts) of the sense and antisense reads with the alignment window of 25 bp over the NIH2624 genome, and (*iii*) quantification of the aligned reads resulting into a pooled value for the number of fragments per kilobase of exon per million reads mapped (pooled FPKM) (Figure 8 on page 32, full FPKM list is in Publication III, File S2, available online at http://www.mdpi.com/2076-2607/5/1/12). In the transcript-specific analyses several web servers were used to confirm the predicted gene structures, translate into proteins as well as search and analyse presumed gene orthologues. The phylogenetic studies were conducted using the online web server (Phylogeny.fr [142, 143]) which was set to utilise following programmes and parameters: multiple alignment software MUSCLE, PhyML programme with maximum likelihood method, similarity matrix type WAG and bootstrapping. The used software and web servers are summarised in Table 4 and detailed in Publication III, File S1.

Table 4: Used software and web servers in the transcriptome sequencing, assembly and further analysis.

Software or Web Server	Version	Function	Ref.
Illumina's Pipeline Analysis	NA	Base-calling and quality determination	NA
FASTX-Toolkit	0.0.13	Read trimming	[144]
FastQC	0.10.1	Read quality evaluation	[145]
Trinity *de novo*	trinityrnaseq _r2012-03-17	Transcriptome *de novo* assembly	[146–148]
Genome-Guided Trinity	_r2013-02-25	Genome-guided transcriptome assembly	[147–149]
GSNAP	2012-07-20 v3	Genomic alignment of reads	[150, 151]
GMAP	2012-07-20 v3	Genomic alignment of transcripts	[150, 152]
PASA Pipeline	PASA2-r20130605	(i) Assembly of the aligned transcripts (ii) Concatenation with *de novo* transcripts	[153–155]
BLAT	35×1	Stringent genomic re-alignment of the concatenated transcripts	[156, 157]
Minimo Assembler of AMOS Assembler Pipeline	3.1.0	Re-assembly of the re-aligned and concatenated transcripts	[158, 159]
Integrative Genomics Viewer (IGV)	IGV_2.3.26	Visualisation of the genomic alignment of transcripts and reads	[160–162]
Igvtools of IGV	IGV_2.3.26	Calculation of read coverage median (counts)	[160–162]
Cuffquant and Cuffnorm of Cufflinks Tools	2.2.1	Quantification and normalisation of the mapped reads (FPKM)	[163]
GENSCAN Web Server	NA	Exon-intron structure prediction	[164–166]
Web Server of ExPASy	NA	Putative protein translation	[167, 168]
NCBI CDD, InterProScan Web Servers	NA	Search of predicted protein domains	[169–173]
NCBI BLASTP Web Server	NA	Amino acid-based alignment search tool	[174]
Web Server Phylogeny.fr	NA	Construction of phylogenetic trees on protein level	[142, 143]
PKS/NRPS Analysis Website	NA	Domain extraction of PKS and NRPS enzymes	[175]

Results

4.1 Butyrolactone I biogenesis

The chromatographic quantitative results are described in detail in Publication II.

4.1.1 Identification of butyrolactone I

Butyrolactone I was consistently identified in all of the samples of *A. terreus* submerged culture (both mycelial cell pellets and culture supernatants, taken at 24, 48, 96, 120, 144 and 216 hours p.i.) as determined using tandem mass spectrometry (LC-ESI-MS/MS) in order to fragment the molecule into a pattern specific to butyrolactone I. The protonated ion form of butyrolactone I (*m/z* 425) was found to be fragmented into four main daughter ions (*m/z* 307, 331, 363 and 393), two of which presumably correspond to a cleavage of hydroxyphenyl or O-methyl group from butyrolactone I (Figure 6). The distinct signals of the four fragments [Figure 1 in Publication II] were used in the subsequent butyrolactone I quantification in all of the samples and biological replicates along with external butyrolactone I standards.

4.1.2 Quantification of the intracellular butyrolactone I production

While the butyrolactone I was quantified with both HPLC as well as LC-ESI-MS/MS instruments, more descriptive intracellular results were revealed using HPLC. They had a lower level of observed variance among the biological replicates. The reason behind the obtained larger variance in the MS/MS-based quantification may be matrix interference (ion enhancement/suppression phenomena). However, the consistent MS/MS fragments confirmed the concentration range obtained by HPLC. The observed intracellular butyrolactone I concentrations revealed supplementation time point-specific enhancement patterns in the butyrolactone I production: the concentration was statistically significantly increased after 24 hours of the supplementation in all of the treated cultivation sets (1, 2 and 3). Furthermore, the enhanced production continued up to or at least for 96 hours long periods when butyrolactone I had been added at 24 hours or 120 hours p.i., respectively, resulting into 2-fold respective 1.8-fold statistically significant increases, at the most, in the butyrolactone I concentration. When butyrolactone I was added at 96 hours p.i. the statistically significant increase was 1.6-fold 24 hours after the supplementation. The endogenous butyrolactone I concentration in the untreated control set remained approximately on the same level (between 100 and

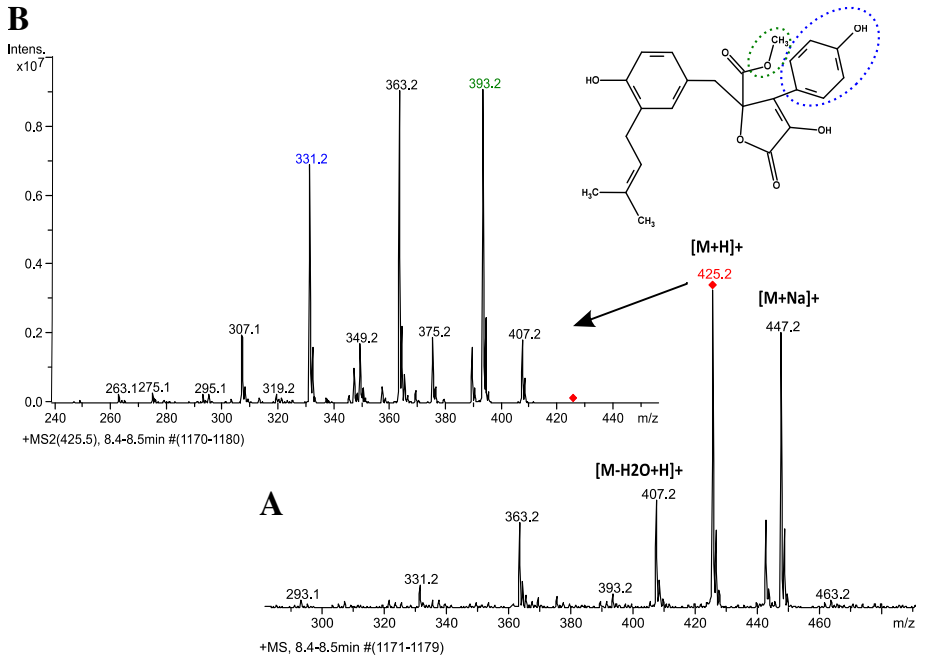

Figure 6: (**A**) MS and (**B**) MS/MS spectra for butyrolactone I identification. The calculated and indicated cleavage groups of butyrolactone I correspond to fragments *m/z* 331 and 393, and were used in the butyrolactone I quantification together with two additional fragments *m/z* 307 and 363 [modified Figure 2 of Publication II].

126 µg/ml) throughout the cultivation after achieving its constant level at 48 hours p.i. (Figure 7).

4.1.3 Quantification of the secreted butyrolactone I

All of the extracellular butyrolactone I concentration patterns measured with HPLC and LC-ESI-MS/MS are very similar regardless of the supplementation time point (Figure 7B and Publication II, Figure 3A), whereas they appear to be characteristically different when compared to the butyrolactone I concentration patterns derived from mycelia (Figure 7A). A distinct time point-specific concentration peak occured at 48 hours p.i. in each of the cultivation sets, including the control with values *ca*. 49 µg/ml and 42 µg/ml measured with LC-ESI-MS/MS and HPLC, respectively. Nevertheless, remarkable and statistically significant increases in the butyrolactone I secretion (3.5- and 2.3-fold) that resulted from exogenous butyrolactone I supplementation at 24 and 120 hours p.i. occured during the stationary growth phase (at 120 and 144 hours p.i., respec-

tively) (Figure 7B and Publication II, Figure 3A).

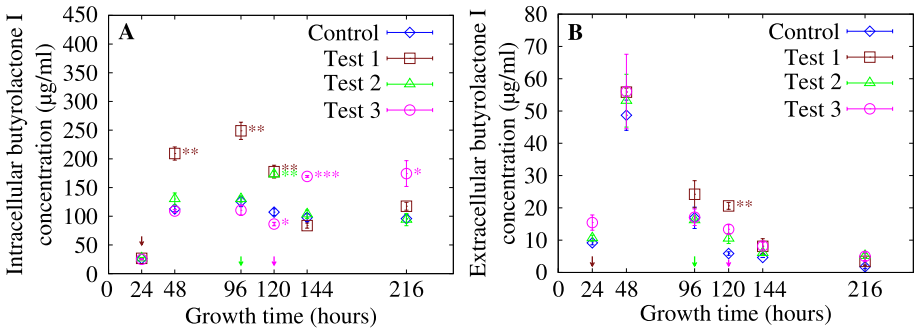

Figure 7: Butyrolactone I concentration in the (**A**) mycelia pellets and (**B**) culture supernatants as measured with (**A**) HPLC and (**B**) LC-ESI-MS/MS. Butyrolactone I was supplemented at 24 h (Test 1), 96 h (Test 2) and 120 h p.i. (Test 3) as indicated with arrows. The error bars represent the standard error of the mean of three biological replicates. Statistically significant difference levels are denoted as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The slight decrease in set 3 at 120 hrs p.i. is likely caused by biological variation. [modified Figures 3 and 4 of Publication II]

4.2 *Aspergillus terreus* transcriptome

The detailed gene expression and transcriptomics results are described in Publications I, III and IV, and in the including supplementary materials. Publication I contains the preliminary microarray gene expression results, whereas Publications III and IV contain the final microarray results improved with the sequenced transcriptome results.

4.2.1 Transcriptional influence of butyrolactone I

Overview of aligned transcript quantities

Further analysis of the assembled transcripts (obtained from the pooled samples of test set 3) through normalisation and quantification resulted into a dominative accumulation range of $0 - 500$ FPKM with 69 % of the annotated (*A. terreus* strain NIH2624) genes being expressed (FPKM ≥ 1) at least at one of the six sampling time points of the set 3 cultivation. There is however an extensive deviation in the FPKM range: one highly enriched transcript at the quantile percentile 1, amounting to 5 % of the total number of expressed transcripts (Figure 8). This explains at least partially the calculated read quality results indicating high read duplication level, while no overrepresented reads were present (Table 2 on page 25 and Table S1 in Publication III).

