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Dissimilar pigment regulation in *Serpula lacrymans* and *Paxillus involutus* during inter-kingdom interactions

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Abstract

Production of basidiomycete atromentin-derived pigments like variegatic acid (pulvinic acid-type) and involutin (diarylcyclopentenone) from the brown-rotter *Serpula lacrymans* and the ectomycorrhiza-forming *Paxillus involutus*, respectively, is induced by complex nutrition, and in the case of *S. lacrymans*, bacteria. Pigmentation in *S. lacrymans* was stimulated by 13 different bacteria and cell-wall-damaging enzymes (lytic enzymes and proteases), but not by lysozyme or mechanical damage. The use of protease inhibitors with *Bacillus subtilis* or heat-killed bacteria during co-culturing with *S. lacrymans* significantly reduced pigmentation indicating that enzymatic hyphal damage and/or released peptides, rather than mechanical injury, was the major cause of systemic pigment induction. Conversely, no significant pigmentation by bacteria was observed from *P. involutus*. We found additional putative transcriptional composite elements of atromentin synthetase genes in *P. involutus* and other ectomycorrhiza-forming species that were absent from *S. lacrymans* and other brown-rotters. Variegatic and its precursor xerocomic acid, but not involutin, in return inhibited swarming and colony biofilm spreading of *Bacillus subtilis*, but did not kill *B. subtilis*. We suggest that dissimilar pigment regulation by fungal lifestyle was a consequence of pigment bioactivity and additional promoter motifs. The focus on basidiomycete natural product gene induction and regulation will assist in future studies to determine global regulators, signalling pathways and associated transcription factors of basidiomycetes.

INTRODUCTION

Basidiomycetes play a critical role in element cycling and lignocellulose disintegration, yet they have also entered the spotlight for their unprecedented capacity to make an array of natural products. These small and often highly functionalized molecules may serve local or global processes (e.g. defence or carbon cycling, respectively). The terphenylquinone atromentin is a widespread pigment and precursor to numerous compounds depending on the cleavage of the benzoquinone ring and subsequent various modifications (Fig. 1; [1, 2]). Atromentin-derived compounds, variegatic acid from *Serpula lacrymans* and involutin from *Paxillus involutus,* were inducible under nutritional cues, and *in vitro* evidence identified them as Fe^{3+}-reductants in Fenton chemistry for lignocellulose degradation, thus highlighting their involvement in carbon cycling [3, 4]. The brown-rotter *S. lacrymans* and the ectomycorrhiza-forming *P. involutus* are taxonomically related (*Boletales*), but live different lifestyles. The former is recognized as an economic burden because it degrades timber whereas *P. involutus* is an important symbiont that promotes tree health by nutrient exchange [5–7].

We previously reported that the enzymes involved in the production of atromentin are encoded within a cluster that is widely orthologous in basidiomycetes, and the promoters of the two essential genes (encoding an atromentin/quinone synthetase and aminotransferase) have a conserved genetic promoter motif [8–10]. Additionally, three fungal–bacterial co-incubations led to gene cluster induction and subsequent pigment accumulation in the model *S. lacrymans* [9]. Here, using both *S. lacrymans* and *P. involutus*, we expanded our understanding of atromentin-derived pigments and its regulation during co-incubation with bacteria. We questioned (i) how universal pigment induction was in *S. lacrymans*