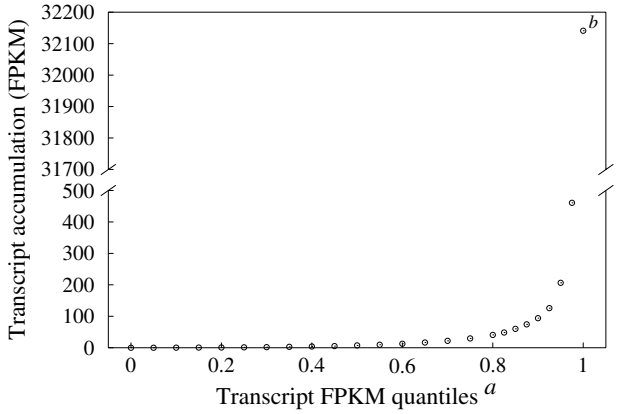

Figure 8: Distribution of the transcript accumulation (FPKM) over the quantile percentiles of the number of sequenced gene transcripts [modified Figure S1 of Publication III]. [a] The quantile intervals were 0.05 up to the percentile 0.8, after which the intervals were 0.025 up to the percentile of 1. [b] 5 % (32141 FPKM) of the total sum of the normalised amount of transcripts (633388 FPKM).

Overview of the large-scale gene expression

Comparison of the differential expression results of the separate pathways (*i*) and (*ii*) The statistically significant differential gene expression results of the preliminary analysis pathway (*i*) contained 2155 probes that displayed statistically significant differential gene expression at 24 hours after butyrolactone I supplementation in at least one of the test sets 1, 2 or 3, where butyrolactone I had been supplemented at 24, 96 and 120 hours p.i., respectively. Approximately 6 % (123 probes) of these showed statistically significant regulation in at least two of the test sets as visualised in the Figure 9, diagram A. In a similar manner with the same statistical significancy criteria and time points, the more reliable, final gene expression analysis pathway (*ii*) revealed a smaller amount of probes (1066) showing differential, statistically significant gene expression in at least one of the test sets at 24 hours post-supplementation. Of these approximately 3 % (30 probes, 19 genes) displays regulation in two test sets at 24 hours after butyrolactone I supplementation (Figure 9B). A closer look on the predicted domains of these translated, significantly regulated genes revealed several categories for their predicted functions. The most numerous categories in both of the analysis pathways (*i* and *ii*) included membrane-associated roles, protein degradation, secondary metabolism and transcriptional factors (Table 5).

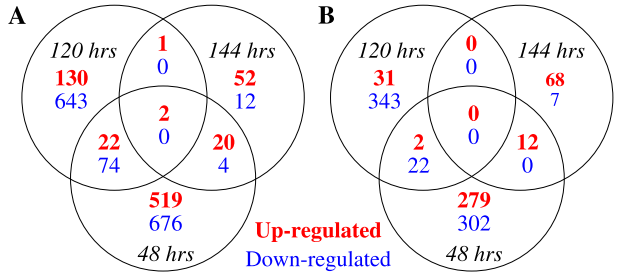

Figure 9: Growth phase related distribution of genes showing statistically significant differential expression (adjusted p-value ≤ 0.05 and $|\log_2 FC| \geq 1$) at 24 hours after butyrolactone I addition, i.e. at 48 hours (set 1), 120 hours (set 2) and 144 hours (set 3) p.i. These diagrams are based on the gene expression results obtained through (**A**) the preliminary (*i*) and (**B**) the final (*ii*) analysis pathways. The diagram **A** is a corrected version of the Figure 5 of Publication I whereas the diagram **B** is based on the gene expression and sequencing results presented in Publication III.

Table 5: Categories of the inferred protein functions of the differentially expressed genes (visualised in the Figure 9) with statistical significance under the influence of butyrolactone I (adjusted p-value ≤ 0.05 and $|\log_2 FC| \geq 1$) in at least two test sets and at 24 hours post-supplementation. This table is based on the Table 2 of Publication I (***i***) and the gene expression results of Publication III (***ii***).

Category	**(*i*) Preliminary analysis**		**(*ii*) Final analysis**	
	Number of		**Number of**	
	probes	**genes**	**probes**	**genes** [a]
Membrane-associated	21	15	5 [b]	3 [b]
Nucleocytoplasmic transport	-	-	2	1
Post-transcriptional processes	4	3	-	-
Primary metabolism	-	-	7	4
Protein degradation	11	7	4	4
Protein processing	-	-	2	1
Pseudouridinylation/ telomere-related	8	5	-	-
Ribosome-related	17	8	1	1
Secondary metabolism	6	4	2	1
Transcriptional factors	7	5	4	2
Virulence	6	5	-	-

[a] Some of the genes showed alternative splicing
[b] Plasma membrane transport/trafficking

4.2.2 Effects of butyrolactone I on the regulators of conidiation and secondary metabolism

The key conidiation regulating gene orthologues of *A. terreus* — *brlA*, *abaA* and *wetA* — showed similar gene expression profiles regardless of the growth phase, at which exogenous butyrolactone I was supplemented, exponential (set 1), transition (set 2) or stationary (set 3) (Figure 10 and Publication III, Figure S5). The remarkable pattern of the profiles can be seen during the stationary growth phase, i.e. after 120 hours p.i., culminating at the pre-autolysis where the last sample snapshot (at 216 hours p.i.) appears to be the most significant in all of the test sets. The first of the regulator genes — *brlA* — encoding a transcription factor involved in the first phase of conidiation is increasingly downregulated by butyrolactone I and reaches the highest state of downregulation at the pre-autolysis phase with a statistically significant $\log_2$ fold change below -3 in set 1 (Figure 10). The transcripts of *brlA* were also expressed and amounted to a lower level (1.5 FPKM) in the snapshots of set 3 when compared to the following conidiation regulators encoding genes *abaA* and *wetA*. The second key regulator encoding gene — *abaA* — was statistically significantly upregulated at the same time point (216 hours p.i., all sets) as *brlA* was downregulated, and the *abaA* transcripts amounted also to a higher level with pooled FPKM value of 7 (set 3). The gene that encodes a conidial

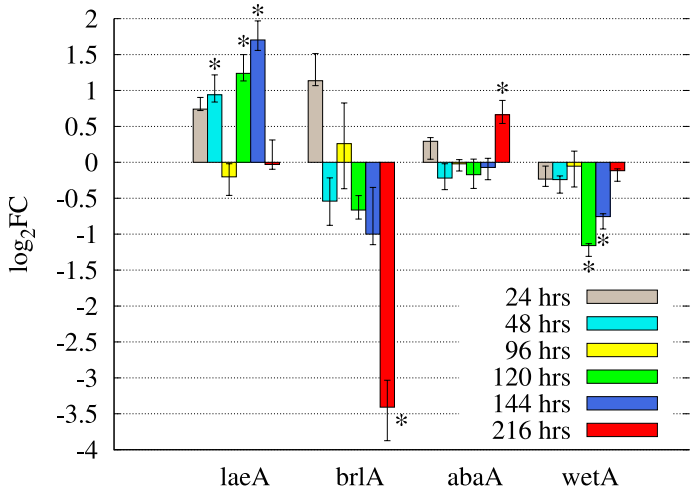

Figure 10: Butyrolactone I affects the gene expression of the global regulator *laeA* and the conidiation key regulators *brlA*, *abaA* and *wetA* when supplemented at 24 hours p.i. in final concentration of 100 nM (test set 1) [modified Figure 6 of Publication III]. * Adjusted p-value ≤ 0.05 and $|\log_2 \mathrm{FC}| \geq 0.5$.

maturation regulator — *wetA* — reached an unregulated stage after the preceding repression during the stationary growth phase (all sets). Moreover, the transcripts of *wetA* and the gene encoding a conidia viability improving enzyme — *tpsA* — amounted to even higher levels of FPKM, 13 respective 270 (set 3), amongst these genes of the key regulators and conidiation improving enzymes (Figure 10 and Table 6).

The gene encoding the global regulator orthologue — *laeA* — that has been shown to play a role in the secondary metabolism and mycelial development in *A. nidulans* [55, 58, 90] was positively regulated during the stationary phase in all sets, showing statistical significance at 120 hours (sets 1 and 2) and at 144 hours p.i. (set 1) with a $\log_2$ fold change above 1.5. Remarkably, the gene expression at the transition and pre-autolysis growth phases appears to remain rather unregulated or slightly up- or downregulated by butyrolactone I in each of the sets (1, 2 and 3). The expression during transition phase shows similarity to the key conidiation regulators, whereas both stationary and pre-autolysis phases display a contrasting pattern (Figure 10 and Publication III, Figure S5)

Table 6: The total amount of pooled transcripts regarding the genes involved in the conidiation and secondary metabolism (SM) regulation under the influence of butyrolactone I in test set 3 [modified Table 3 of Publication III].

Gene	Pooled FPKM	Pooled counts [a] max		Presumed cellular function [b]
		sense	antisense	
brlA	1.5	0.78	0.00	vesicle and metulae budding
abaA	7.0	2.9 [c]	0.11	phialide formation
wetA	13	3.8 [c]	0.25	conidia maturation
tpsA	270	77 [c]	0.98	conidia viability improvement
laeA-α	6.8	2.1 [c]	0.12	global SM regulator [d,e]
veA	0.83	0.45	0.057	*velvet* family member [e]
velB	120	62 [c]	0.64	*velvet* family member [e]
velC	NA	1.3 [c]	0.062	*velvet* family member [e]
vosA-α	110	73 [c]	0.66	*velvet* family member [e]

[a] represents coverage medians of pooled RNA samples with a 25 bp window over the NIH2624 genome
[b] as described for *A. nidulans* [55, 58, 82–87, 89–92, 94, 95]
[c] complete coverage
[d] reported for *A. terreus* and *A. nidulans* [55]
[e] involved in the conidiation and SM regulation
NA Not available

4.2.3 Transcript variance under the influence of butyrolactone I

The sequenced transcripts revealed intriguing structures with alternative splicing both inside and upstream of the open reading frames (ORF). These splice variants appeared to occur especially amongst the orthologues of conidiation and secondary metabolism related regulators — *veA, velB, velC, vosA* and *laeA*. A further analysis of these transcripts revealed several updates to the annotation of these genes in the strain NIH2624. Regarding the global regulator encoding gene *laeA*, the first intron inside the ORF showed alternative splicing with *ca.* 36 % presence of the intron (Figure 11) leading to two transcripts with ORFs of different length. Within both of these ORFs an absence of a second intron (present in the annotation of strain NIH2624) and a one-nucleotide deletion were observed. In addition, there appears another intron showing alternative splicing in the upstream untranslated region (UTR) of both *laeA*-α and *laeA*-β (Figure 11A) [Publication III].

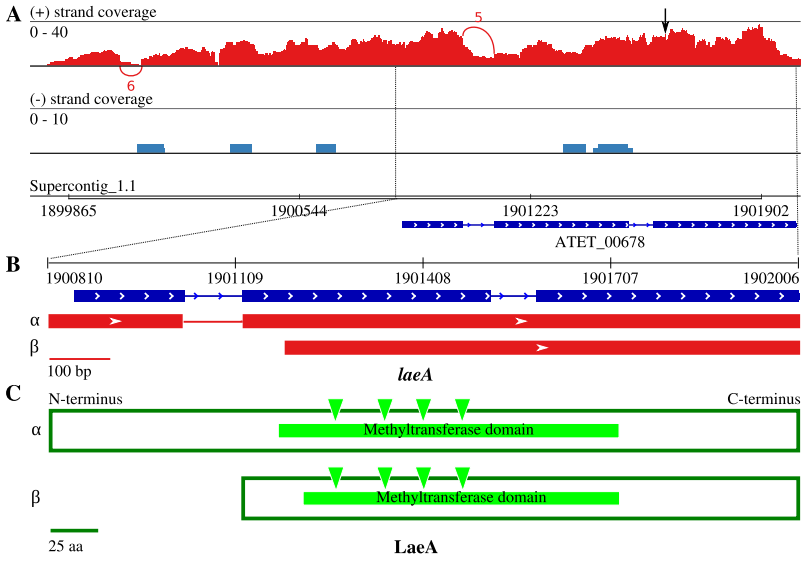

Figure 11: Splice variants and the resulting ORFs of the global regulator *laeA* orthologue. (**A**) Aligned read coverage over the sense and antisense strands of the supercontig_1.1 of strain NIH2624 genome. The curved junctions give the number of reads supporting the presence of an intron. The black arrow shows the location of a 1 bp deletion. (**B**) An enlarged scheme of the two resulting ORFs, *laeA*-α and *laeA*-β. (**C**) A scheme of the translated *laeA* splice variants with the predicted protein motif with the SAM-binding sites indicated with triangles [modified Figure 5 of Publication III].

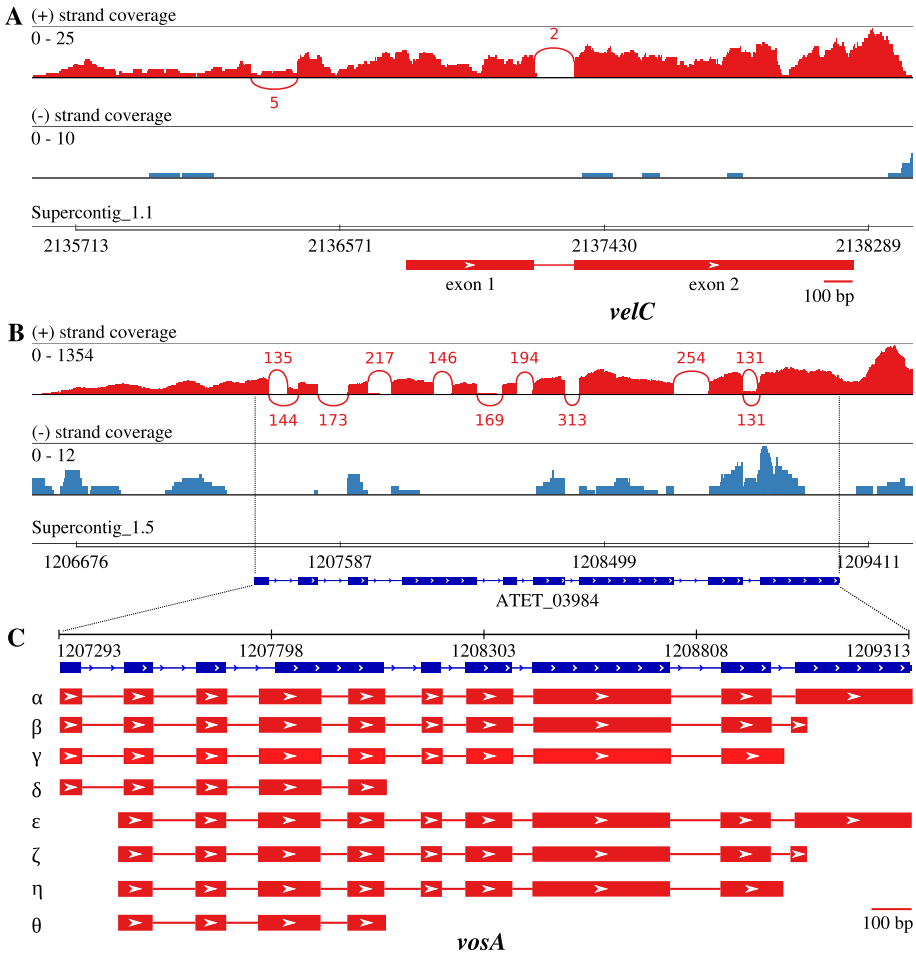

Figure 12: (**A**) One of the *velvet* family members, *velC*, was revealed to be located on the supercontig_1.1 of *A. terreus* NIH2624 genome. (**B**) The numerous splice variants of the *velvet* family member *vosA*. The read coverages of both sense and antisense strands are shown above the corresponding genomic region. The curved junctions indicate the number of reads supporting the presence of an intron. (**C**) An enlarged schematic figure describing the splice variants of the *vosA* trancripts [modified Figures 2 and 4 of Publication III].

Regarding the *velvet* family members — *veA, velB, velC* and *vosA* — the sequenced transcripts revealed structural differences between the corresponding annotated genes of *A. terreus* strain NIH2624, whereas the orthologues of the specific conidiation related genes — *abaA, wetA* and *tpsA* — show similar transcript structures to the strain NIH2624. One of the *velvet* family members, *velC*, was expressed, and therefore revealed

in the cultivation conditions used in this thesis, leading to an annotation update of the *A. terreus* genome (Figure 12A). Another member, *veA*, appears to have a shorter ORF than annotated due to the sequence coverage over a predicted intron, while *velB* displays a minor transcript update in its ORF length [Figures 2A, B and 4A in Publication III]. Of the *velvet* family members, *velC* and *velB* show alternative splicing on their upstream UTR regions thus having no effect on their ORF lengths (Figure 12A on page 37 and Figure 4A in Publication III). The most complicated structure amongst the members was discovered for the regulator *vosA*, leading to eight possible splice variants (α, β, γ, δ, ε, ζ, η and ϑ). Approximately the variants *vosA*-α and *vosA*-β are the most prevalent forms within the *A. terreus* cultivations in this thesis study (Figure 12B, C) [Publication III].

4.2.4 The effect of butyrolactone I on a new NR-PKS gene cluster and Asp-melanin biogenesis genes

Within the assembled transcriptome results, one only partially annotated PKS gene cluster was expressed in the submerged cultivation conditions applied throughout the studies. The cluster contained nine genes, seven of which encode potential tailoring enzymes (genes *pgmB*, *pgmC*, *pgmD*, *pgmE*, *pgmF*, *pgmG* and *pgmH*) as well as one putative transcription factor (gene *pgmR*) and a suggested core PKS gene *pgmA*. Although this PKS gene was only partially covered, the wide genomic alignment region indicated a significantly longer gene than was annotated for the strain NIH2624 (Figure 13). The observed length was later computationally confirmed.

Furthermore, the surrounding clustered genes were expressed on a higher level, as revealed by the pooled FPKM values ranging from 1.1 up to 290, except for the putative transcription factor (gene *pgmR*) (Table 7). A few of the sequenced transcripts of the surrounding cluster genes revealed also annotation updates: *pgmC* was indicated to code a longer ORF together with the computationally predicted transcription factor *pgmR* while *pgmG* was revealed to contain an additional intron with no effect on the ORF length [Figures S1 and S2 in Publication IV]. The translation of the suggested ORF of the *pgmA* gene resulted into a nonreducing PKS with a non-canonical protein domain architecture amongst *Aspergillus* spp., with the following motifs: Starter unit:ACP transacylase (SAT), beta-ketoacyl synthase (KS), acyl transferase (KS), acyl transferase (AT), polyketide product template (PT), two successive acyl carrier domains (ACP) and thioester reductase (R) domain (Figure 13B) [Publi-

cation IV].

Within these updated *pgm* cluster genes, several of them appeared to be regulated by exogenously added butyrolactone I in an intriguing pattern: a specific response culminating in the pre-autolysis phase in each of the test sets (1, 2 and 3) in a similar manner to the gene expression profiles of the key conidiation regulator genes *brlA, abaA* and *wetA*. The majority of these previously unstudied genes of the PKS cluster (*pgmB*, *pgmD*, *pgmE*, *pgmF* and *pgmH*) show statistically significant upregulation at 192 hours after the butyrolactone I supplementation, i.e. at 216 hours p.i. (Figure 10 on page 34, Figure 14 on page 40, Figure S5 in Publication III and Figure 3 in Publication IV).