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Abbreviations: HPLC, high performance liquid chromatography; MEP, malt extract peptone; MEME, Multiple Em for Motif Elicitation.
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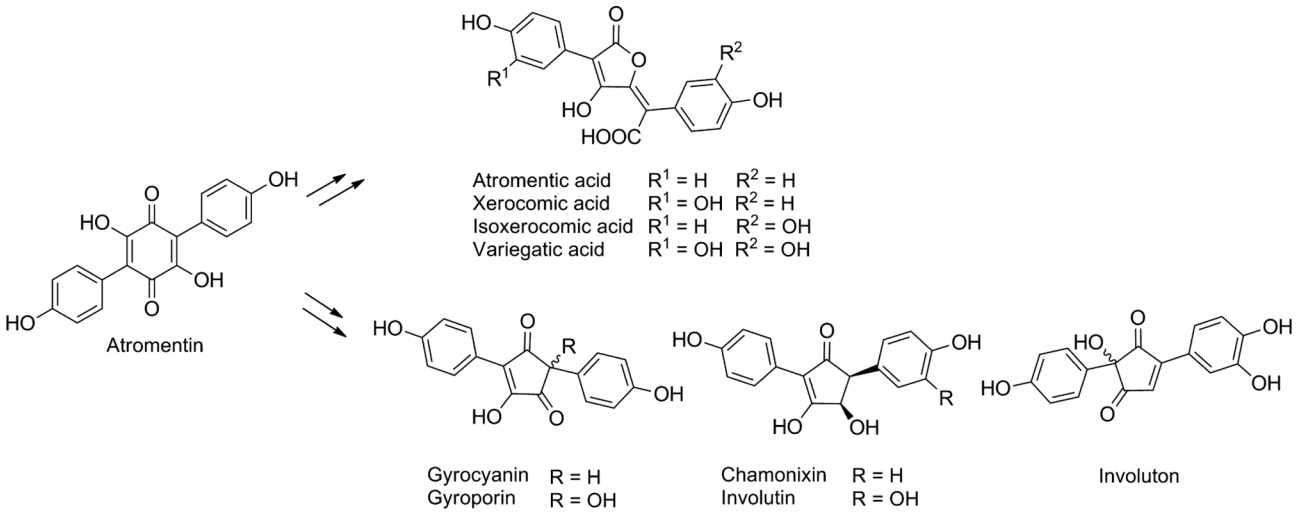

Fig. 1. Chemical structures of atromentin and congener pigments.

and whether related fungal species that follow a different lifestyle would be similarly stimulated; (ii) what the underlying eliciting factor was; and (iii) what local function the pigments might have during co-incubation. We show that although pigmentation by bacteria appeared ubiquitous in *S. lacrymans*, this was not the case in *P. involutus*, and that certain pigments from *S. lacrymans* impacted biofilm spreading and swarming motility.

METHODS

Organisms and growth conditions

For *Serpula lacrymans* S7.9 [3] co-incubations, we followed a published protocol with some modifications [9]. A synthetic medium agar plug containing *S. lacrymans* mycelium was inoculated atop a fresh synthetic agar plate (KH_2PO_4 500 mg l^{-1}, NH_4Cl 200 mg l^{-1}, $MgSO_4$ ×7 H_2O 150 mg l^{-1}, $CaCl_2$ ×2 H_2O 50 mg l^{-1}, NaCl 25 mg l^{-1}, $FeCl_3$ ×6 H_2O 12 mg l^{-1}, Thiamin 1 mg l^{-1}, D-glucose monohydrate 5 g l^{-1}, 1.8 % w/v agar, pH 5.6 by NaOH) and grown axenically for 13–14 days at ambient temperature. For all bacteria except *B. subtilis* to be inoculated atop the fungus, an overnight culture from a 25 % glycerol stock was grown in LB (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 5 g l^{-1}) or German Collection of Microorganisms and Cell Culture M79-Medium 426 (D-glucose monohydrate 10 g l^{-1}, bacto peptone 10 g l^{-1}, casamino acids 2 g l^{-1}, yeast extract 2 g l^{-1}, NaCl 6 g l^{-1}, pH 7.8) with agitation at 28 °C. The culture was pelleted, washed three times with autoclaved water in 50 ml tubes and resuspended in water to make a 200-fold concentrate from the initial culture. Two 500 µl and two 250 µl droplets were then added atop a fungal mycelial bed which started the co-incubation. *B. subtilis* 3610 was grown in LB with agitation at 37 °C and concentrated 100-fold. For *B. subtilis*, rinsed and suspended bacteria were split and tested under three conditions: (i) as is, with ca. 3.8×10^9 c.f.u. per 250 µl droplet; (ii) autoclaved; or (iii) incubated with one EDTA-free protease inhibitor cocktail tablet (Sigmafast, Sigma) before being introduced to the fungus. Separately, enzymatic damage to the fungus was performed. Here, two 500 µl droplets (50 mg ml^{-1} solution in PBS) of lysing enzymes from *Trichoderma harzianum* (Sigma), proteases (from *Streptomyces griseus*, Sigma), or lysozyme from chicken egg white (Sigma) were inoculated atop the fungal mycelial bed. As controls, mechanical damage to the mycelia was carried out by scalpel wounding, and all cultures were run alongside water or PBS droplet controls. Co-cultures, enzymatic assay cultures or controls were run at ambient temperature in darkness in duplicates and repeated twice. Co-cultures were extracted after 7 days, *B. subtilis*-related co-cultures after 2 days, and enzymatic assays after 3 days. *O. olearius* [11] co-cultures with *B. subtilis* or *P. putida* were executed the same as for *S. lacrymans*, except cultures were extracted after 3 days of growth. Co-cultures of *Suillus bovinus* JMRC: SF013586 with *B. subtilis* were also performed as described for *S. lacrymans*, but using an 11-day-old axenic culture prior to adding *Bacillus*.