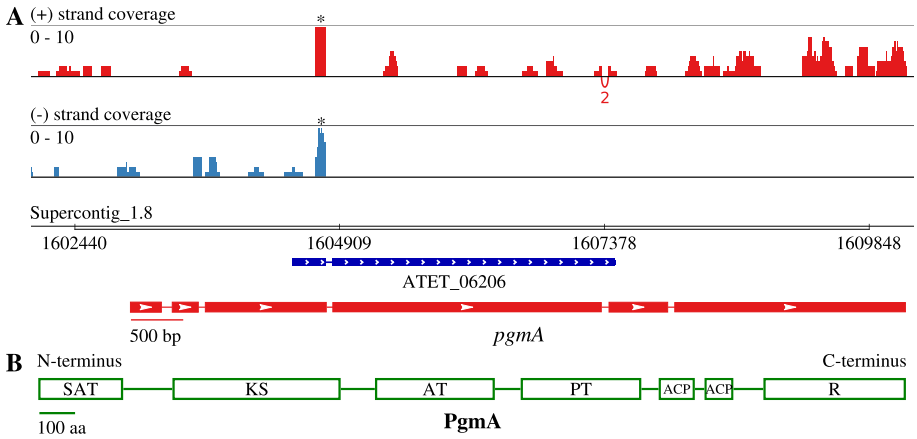

Figure 13: (**A**) Strand-specific read coverage of the suggested transcript of NR-PKS *pgmA* over the genomic region of *A. terreus* strain NIH2624. The curved junction indicates the number of reads supporting the presence of an intron. (**B**) Predicted protein domain architecture of the translated *pgmA* gene: Starter unit:ACP transacylase (SAT), beta-ketoacyl synthase (KS), acyl transferase (KS), acyl transferase (AT), polyketide product template (PT), acyl carrier (ACP) and thioester reductase (R) domain. *An incorrect alignment of a short part of a longer transcript of another gene located on the same supercontig of *A. terreus* NIH2624 genome [modified Figure 1 of Publication IV].

While *abaA* showed upregulation by butyrolactone I at the pre-autolysis phase, the known in the conidiation involved Asp-melanin producing genes *melA* and *tyrP* [66, 108] showed statistically significant downregulation at the same time point (at 216 hours p.i.), and were down- or unregulated throughout the six sampling time points of the test set 1 (Figure 14). However, the total transcript amounts of these genes with pooled FPKM values of 6.0 (*melA*) and 1.3 (*tyrP*) indicate these genes to been expressed at least at one of the sampling time points of the test set 3 (Table 7 and detailed results in Publications III and IV).

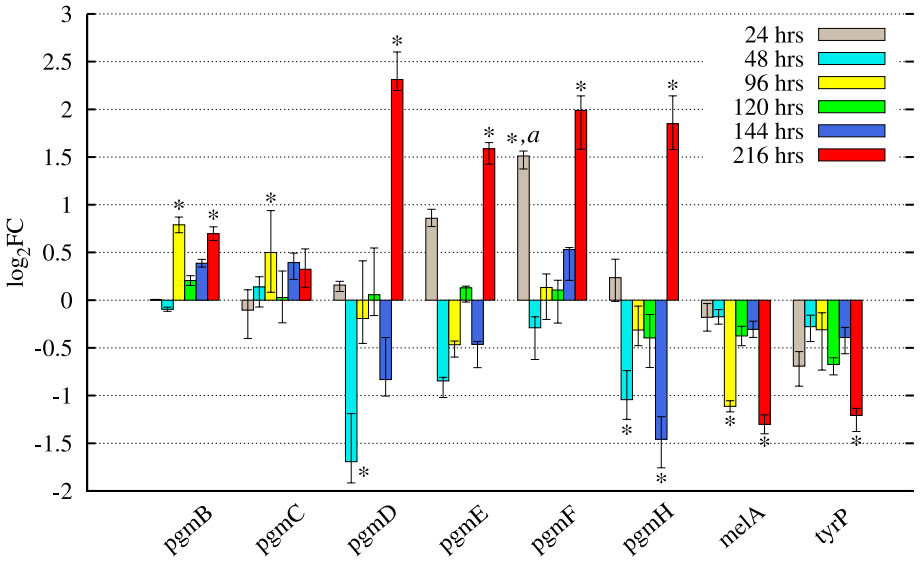

Figure 14: The gene expression patterns of *pgm* gene cluster and Asp-melanin biosynthesis genes *melA* and *tyrP* under butyrolactone I influence in submerged cultivation conditions. Butyrolactone I was supplemented at 24 hours p.i. in final concentration of 100 nM (test set 1) [modified Figure 3 of Publication IV]. * Adjusted *p*-value $\leq$ 0.05 and $|\log_2 FC| \geq 0.5$. [a] The apparently statistically significant upregulation at 24 hours p.i. may not be biologically significant due to the low cultivation density at the start of exponential growth.

Table 7: The total transcript amount of *pgm* cluster genes and the known Asp-melanin producing genes *melA* and *tyrP* under the influence of enhanced butyrolactone I biogenesis in test set 3, and the suggested protein functions based on the domain prediction [modified Tables 1 and 2 of Publication IV].

Gene	Pooled FPKM	Pooled counts max. [a]		Predicted molecular function
		sense	antisense	
pgmB	3.9	1.3 [b]	0.00	O-methyltransferase
pgmC	1.1	0.34	0.00	Cytochrome p450 monooxygenase
pgmR	0.13	0.12	0.095	Aflatoxin biosynthesis regulatory protein-like
pgmA	0.65	0.45	0.25	Nonreducing polyketide synthase; NR-PKS
pgmD	4.7	2.5 [b]	0.11	Short-chain dehydrogenase/reductase
pgmE	15	4.8 [b]	0.19	SAM-dependent methyltransferase
pgmF	290	95 [b]	4.4	Quinone reductase
pgmG	56	4.2 [b]	0.65 [e]	MFS family permease
pgmH	12	18 [b]	0.42	FAD/FMN-binding CO dehydrogenase
melA	6.0	2.1 [b]	0.23	Nonribosomal peptide synthetase-like (NRPS) [c]
tyrP	1.3	0.69	0.00	Tyrosinase [d]

[a] represents coverage medians of pooled RNA samples with a 25 bp window over the NIH2624 genome
[b] complete read coverage
[c] shown by Guo et al. [66] and Geib et al. [108]
[d] shown by Geib et al. [108]
[e] due to the overlapping 5' end of *pgmF* transcript (see Fig. S2 of Publication IV)

4.2.5 The similarity of the Pgm cluster members to the pigment biogenesis cluster of *Fusarium fujikuroi*

In Publication IV this uncharacterised NR-PKS enzyme, PgmA, was revealed to have significant similarity (59 % ID) to a known NR-PKS (Fsr1) that produces a perithecium pigment fusarubin of an *Ascomycete Fusarium fujikuroi* [98]. A following phylogenetic study of the similarity of PgmA to other NR-PKS enzymes amongst *Ascomycota* indicates PgmA to belong to a special NR-PKS group of similar enzymes within *Fusarium* spp., which are known to produce perithecial pigments and share the same domain architecture (SAT-KS-AT-PT-ACP-ACP-R) as is predicted for PgmA (Figures 13 on page 39 and 15 on page 43). Furthermore, *F. fujikuroi* contains also a gene cluster of six genes (*fsr1-6*) involved in the fusarubin pigment biosynthesis [98], three of which show similarity with the *pgm* cluster genes on protein level. Specifically the putative O-methyltransferase PgmB, quinone reductase PgmF and the predicted transcription factor PgmR show amino acid identities of 41, 26 and 31 % with the corresponding tailoring enzymes Fsr4, Fsr6 and Fsr2 of fusarubin cluster, respectively. Two of the putative cluster tailoring enzymes, cytochrome p450 monooxygenase (PgmC) and short-chain dehydrogenase/reductase (PgmD), share predicted domains or motifs with Fsr3 respective Fsr5 although no notable similarity on amino acid level was observed [Table 2 in Publication IV].

Taken together, the domain structures of this *A. terreus* Pgm cluster and the *F. fujikuroi* perithecium pigment cluster Fsr, along with the several NR-PKS enzymes involved in the perithecium pigmentation of *Fusarium* spp. (Figure 15) show mutual similarity and, furthermore, the gene expression profiles of both the core conidiation regulator genes (Figure 10 on page 34) and the *pgm* cluster genes (Figure 14 on page 40) show mutual similarity as well.

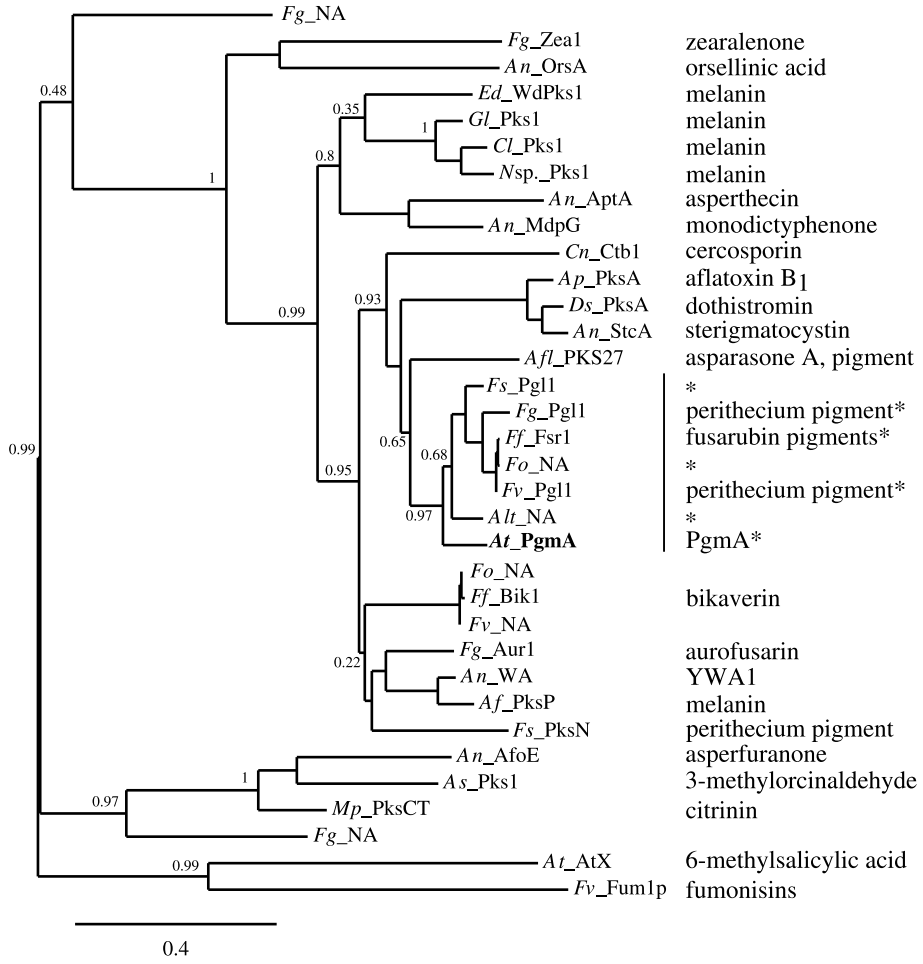

Figure 15: A phylogenetic tree of various 34 NR-PKS enzymes of *Ascomycota* phylum based on the predicted and extracted KS domains of the translated proteins. The biogenesis products are given on the right side of the corresponding NR-PKS. The organisms presented in the phylogram: *Af; A. fumigatus, Afl; A. flavus, Alt; A. lentulus, An; A. nidulans, Ap; A. parasiticus, As; Acremonium strictum, At; A. terreus; Cl; Colletotrichum lagenaria, Cn; Cercospora nicotianae, Ds; Dothistroma septosporum, Ed; Exophiala dermatitidis, Ff; Fusarium fujikuroi, Fg; F. graminearum, Fo; F. oxysporum, Fs; F. solani, Fv; F. verticillioides, Gl; Glarea lozoyensis, Mp; Monascus purpureus, N*sp.; *Nodulisporium* spp. strain ATCC74245. * PKS domain structure is: SAT-KS-AT-PT-ACP-ACP-R. NA; No specific annotated gene name available. Figure 2 in Publication IV contains the accession numbers for the NR-PKS enzymes included in this schematic figure.