P. involutus ATCC 200175 [6] was grown at ambient temperature in darkness. For *P. involutus* co-culturing, conditions I–III (below) were tested, each based on established growth methodologies utilizing glass beads submerged in liquid media whereby the fungus remained stationary within the same petri dish, and liquid media can be exchanged [12–14].

Condition I: the fungus was grown axenically on 15 ml synthetic broth for 14 days. Then, 150 µl pre-rinsed bacteria listed in Table 1 was spotted atop the mycelia (similar to above with *S. lacrymans*' co-culturing), and co-incubated for 3 days until extraction.

Table 1. Bacteria used in co-incubation experiments with *S. lacrymans* and *P. involutus*

Bacteria were obtained from previous works or the Jena Microbial Resource Collection.

Bacterium	Taxonomy	Strain/reference
Bacillus subtilis 3610*	*Bacilli*	[9]
Lysinibacillus fusiformis M5	*Bacilli*	[28]
*Streptomyces iranensis**	*Actinobacteria*	[9]
Arthrobacter spp.	*Actinobacteria*	[59]
Micrococcus luteus	*Micrococcales*	[59]
Acetobacter pasteurianus subsp. *pasteurianus*	*Alphaproteobacteria*	ATCC 9433
Sphingomonas spp.	*Alphaproteobacteria*	ST027129
Methylobacterium mesophilicum	*Alphaproteobacteria*	ATCC 29983
*Pseudomonas putida**	*Gammaproteobacteria*	[9]
Escherichia coli XL1-Blue	*Gammaproteobacteria*	Stratagene
Pseudomonas tolaasii	*Gammaproteobacteria*	[60]
Pseudomonas fluorescens	*Gammaproteobacteria*	[59]
Acinetobacter spp.	*Gammaproteobacteria*	[59]

*Indicated initial bacteria tested that set the basis for testing various other strains [9].

Condition II: the fungus was axenically grown for 9 days on 15 ml synthetic broth, whereby the broth was then discarded and the petri was replaced with fresh synthetic media. Then, 3 days later, soil-isolated bacteria, each set to an OD_{600}=1.8 by water from pelleted and rinsed bacterial pre-cultures, were inoculated (1 ml each or 1 ml from equally pre-mixed consortia) into the media and atop of the fungus. The co-culture was terminated after 7 days.

Condition III: an agar plug containing mycelium (mycelium facing aerially) was placed atop glass beads submerged in 15 ml synthetic broth and the fungus grown for 9 days. Then, the broth was removed, the mycelia and beads washed with autoclaved dH_2O, and 15 ml synthetic broth without inorganic nitrogen was added. After 24 h, the broth was discarded from the N-starved fungal mycelia, and then introduced to 15 ml MEP (malt extract peptone medium; malt extract 30 g l^{-1}, soytone 3 g l^{-1}) amended with D-glucose monohydrate (5 g l^{-1}). The day before the onset of co-culturing, soil-isolated *Bacilli* (isolation described later) were grown overnight at 37 °C with agitation (180 r.p.m.) in 400 ml LB and used as a seed culture in fresh LB, which was then grown to an OD_{600}=0.20–0.25. Then, 0.2 ml of bacteria suspension was added to the fungal MEP culture. For the bacterial consortia, 0.2 ml of each bacterium was added. As a negative control, 0.6 ml of blank LB was added to axenic fungal cultures. After 7 days, the conditioned broth was extracted for chromatography (below).

Soil samples for quantification and isolation

Soil was collected from directly underneath *P. involutus* mushrooms. 16S and 28S rDNA was amplified from the soil samples after gDNA nucleic acid extraction (cetyltrimethylammonium bromide and phenol:chloroform:isoamyl alcohol methodology), using taxonomic-specific or universal primers for bacteria, fungi, or archaea (detailed in Table S1 and Fig. S3; available in the online version of this article). Isolation of spore-forming bacteria from soil samples was performed by suspending 1 g wet soil samples in 10 ml PBS, incubating at 80 °C for 15 min, creating a 1 : 10 dilution series using PBS, spreading 100 µl of the suspension onto MEP or synthetic agar, and incubating at 28 °C or ambient temperature for up to 2 weeks until colony growth was observed. Bacteria were re-streaked until single colonies were isolated and chosen by distinct colony morphology. Colonies were grown in liquid LB or M79, and their 16S rDNA was sequenced using 27F and 1492R universal primers (Table S1, [15, 16]). Soil isolates were deposited at the Jena Microbial Resource Collection (Table S2).

Sequence collection and motif search

Sequence data were downloaded from the JGI MycoCosm portal [17]. A list of the 23 atromentin-producing species is provided in Fig. 4 [2, 3, 6, 11, 18, 19]. Promoter sequences of *Tapinella panuoides* and *Suillus grevillei* were collected from a cosmid library [2, 9, 10]. The promoter region was selected as −1000/+2 bp around the transcription start site [20]. *De novo* motif prediction was performed by the MEME (Multiple Em for Motif Elicitation) software [21, 22]. The negative promoter set used for discriminative mode had been established and reapplied here [9]. In short, it contained 41 promoters including (i) promoters of genes flanking cluster regions, and (ii) promoters of non-secondary metabolite genes. The parameters for the MEME search were: motif length: 8–12 bp; and 0 or 1 motifs per sequence. Analyses were grouped as follows: (i) 23 fungi in total; (ii) 'larger *Paxillus*' and '*Serpula*' groups; and (iii) within the '*Paxillaceae*.' The generic name *NPS* was used for annotated or characterized atromentin/quinone synthetases, *ADH* for alcohol dehydrogenases/oxidoreductases, and *AMT* for aminotransferases.

Sequence alignment and phylogeny reconstruction

Alignments were performed in MUSCLE with standard parameters [23, 24]. The phylogenetic trees were built by PhyML v3.0.1 [25], and statistical branch supports were computed with a Bayes likelihood-based method. The best evolutionary model for the ML analysis was selected by the Smart Model Selection (SMS) in PhyML [26], and the best substitution models according to the selection criterion AIC (Akaike information criterion) were HKY85 for nucleotide sequences and Le-Gascuel for proteins. The architectures of the promoter and protein ML trees were compared by tanglegrams that were performed by the EPoS framework for phylogenetic analysis [27].

Pigment isolation and chromatographic analyses

For *S. lacrymans*, *S. bovinus* and *O. olearius* co-cultures, the plates including the inocula and mycelia (two plates made one biological replicate) were soaked in 200 ml ethyl acetate, which was amended to a final volume of 400 ml using dH_2O and glacial acetic acid (1 % v/v), and incubated with intermittent shaking for 1 h. The organic phase was removed, and dried under reduced pressure, followed by lyophilization [9]. For *P. involutus* co-cultures, the conditioned broth (two plates made one biological replicate) was extracted vigorously with twice volume ethyl acetate amended with 1 % (v/v) glacial acetic acid with intermittent shaking or in a rotator, and dried as above.

High performance liquid chromatography (HPLC) analyses followed a published protocol [9]. Authentic standards were isolated from the axenic, aged secretomes of either *S. lacrymans* S7.9 (variegatic, xerocomic, isoxerocomic and atromentic acids; xerocomorubin and variegatorubin), or *P. involutus* (involutin, gyroporin and chamonixin) by semi-preparative HPLC as performed before with some modifications [1, 8, 9], and purity was verified by analytical HPLC. Statistical significance from three biological replicates was determined by one-way ANOVA with a post-hoc Tukey honest significant difference test using the peak area under the curve of known pigments.

Bioactivity assays

We used a described assay to assess the effect that different pigments have on biofilm development and wrinkle formation of *B. subtilis* 3610 [28]. To assess the effect that different compounds have on swarming motility of *B. subtilis* 3610 we used 90 mm diameter LB plates with 0.7 % (w/v) agar that were prepared and dried as described [29]. The antibiotic activity of different compounds was assessed via OD_{590} kinetics of liquid cultures of *B. subtilis* 3610 (final concentration of 0.25 mg ml^{-1}). All bright-field and fluorescence images of colonies were obtained with an Axio Zoom V16 stereomicroscope (Zeiss, Germany) [29]. More details are found in the supplement.