Discussion

5.1 Butyrolactone I — a hormone-like signaling molecule in *Aspergillus terreus*

5.1.1 Autoinduction and potential quenching

Positive feedback loops in the butyrolactone I biogenesis

In bacteria several quorum sensing signalling molecules with selfregulative and autoinducing features contain lactone ring moieties and have been studied in several bacterial microbes including the soil-dwelling *Streptomyces* genus. The 13-carbon γ-butyrolactone (Figure 2E on page 9) A-factor of *S. griseus* has been found to be autoinduced during the exponential growth phase and to regulate the production of a secondary metabolite streptomycin and the morphology of the filamentous bacteria in question [70, 71, 116, 176, 177], reviewed by Horinouchi and Beppu [72]. Similarly, the quantification of butyrolactone I in Publication II revealed an increase during the exponential growth phase, both in the extra- and intracellular fractions of the control and supplemented cultivations. In contrast to the decrease in the extracellular butyrolactone I concentration in all sets when the growth is turning into the transition phase, the intracellular concentration increased remarkably and remained on the achieved level in all sets, except for the set 1 where butyrolactone I showed even higher statistically significant increase during the shift to the transition phase (see Figure 7 on page 31). The exogenous supplementation at the transition (set 2) and stationary (set 3) growth phases resulted into a subsequent intracellular increase 24 hours after the supplementation, both during the stationary growth. All of this indicates (*i*) the autoinduction of butyrolactone I biogenesis and (*ii*) occurrence within all of the mycelial differentiation stages preceding autolysis of the submerged cultivation of the filamentous fungus *A. terreus*.

The length of autoinduction *versus* growth phase. The length of the artificial butyrolactone I autoinduction stage appeared to be dependent on the growth phase at which it was supplemented: when added within the exponential or stationary growth it continued for 96 hours at least, and may thus indicate elongated positive feedback loop for the butyrolactone I biogenesis. The increase in the butyrolactone I production was more transient in the beginning of the stationary phase (Figure 7A on page 31) when butyrolactone I had been supplemented during the transition from the coagulated pellet growth to the maturation and maintenance of the core pellet hyphae.

In bacteria the autoinducer accumulation threshold correlates with the achieved cell density and induces the subsequent regulatory cascade (investigated in *Streptomyces* spp., for instance [reviewed in 72]). In *A. terreus* the enhanced early autoinduction stage continues over the transition phase until the beginning of the stationary growth phase, and ends simultaneously with the more transient artificial autoinduction stage. This emphasises the importance of the achieved mycelial density and growth phase in the potential butyrolactone I feedback regulation and in the subsequent change(s) in the signalling cascade(s) under these growth conditions (Figure 7A on page 31).

What initiates the rapid decrease in the extracellular butyrolactone I amount?

The important role of the autoinducers in the quorum sensing phenomenon amongst bacteria is to represent the species-specific cell density and it has been reported to occur through diffusion or secretion of the continuously produced autoinducer molecules to the cellular environment [178]. While the intracellular butyrolactone I concentration profiles (Figure 7A on page 31) imply the significant autoinduction activity in all of the growth phases, the measured extracellular profiles of both control and supplemented test sets demonstrate that the actual mycelial density maximum has been reached at 48 hours post-inoculation (Figure 7B on page 31), in accordance with the corresponding biomass accumulation. These extracellular profiles are also reminiscent of the bacterial quorum sensing autoinducer production profiles of *S. griseus, A. tumefaciens* and the supernatant curve of *E. carotovora* submerged culture [71, 179, 180]. Furthermore, the concentration range of quantified extracellular butyrolactone I, both in the control and supplemented sets, appeared to be remarkably lower than the corresponding intracellular range. The extracellular butyrolactone I amount of the control cultivation was revealed to contain 27 % of the total amount of butyrolactone I during the exponential growth while the amount during the stationary phase was < 10 % (Table 8). This might indicate inhibition of the butyrolactone I secretion by the extracellular butyrolactone I. However, supplementation during the exponential and stationary growth phases (i.e. at 24 and 120 hours p.i.) resulted in statistically significant but transient increase in the butyrolactone I secretion during the stationary growth phase, analogously to the intracellular autoinduction phases, during which positive feedback presumably occurs and may cause an excess in the butyrolactone I pro-

duction kinetics (Figure 7 on page 31 and Figure 3 in Publication II).

Table 8: The ratio (%) of the extracellular butyrolactone I concentration to the total (intra- and extracellular) concentration per sampling time point in the untreated control cultivation [based on Publication II].

Sampling time point (p.i.)	24 hrs	48 hrs	96 hrs	120 hrs	144 hrs	216 hrs
Extracellular conc. / Total conc.	27 %	27 %	8 %	7 %	5 %	5 %

Quorum quenching? The reason behind the difference between the butyrolactone I intra- and extracellular concentration profiles and levels, both in the control and supplemented cultivations, is currently not known. However, the low extracellular concentration level of butyrolactone I may indicate a quorum sensing-related behaviour, quorum quenching. In bacteria the circumstances that lead to or appear as quorum quenching activity concerning autoinducers with a lactone moiety include at least: (*i*) secretion or diffusion of the autoinducer (e.g. *Pseudomonas aeruginosa* [178]), (*ii*) enzymatic degradation of the autoinducer (lactonase-mediated) as observed in *A. tumefaciens* [179, 181] and the concurrent intracellular prevention through receptor binding [182] together with the suggested moderation of the autoinducer accumulation [183], (*iii*) enzymatic extracellular interspecies degradation (AHL-acylase mediated as reported for one strain of *Streptomyces* spp. [184]), (*iv*) inhibition of the autoinducer biogenesis (as observed in *S. griseus* [177]) and (*v*) non-enzymatic degradation, e.g. lactonolysis (i.e. opening of the lactone ring) caused by alkalic conditions and/or high temperature (e.g. *P. aeruginosa, Yersinia pseudotuberculosis* and *E. carotovora* [180, 185]).

Secretion or enzymatic degradation? Two of these factors, (*i*) secretion or diffusion, and (*ii*) the enzymatic degradation together with a simultaneous receptor binding may be involved in the submerged cultivation of *A. terreus* as indicated by the observed butyrolactone I concentration profiles. The cooperation of these factors (*i*) and (*ii*) might result into a signalling cascade including positive feedback loops, regulation of secondary metabolism, conidiation as well as suggested pigmentation (described further in the forthcoming sections and in Publications III and IV).

In accordance with the factors (*i*) and (*ii*), the whole-genome microarray gene expression analysis revealed four transport-related genes, one secondary metabolism-related gene and two transcriptional factors to be statistically significantly regulated 24 hours after butyrolactone I supplementation simultaneously in two test sets (at 48 and 120 hrs p.i. or at 48 and 144 hrs p.i.) as displayed in Figure 9B on page 33 and in Table 5(*ii*) on page 33. All of this would explain the apparent depletion of extracellular butyrolactone I if the receptor binding occurred intracellularly, and would thus inhibit the possible enzymatic degradation (*ii*) whereas the secreted or diffused butyrolactone I would be degraded. The active secretion (*i*) might also be required as has been observed to occur regarding a known autoinducer (3-oxo-C12-homoserine lactone) of *P. aeruginosa* while the known other autoinducer (C4-homoserine lactone) with a shorter acyl chain displayed free diffusion in the same species [178].

Biogenesis inhibition or non-enzymatic degradation? The autoinducer biogenesis inhibition (*iv*) seems to be less likely due to the indicated positive feedback loops, which begin either at the exponential or stationary phase and continue for 96 hours (Figure 7A on page 31). The rather stable intracellular butyrolactone I concentration level of the control cultivation ($>$ 95 µg/ml from the exponential growth onwards) indicates continuous butyrolactone I production, thus contradicting the alternative of biogenesis inhibition. Regarding the non-enzymatic degradation possibility (*v*) the cultivation conditions remained at favorable levels: temperature was set to 27 °C and pH remained slightly acidic throughout the cultivation regardless of the exogenous butyrolactone I supplementation, thus it appears to be unlikely at least concerning the lactone ring [Publications I and II]. The possible interspecies quorum quenching was left out of these speculations due to the one-species cultivation of *A. terreus* and because of a presumption that the interspecies quorum quenching is assumed to target other species.

Signalling suppression? The suggested cooperation of the factors (*i*) and (*ii*) could be interpreted as regulated secretion of a degradative enzyme (a quorum quencher) which was initiated by exogenous addition of butyrolactone I and indicates extracellular signalling suppression. The butyrolactone I secretion would be then restricted to occur only during the exponential growth, thereby displaying the mycelial density plateau to be reached (represented by the cell dry weight profile [Publication I, Additional File 2]) and enabling the indicated intracellular positive feedback

loops as well. This resulting early decrease in the extracellular butyrolactone I concentration might indicate a possibility for an extracellular signalling turnover to occur through restoring the signalling system after its off-switching.

Taken together, the extracellular butyrolactone I concentration results of Publication II are not fully consistent with the corresponding results of Publication I especially concerning the supplementation during transition phase; no significant effect was observed in Publication II and, furthermore, the intracellular increase during that growth phase was only transient, thereby emphasizing the role of transition from exponential to stationary growth phase in submerged cultivation conditions. The observed extensive autoinduction together with the low level secretion (regardless of the quenching factor), combined with the notably low final concentration (100 nM) of the exogenously supplemented butyrolactone I, allows a suggestion of a hormonal-like role in the *A. terreus* signalling. Supporting this, the studies characterising the self-regulatory role of A-factor in *S. griseus* were performed with the effective nanomolar concentration scale as well, emphasizing this presumed hormonal feature [116].