RESULTS AND DISCUSSION

Cell-wall-damaging enzymes induced pigmentation in *S. lacrymans*

We used our established *S. lacrymans*-bacterium co-incubation system to screen for additional organisms or mechanisms that could also induce pigmentation as examined by HPLC [9]. We analysed pigmentation intensity by focusing on the signals (area under the curve) for the main pulvinic acid-type pigments (variegatic, xerocomic, isoxerocomic and atromentic acids; Fig. 1; [1]). Additionally, other variants of pulvinic acid-type pigments were detected. We also identified oxidized variants of variegatic acid and isoxerocomic acid, which are formed from the production of a second lactone ring from the carboxylic acid to produce variegatorubin and xerocomorubin, respectively.

In total, a set of 13 different bacteria were tested (Table 1), all of which induced pigmentation after 72 h when the fungus was grown on non-inducing media. As a control, cultures were compared to axenic fungal cultures that were exposed to water droplets *in lieu* of bacteria. Accumulation of pulvinic acid-type pigments from co-culturing was observed in all cases by HPLC (Fig. S1). As an exemplar co-incubation, *S. lacrymans* – *Sphingomonas* sp. showed intense pigmentation (Fig. 2a). As different bacteria were able to induce pigmentation, we then considered that a common inducing mechanism may be shared amongst many bacteria. Numerous bacteria release degrading enzymes, such as general proteases or oxidoreductases and hydrolases (reviewed in [30, 31]). Thus, we hypothesized that hyphal damage may represent such a common factor. We tested this hypothesis and exposed *S. lacrymans* to fungal cell-wall-lysing enzymes (containing β-glucanase, cellulase, protease and chitinase activity) or general proteases. Pulvinic acid-type pigments were observed in the chromatograms for both instances (Fig. 2b). For controls, we tested lysozyme that does not target fungal cell-walls, and mechanical damage by scalpel. Mechanical damage was shown to induce the biosynthesis of polyene defence compounds in a stereaceous mushroom [32]. Various other controls that did not result in pigmentation were run in parallel (excessive inorganic nitrogen, water or PBS). Based on the enzymatic assays we hypothesized that an eliciting factor from the bacterial partner, like a protease, was secreted which damaged the fungal cell-wall. For example, proteases are involved in mycoparasitism [33]. Therefore, we grew the fungus in the presence of pre-rinsed and water-resuspended *B. subtilis* NCIB 3610 (hereafter 3610) amended with a protease/metalloprotease inhibitor cocktail, in addition to testing heat-killed *B. subtilis*. The inhibition of proteases or introduction of dead bacteria showed a significantly reduced fungal pigment response compared to the alive *B. subtilis* 3610 co-incubation (*P<0.01; Fig. 2c). Nevertheless, the amended or heat-killed *B. subtilis* caused slight pigmentation. We cannot exclude that the protease inhibitor cocktail would fail to abolish all protease activity (for example, *B. subtilis* 168, the derivative of 3610, is known to produce eight different