5.2 Upregulation of secondary metabolism via LaeA

Schimmel et al. [60] demonstrated exogenous butyrolactone I to increase the production of secondary metabolites lovastatin and sulochrin during submerged cultivation of *A. terreus*. Bok and Keller [55] reported the global regulator, DNA methyltransferase LaeA, to regulate secondary metabolism including penicillin and sterigmatocystin production in *A. nidulans* and gliotoxin production in *A. fumigatus*, whereas heterologous overexpression of *A. nidulans laeA* in *A. terreus* was observed to increase the lovastatin biogenesis.

In Publication III the revealed *laeA* gene that encodes an orthologue to *A. nidulans* LaeA was observed to be transcripted and, furthermore, upregulated in a statistically significant manner during the exponential and stationary growth phases as a result of the exogenous butyrolactone I supplementation (Figure 10 on page 34 and Figure S5A in Publication III). In parallel with this, lovastatin was increasingly produced during the stationary growth phase after butyrolactone I supplementation during the transition phase [Figure 2A in Publication I], in accordance with the results of Schimmel et al. [60].

In the filamentous bacterium *S. griseus* the regulatory cascade induced by the autoinducer A-factor has been reported to occur via derepression

of a global regulator AdpA through binding of the A-factor to its receptor (ArpA), and thereby enabling the transcription of the regulator AdpA and initiation of the secondary metabolism that includes the antibiotic streptomycin, for instance [177]. In addition to secondary metabolism, AdpA has been observed to induce the aerial mycelium formation [177] leading to spore formation [176] and was presumed to induce also the production of a brown HPQ melanin with a tetrahydroxynaphthalene (THN) intermediate (initiated by a type III polyketide synthase) [186, 187].

The metabolite production and gene expression schedule *butyrolactone I – laeA – lovastatin – mycelial development* that was observed in Publications I–III is reminiscent of this reported signalling cascade of *S. griseus* (AdpA regulon under the control of A-factor), although no butyrolactone I receptor has been reported. Taken together, these results suggest a presumably indirect regulative role for butyrolactone I in the secondary metabolism via the suggested global regulator LaeA (see Figure 16 for a scheme).

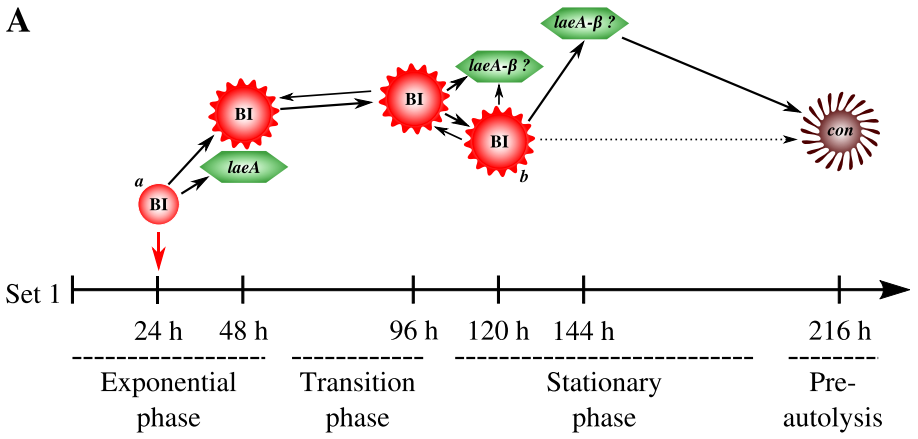

Figure 16: Continues on next page.

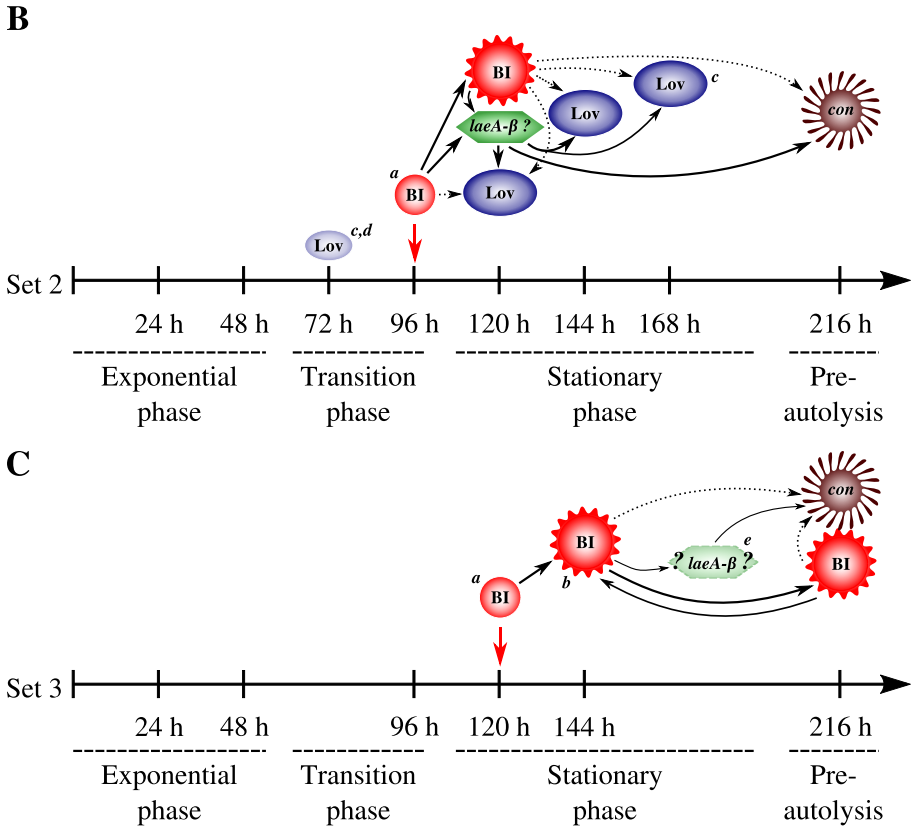

Figure 16: A schematic figure describing the observed influence of butyrolactone I during submerged cultivation of *A. terreus* in the three test sets focused on in this thesis. Exogenous butyrolactone I was supplemented at (**A**) 24 hrs, (**B**) 96 hrs and (**C**) 120 hrs p.i. The vertical positions of the gene and secondary metabolite symbols give a rough estimate of the corresponding positive levels of the statistically significant differences in concentration or gene expression *versus* the control set values. *con* represents a rough average of *abaA* and four of the *pgm* cluster genes (*pgmD, pgmE, pgmF, pgmH*), BI with a surrounding polyconic circle indicates biosynthesised intracellular butyrolactone I and Lov indicates lovastatin. Lovastatin was quantified as total sample concentration in the test set 2 [Publication I]. Solid arrows indicate the suggested regulation pathways, while the dashed arrows indicate possible but less likely pathways. This figure is based on Publications I–IV. [a] Indication of butyrolactone I supplementation; no increase was observed in butyrolactone I concentration. [b] An extracellular increase was observed as well. [c] Only lovastatin was quantified at 72 hours and 168 hours p.i. [Publication I]. [d] Lovastatin quantification showed no differential biogenesis at this time point [Publication I]. [e] The observed increase in *laeA* gene expression was not statistically significant in set 3.

5.2.1 Transient patterns of enhanced butyrolactone I production and *laeA* expression

In the genome-wide transcription scale the distribution of the differentially expressed genes between the three test sets, specifically at 24 hours after butyrolactone I supplementation indicates growth phase-dependent regulation. The supplementation during transition or stationary growth phases resulted into statistically significant differential gene expression simultaneously with the exponential growth phase only (Figure 9B on page 33). In parallel, the exogenous butyrolactone I supplementation during the exponential phase resulted into a continuous upregulation of *laeA* gene expression until the middle of stationary phase was reached, with the exception of the transition phase (Figure 10 on page 34). In contrast, the supplementation during the transition phase led to only transient upregulation occurring in the beginning of the stationary phase [Figure S5A in Publication III]. Concomitantly, the enhancement in the endogenous butyrolactone I production appeared also to be only transient and, furthermore, with extracellular absence during the same supplementation schedule i.e., when supplemented during the transition phase (Figure 7 on page 31). Nevertheless, the exogenous supplementation of butyrolactone I during this transition phase resulted in an increase in lovastatin production. All of these observations raise a question whether the transition phase especially at 96 hours post-inoculation has a specific role in the secondary metabolism under submerged cultivation.

5.3 LaeA and mycelial development

Schimmel et al. [60] revealed exogenous butyrolactone I to have morphological effects on *A. terreus*, in addition to the increase in secondary metabolism during submerged cultivation. Both hyphal branching and conidiation were observed to be increased; when butyrolactone I was supplemented during the exponential growth phase the branching was observed to increase rapidly, while the submerged conidiation was observed to be enhanced during the 192 hours lasting cultivation [60]. In *A. nidulans* the global regulator LaeA has been reported to be involved in the regulation of both conidiophore and cleistothecium formation in addition to secondary metabolism and pigmentation [55, 58, 90]. *A. terreus* has been shown to form cleistothecia [76] and unique accessory conidia [79] in addition to conidiophores while the role of the *A. terreus* LaeA orthologue in conidia and cleistothecia formation, and pigmentation has not been studied.

5.3.1 Asexual conidia, ascospores or accessory conidia?

The displayed upregulation of *laeA* in the microarray gene expression study redirected the focus to reveal the regulation patterns of the genes that encode the orthologues of the *A. nidulans* conidiation key regulators *brlA* (metulae budding), *abaA* (phialide emergence) and *wetA* (conidia maturation) [82–87] to be differentially expressed during the stationary and pre-autolysis phases. In short, *brlA* was increasingly downregulated at the sample snapshots while *abaA* showed upregulation in contrast to *brlA* during pre-autolysis and the downregulation of *wetA* decreased towards the pre-autolysis, during which it showed no differential expression (Figure 10 on page 34). Moreover, the *tpsA* orthologue of *A. nidulans* that encodes the trehalose synthase involved in conidia viability and maturation [88, 89] revealed high transcript amount in the pooled samples of the test set 3, where butyrolactone I had been supplemented during the stationary growth phase (Table 6 on page 35).