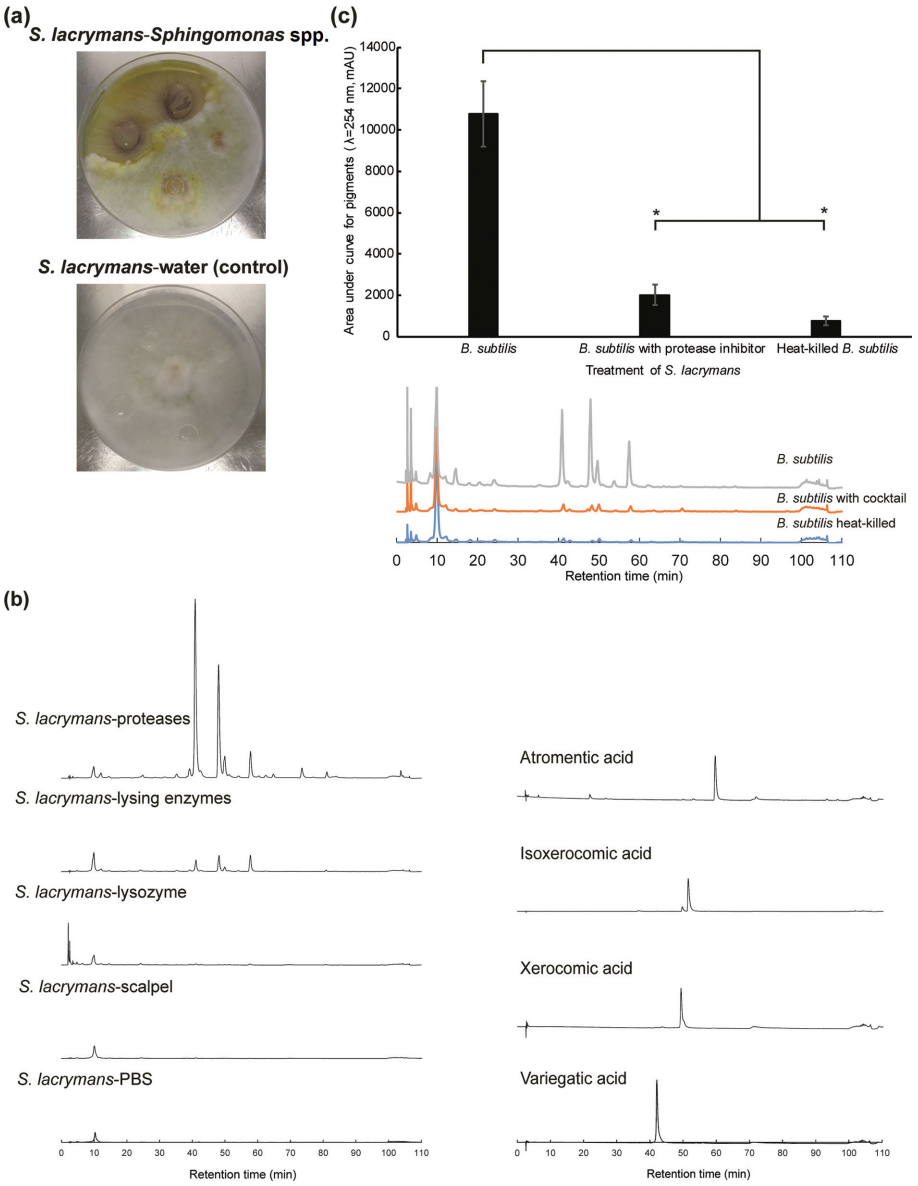

Fig. 2. (a) Representative example of strong pigment induction of *S. lacrymans* by *Sphingomonas* spp. The two 500 µl inocula had a greater observed effect than the 250 µl inocula. (b) Representative chromatograms (λ=254 nm) of enzymatic assays that show pigment induction by proteases and lysing enzymes, but not in the controls. Chromatograms are proportionally scaled. Further, chromatograms of authentic standards are shown: variegatic acid (t_R=42.1 min), xerocomic acid (t_R=49.2 min), isoxerocomic acid (t_R=51.4 min) and atromentic acid (t_R=59.5 min). (c) Cumulative compound titres (as assessed by the signal area under the curve at λ=254 nm) of the four main pigments (variegatic, xerocomic, isoxerocomic and atromentic acid) showing statistical difference (*P<0.01) between *S. lacrymans* co-incubated with alive *B. subtilis*, and *B. subtilis* amended with a protease inhibitor cocktail or heat-killed *B. subtilis*. The arithmetic mean and standard error from three biological replicates are shown, as well as representative chromatograms of each condition.

proteases [34–36]). Released peptides or cell-wall components from the fungus itself or exo-proteins due to the action of proteases or lysing enzymes may trigger pigmentation, and would draw comparisons that organic, but not inorganic, nitrogen also induced pigmentation [3]. Nor could we eliminate the idea that the fungus may be stimulated by other bacterial factors, such as secreted peptides, secreted low molecular weight compounds, competition for nutrition, or intimate physical contact [37, 38]. Degrading exo-enzymes from bacteria such as proteases, lysing enzymes or oxidases that harm the fungus may be a direct consequence of the co-incubation or merely the bacterium modifying the environment to, for example, secure food. This remains to be studied by focusing on the bacterial partner's response during co-incubation using proteomics and/or transcriptomics. Due to the lack of genetic tractability of

basidiomycetes, the global transcriptomic response of the fungal partner is of additional interest which will help deduce whether bacterium-induced pigmentation correlates with cell-wall repair, stress-related or nitrogen metabolism genes, or whether these responses are independent. Our bacteria-induced pigmentation was also applicable for another atromentin-producing, wood-rotting basidiomycete, *Omphalotus olearius*, whereby significant brown pigmentation occurred after the introduction of either *B. subtilis* 3610 or *P. putida*. The pigment was verified to be atromentin by HPLC (Fig. S2). All in all, our results showed that pigment induction was triggered, although not definitively correlated, by an enzymatic degradation of the fungal cell-wall.