Altogether, these gene expression patterns and the moderate to abundant transcript amounts imply phialides to be increasingly developing and the conidia emergence begun during the pre-autolysis growth phase presumably leading to increased submerged conidiation. The low transcript amount and the observed decrease in *brlA* gene expression, especially during pre-autolysis, indicate perhaps the conidiation initiation to occur during the stationary growth phase prior to the last sampling at 216 hours p.i. However, morphologically reduced conidiation cannot be ruled out as it has been observed during submerged cultivations of *A. nidulans* with low-glucose culture media [188, 189] and *Aspergillus niger* under carbon starvation [75]. Production of accessory conidia seems unlikely due to the observed upregulation of *abaA* indicating enhanced activity in the phialide emergence, which is required for asexual conidiation although the biogenesis pathway of accessory conidia is not known.

The suggested conidiation appeared to be enhanced during the pre-autolysis regardless of the supplementation time point and, furthermore, to occur subsequent to the end of *laeA* upregulation when exogenous butyrolactone I was supplemented during the exponential and transition growth phases, while the supplementation during the early stationary phase resulted into upregulation as well, although not statistically significant (Figure 10 on page 34 and Figure S5 in Publication III). The constant and significant regulation of the *brlA* and *abaA* genes at the pre-autolysis, together with the constant *laeA* regulation pattern implies *laeA* to be upregulated during the later non-sampled stationary growth phase as well

(i.e. prior to pre-autolysis) when butyrolactone I was supplemented after transition phase (i.e. in set 3) (see Figure 16). In accordance with this, *abaA* gene expression has been reported to be dependent on LaeA presence in *A. nidulans* [90].

5.3.2 Pigment biogenesis involving NR-PKS enzymes in *Ascomycota*

Ahuja et al. [47] presented seven phylogenetically different NR-PKS enzyme groups containing enzymes known to be involved in secondary metabolite biogenesis and pigment production among *Ascomycota* phylum, including *Aspergillus* spp. Two core enzymes of the well studied pigment biogenesis pathways of *Aspergillus* spp. (*A. fumigatus* PksP and *A. nidulans* WA) were located in one of these seven groups together with two known enzymes involved in pigment biogenesis of *Fusarium* spp. (Bik1 and Aur1) among others, whereas no core PKS enzyme for pigmentation in *A. terreus* was reported. In the genus *Fusarium* Studt et al. [98] revealed a perithecium pigment fusarubin to be biosynthesised by a pathway with a core NR-PKS enzyme (Fsr1) which was not included in this phylogenetic tree presented by Ahuja et al. [47]. However, the phylogenetic tree represented by Studt et al. [98] contained also NR-PKS enzymes of *Aspergillus* spp., and revealed the fusarubin synthesising core enzyme to belong to a separate phylogenetic family of similar enzymes of *Fusarium* spp.

In Publication IV these two phylogenetic trees were combined and analysed together with the revealed and translated *pgmA* gene (Figure 13 on page 39 and Figure 15 on page 43) to build a more comprehensive phylogram (Figure 17). It revealed an additional enzyme group to contain the discovered, predicted PgmA [Publication IV], one core enzyme of asparasone pigment biosynthesis in *A. flavus* [190] as well as one NR-PKS enzyme of *Aspergillus lentulus* in addition to the enzymes involved in the fusarubin-like perithecium pigment biosynthesis in *Fusarium* spp. [Figure S5 in Publication IV]. The majority of these enzymes contain a predicted or known reductase domain on their C-termini together with two acyl carrier protein-like domains revealing a thus far uncommon NR-PKS domain structure in genus *Aspergillus*. The predicted protein level similarities [Table 2 in Publication IV] of the five tailoring enzymes of the Pgm cluster with the corresponding *F. fujikuroi* fusarubin cluster enzymes, together with the high similarity of the core enzymes — PgmA and Fsr1 — raises a hypothesis of pigment biogenesis for the Pgm cluster as was shown for the fusarubin cluster [98].

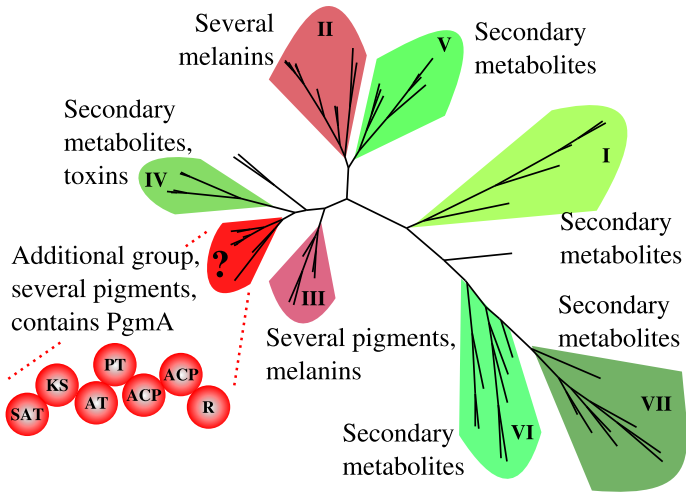

Figure 17: A phylogram with the eight groups of 62 NR-PKS enzymes of *Ascomycota*. The formed groups are based on the extracted KS domain sequences of the NR-PKS proteins included in the studies by Studt et al. [98] and Ahuja et al. [47]. Modified from the Figure 4 and is detailed in the Figure S5 of Publication IV.

5.3.3 Submerged conidial pigmentation in *Aspergillus terreus*?

Followed by the *pgm* cluster update and phylogenetic analysis, the obtained gene expression results revealed the differential gene expression of this cluster to occur in parallel with the key conidiation genes *brlA, abaA* and *wetA* (Figure 10 on page 34 and Figure 14 on page 40). These gene expression profiles indicate simultaneous increased activity of the tailoring enzymes of the Pgm cluster with the probable phialide emergence and initiation of conidia maturation. The low amount of *pgmA* transcripts (Table 7 on page 41) indicates perhaps the initiation of this suggested pigmentation to occur during the 72 hours long non-sampled period of the stationary growth phase as was hypothesised regarding the conidiation initiation by BrlA. In *A. niger* a NR-PKS encoding gene (*fwnA*) together with the tailoring enzymes encoding genes, all of which are involved in conidial pigmentation, were reported to be increasingly expressed towards conidiation (the latter of which was shown both morphologically and transcriptionally with *brlA, abaA* and *wetA* orthologues) under submerged cultivation conditions including carbon starvation as well [75, 191, 192]. In *A. nidulans* a *laeA* deletion mutant revealed the requirement of LaeA in mycelial pigmentation in solid cultivation conditions as well as in pigmentation of both conidia and cleistothecia with an

intact *veA* gene in both light and dark solid cultivation conditions [55, 90]. These observations indicate a positive role for LaeA in the regulation of pigmentation at least in solid cultivation conditions.

Conidial or cleistothecial pigment?

In contrast to this suggested conidial Pgm pigmentation, the fusarubin pigment of *F. fujikuroi* was reported to be produced in perithecia (i.e. ascospores) during solid cultivation conditions, while it was also observed in the culture broth under specific cultivation conditions [98]. In *A. terreus* a non-canonical type of pigment Asp-melanin has been shown to be produced by a non-ribosomal peptide synthetase MelA together with a tyrosinase TyrP during conidiation on solid cultivation conditions [66, 108] contradicting the suggested role of Pgm cluster in conidial pigmentation [Publication IV]. However, in addition to the suggested increase in conidiation, as indicated by the gene expression profiles of the conidiation regulators (*brlA, abaA* and *wetA*), the obtained gene expression profiles of both *melA* and *tyrP* show downregulation throughout the sampling snapshots indicating that this type of conidial melanisation is decreased in these submerged cultivation conditions with exogenous butyrolactone I supplementation (Figure 10 on page 34 and Figure 14 on page 40). As brought out in a comment by Geib and Brock [193] the suggested Pgm pigmentation could also occur during accessory conidia or ascospore formation. Nevertheless, the *A. terreus*-specific accessory conidia has been reported to be non-pigmented [79] while exogenous butyrolactone I supplementation has been reported to increase conidiation [60] in accordance with the gene expression profiles for the key conidiation regulators as described in Publication III. In spite of these asexual conidiation confirming observations the transcripts of few sexual development regulators were expressed also under these submerged conditions utilised in this thesis (unpublished data) emphasising the necessity of morphological and further transcriptomic studies [194].

The type of suggested pigment

The different melanins of *Ascomycota* are generally classified in two types, DHN or *L*-DOPA, based on their biogenesis pathways; either on the specific intermediates or on the enzymes involved in the synthesis of the intermediates [defined in the review 96]. In *A. terreus* the defined conidial Asp-melanin belongs to neither of these classes as emphasised by Geib and Brock [193]; being synthesised by a pathway that contains both

a non-canonical intermediate and enzyme [108]. Regarding the suggested Pgm pigment [Publication IV], the classification is more questionable due to the diverse effects observed through adding two different enzyme inhibitors, tricyclazole [109] and pyroquilone [193] in different concentrations and culture media. Both of these inhibitors have been observed to inhibit the reductase enzyme(s) of the DHN-type of pathway in at least three *Aspergillus* spp. (including *A. clavatus, A. fumigatus* and *A. sydowii*) and eleven *Penicillium* species [103]. An exception has been observed in *A. flavus, A. niger* and *A. terreus* pigmentation; no inhibition was observed with either tricyclazole or pyroquilone on lower concentrations (8−30 µg/ml*) and on diverse culture media (including potato-dextrose agar for *A. flavus* and *A. niger*, for *A. terreus* glucose containing minimal medium only) [103, 193]. In accordance, even a higher amount of tricyclazole (100 µg/ml*) showed no inhibition on *A. flavus* and *A. niger* whereas this high amount was reported to be the lowest concentration to have an inhibitory effect on *A. terreus* pigmentation on potato-dextrose agar [109]. However, the pigmentation of *A. fumigatus* was shown to be inhibited with as low as 8 µg/ml* of tricyclazole and 20 µg/ml* of pyroquilone [103, 193] indicating perhaps the high sensitivity when both of the canonical enzymes (scytalone and hydroxynaphthalene reductases [103, 104]) of DHN pigment pathway are involved; or was due to the influence of used culture medium as was observed for *Penicillium italicum* between different agar media [103]. However, the proposed type (DHN) of this potential Pgm pigment is purely speculative and requires thorough further experiments.

5.4 The architecture of *velvet* gene family

The *velvet* gene family containing *veA, velB, velC* and *vosA* together with the global regulator gene *laeA* has been studied mainly in *A. nidulans* and was revealed to be involved in the regulation of mycelial development including sexual ascospore and asexual conidia formation, and affecting the pigmentation as well. The members of this family have been observed to form multiple protein complexes with diverse regulatory functions depending on the cultivation conditions, the mycelial location as well as the concurrent growth phase [58, 89–92]. In Publication III, thus far unknown transcriptional structures of *A. terreus velvet* gene family were illuminated, invoking numerous further research topics.

*The final concentration(s) in the culture media, dissolved in ethanol.

5.4.1 Splice variants and various complexes

The transcripts of the *velvet* gene family revealed several splice variants both inside the open reading frames and in the untranslated regions [Publication III]. While the UTR intron in *velB* [Figure 4A in Publication III] and the alternative splicing occurring in UTRs of *velC* and *laeA* showed no effect on the predicted ORF lengths (Figures 12A and 11A on pages 37 and 36), the *laeA* and *vosA* splice variants (e.g. *laeA*-α and -β, *vosA*-α and -η) resulted in ORFs with different lengths (Figures 11B and 12B, C on pages 36 and 37). In *A. nidulans* the observed amounts of *vosA* transcripts as well as *velB* have been reported to uncorrelate with the protein levels both during the sexual and asexual development and, furthermore, LaeA was observed to regulate the VelB-VosA complex on protein level [90]. The splice variants inside the UTR and ORF regions of the *velvet* family members observed in Publication III indicate perhaps an occurrence of translational regulation during the submerged cultivation of *A. terreus*.

The heterotrimeric complex of VelB – VeA – LaeA

In dark, this protein complex was found to promote secondary metabolism and sexual cleistothecia formation of *A. nidulans* in solid cultivation conditions. Specifically, the complex member VeA has been reported to interact with the N-termini of both VelB and LaeA [58]. The sequenced transcripts of *laeA* revealed the N-termini of the translated ORFs of the splice variants α and β to be different in length, while the predicted DNA methyltransferase domain and the SAM-binding sites remained substantially non-affected (Figure 11C on page 36). Thereby the interaction of LaeA with VeA may be disabled, whereas the regulatory function is possibly enabled in an alternative manner as hypothesised in Publication III although no actual protein data is available. In parallel, the low amount of *veA* transcripts and the lower quantity of the longer *laeA*-α variants indicate the absence of secondary metabolism as well as cleistothecia formation (Figure 2B in Publication III, Figure 11 on page 36, Table 6 on page 35), in contrast to the observed enhanced lovastatin [Figure 2A in Publication I] as well as sulochrin production in the similar cultivation conditions [60]. These observations indicate the prevalence of positive regulatory function of LaeA, perhaps LaeA-β variant, in secondary metabolism at least. However, diverse speculations are possible, including transient gene expression of *veA* during the non-sampled period of stationary phase in a similar manner as hypothesised for *brlA*, which

would thus induce secondary metabolism. In this scenario, the resulting ascospore formation remains contradictory with the observed *abaA* up-regulation although VeA has been reported to be required in *A. nidulans* asexual conidiation, to a degree, in addition to LaeA [90] (Figure 10 on page 34).

Dimeric complexes

In *A. nidulans* the Velvet family members have been observed to build several different hetero- and homodimeric complexes in addition to the trimeric complex VelB–VeA–LaeA. All of these have been found to be involved in the direct or indirect gene expression control of key conidia transcription factors *brlA, abaA* and *wetA* as well as cleistothecia regulators [89–92].

VelB – VosA In *A. nidulans* a heterodimeric complex of these two Velvet family members (VelB, VosA) has been reported to suppress asexual conidiation, which is regulated by LaeA in light-dependent manner; asexual conidiation is enabled in light when LaeA decreases the suppressional activity of this complex [90]. Regarding the different mycelial developmental phases, this complex has been reported to have various functions. In phialides both of these members are activated by AbaA on transcriptional level, which is where the complex has been reported to suppress *brlA* expression as well, while in conidia *wetA* expression has been observed to be activated by the complex [89–91]. Although no growth phase-specific data is available in Publication III, the pooled transcript amounts of both *velB* and *vosA* were high, indicating active involvement in the regulation of these mycelial development phases of *A. terreus* in parallel with the observed gene expression profiles of these key regulators of the asexual conidiation (*brlA, abaA* and *wetA*) in the submerged cultivations under the influence of butyrolactone I (Table 6 on page 35 and Figure 10 on page 34).

VelB – VeA and VosA – VelC Both of these two heterodimeric complexes in *A. nidulans* have been observed to be active in the hyphae, and their functions are reported to involve the regulation of sexual development in positive manner [58, 91, 92]. In Publication III the observed low amount of the *veA* transcripts is in accordance with the suggested occurrence of asexual conidiation, while the slightly higher amount of *velC* transcripts (Table 6 on page 35) is perhaps due to the suggested function

of VelC to be involved in the regulation of both asexual conidiation and sexual cleistothecia formation [92].

VelB – VelB and VosA – VosA Analogously with the VelB–VeA and VosA–VelC dimeric complexes, the homodimeric complexes of VelB and VosA have been suggested to be located in the hyphae and, in contrast, to have opposite functions in *A. nidulans*; VelB–VelB activates conidiation while VosA–VosA suppresses the conidiation [90, 91]. Since only pooled transcriptome data was available regarding the *velvet* family genes [Publication III] one can only speculate of the presence of these two complexes, however, both of these members were highly expressed (Table 6 on page 35) indicating perhaps the activity of VosA–VosA complex and subsequent formation of VelB–VelB complex prior to the pre-autolysis growth phase.

Conclusions

The four publications of this thesis have elucidated the functional characteristics of the native metabolite butyrolactone I of the filamentous fungus *A. terreus*, both on the molecular and transcriptional level. The exogenous supplementation of butyrolactone I revealed several regulatory aspects in support for the suggested role in the secondary metabolism of *A. terreus* as presented by Schimmel et al. [60], and uncovered further controlling features which were presented in this thesis.

(A) The production of butyrolactone I was verified and quantified by LC-ESI-MS/MS together with HPLC in the same cultivation conditions as applied throughout this thesis. The time-course patterns of the parallel production and secretion were found to follow the mycelial cell density profile; to indicate autoinduction and signalling suppression (quenching), enabling potential signal turnover; and to proceed in a growth phase-dependent manner [Publication I]. These features are in accordance with the common characteristics of the quorum sensing phenomenon in bacteria.

(B) The genome-wide gene expression experiment combined with whole transcriptome sequencing revealed significant upregulation of the global regulator *laeA* which has a regulative role in the secondary metabolism, and conidia and ascospore formation of the model fungus *A. nidulans* [55, 90]. In accordance with this, the key regulators of conidiation (*brlA, abaA* and *wetA*) displayed gene expression profiles indicating butyrolactone I to have indirect control in *A. terreus* conidiation [Publication III]. Furthermore, this positive regulation of *laeA* is in good agreement with the observed positive effect of exogenous butyrolactone I on lovastatin production [Publication I].

(C) One partially annotated gene cluster was discovered to contain a putative NR-PKS encoding gene *pgmA* that shows high similarity to a group of enzymes, of which 50 % are core enzymes in perithecial pigment biosynthesis, e.g. Fsr1 of *F. fujikuroi* [98]. The surrounding *pgm* cluster genes showed significant upregulation by butyrolactone I in a similar pattern as with the key conidiation regulators, especially *abaA*, thus raising a hypothesis of conidial pigmentation [Publications III and IV]. In contrast, two enzymes (MelA and TyrP) have been shown to produce conidial pigment under solid cultivation conditions [108] albeit they showed significant downregulation in the submerged cultivation [Publication IV].

Therefore, a possible role for *pgm* cluster in ascospore pigmentation *in vivo* growth [194] cannot be ruled out, as supported by the perithecial location of the fusarubin pigment of *F. fujikuroi* [98].

(D) The sequenced transcriptome revealed numerous transcript variants of the *A. terreus velvet* family genes as well as divergent upstream regions indicating translational regulation during submerged cultivation conditions and under the influence of enhanced biogenesis of butyrolactone I. The complex splice variants of *laeA* may also indicate functional differences in the protein complex binding, perhaps. In addition, the thus far unknown genomic locus of *velC*, one of the Velvet complex members, was revealed [Publication III].

(E) Altogether, the parallel gene expression profiles of **(i)** the key regulators of conidiation [Publication III]; **(ii)** the tailoring enzymes of *pgm* gene cluster [Publication IV]; **(iii)** the global regulator *laeA* [Publication III] and **(iv)** the observed increase in lovastatin [Publication I] and butyrolactone I [Publication II] production raise a hypothesis for butyrolactone I to play a positive role in the pigmented conidiation and secondary metabolism via LaeA in *A. terreus* submerged and shaken cultivation. The suggested quorum sensing role for butyrolactone I via LaeA is comparable with the inducing effects of the autoinducer A-factor via regulator AdpA on the mycelial development and secondary metabolism in the filamentous bacterium *S. griseus* [71, 116, 176, 177, 186, 187].

(F) From the methodological point of view the applied LC-ESI-MS/MS in addition to HPLC analysis gave precise information of the butyrolactone I production and distribution between the intra- and extracellular regions. The combination of the two applied genome-wide gene expression methods, microarray analysis and transcriptome sequencing, revealed the criticality of the genome annotation accuracy, nucleotide-level differences between microbial strains and alternative splicing. The *velvet* family members and the *pgm* cluster were found to contain transcriptional features which highlighted the necessity of further studies. These should include quantitative and time-point specific RNA-sequencing to confirm the results obtained using the combined transcriptional methods. Integrating this with diverse cultivation conditions, sampling time points and morphological studies might reveal genes encoding receptors and regulators.

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Elina K. Palonen

The regulatory role of butyrolactone I in the filamentous fungus *Aspergillus terreus*

This thesis gives insight into the regulation of secondary metabolism and fungal reproduction of an industrially and pharmaceutically important fungus, *Aspergillus terreus*, in the aspect of one specific secondary metabolite, butyrolactone I. The studies confirm the enhancing effect of butyrolactone I on the biogenesis of another secondary metabolite lovastatin, which is used in the treatment of cardiovascular diseases as the active ingredient in commercial drugs. The results strongly indicate that the enhancement by butyrolactone I occurs via a global regulator, LaeA, which is known to be involved in the regulation of both secondary metabolism and fungal reproduction. Furthermore, the work revealed a previously unidentified gene cluster the synthesis product of which is thought to be involved in the reproduction of *A. terreus*. Altogether, these results form a starting point for further studies and exploitation of *A. terreus*.

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