Co-culturing with *P. involutus* did not cause pigmentation

To expand our *Serpula*-based study on natural product induction through inter-kingdom co-incubation, we tested if a similar pigmentation response was also valid for atromentin-producing *Boletales* that follow a symbiotic lifestyle. We chose the model ectomycorrhizal fungus *Paxillus involutus*, and focused on the most abundantly secreted pigment under known media-inducing conditions, involutin [4]. The route to biosynthesize involutin via atromentin is redundantly secured by three atromentin synthetase genes [8, 39], which are constitutively expressed at low levels even in non-pigment inducing media (the same with *S. lacrymans*), and overly expressed in high organic nitrogen-containing media [8]. Other atromentin-derived pigments (gyroporin, chamonixin, involuton and atromentic acid) from *P. involutus* are generally produced in insignificant amounts under laboratory conditions [8].

For our co-culturing work with *P. involutus*, we grew the fungus on non-pigment-inducing media [4], and tested a diverse set of bacteria pairwise against *P. involutus* that were used for *S. lacrymans* (most species from Table 1). The bacteria did not cause significant pigmentation when examining involutin titres by HPLC (condition I; Fig. 3a). We also co-incubated *P. involutus* with *B. subtilis* 3610 as performed with *S. lacrymans* by using alive *B. subtilis,* and for control heat-killed *B. subtilis*, and *B. subtilis* incubated with a protease inhibitor. We presumed that the conditions used before may have an opposite effect. Here, we found no significant change in accumulation of involutin between all these conditions.

We presumed that if 'outside, antagonistic' bacteria did not induce pigmentation, then perhaps community-associated bacteria may do so. Such species interactions can be quite specific (discussed below), and thus we wanted to perform a community-guided approach. We first monitored the soil directly underneath a troop of growing *P. involutus* mushrooms at two time points by qRT-PCR using 16S or 28S rDNA (Fig. S3). The isolation of bacteria pertaining to *Actinobacteria* and *Firmicutes* was led by the verification of their highest 16S copy numbers at both measured time points. After isolation by morphology, the following soil-derived bacteria were identified by 16S rDNA sequencing and used for further co-incubation work: *Bacillus aryabhattai*, *Bacillus subtilis* subsp. *subtilis*, *Bacillus subtilis* subsp. *inaquosorum* and *Micromonospora aurantiaca* (Table S2). The bacteria we isolated were deposited in the Jena Microbial Resource Collection.

From the soil-guided approach, we tested the bacteria with *P. involutus* using two further conditions involving multi-partner interactions that may resemble the soil community around fungal hyphae. For condition II, we continued testing *P. involutus* on non-inducing synthetic media. We used pairwise and consortia co-incubations of soil isolates. Similar to the previous results, we observed no accumulation of involutin when compared to the axenic fungal control (Fig. 3b). We then co-incubated the fungus with different consortia of *Bacilli* soil-isolates in a rich medium (malt extract-peptone) that supported both strong growth of bacteria and was also a rich source of organic nitrogen that is known to stimulate the secretion of involutin (condition III; [4]). The multi-partner cultures were carried out with the fungus co-incubated with either one (pairwise), two (tripartite) or three (consortia) different soil-isolated *Bacilli*. We found that the control (i.e. axenic *P. involutus*) had increased involutin titres when compared to the fungus grown on non-inducing medium which was consistent with previous work [4]; concurrently, no signals for the other pigments were found. We looked for even the slightest variations in pigment accumulation. Each co-culture in condition III showed no statistical difference in involutin accumulation when compared to the control, and the only statistical difference was determined to be between pairwise *B. aryabhattai* and two tripartite co-cultures ($^*P<0.01$; Fig. 3c). Representative chromatograms in all cases are shown in Fig. S4. Another ectomycorrhizal-forming basidiomycete, *Suillus bovinus,* that produces atromentin-derived metabolites [1] was also tested for bacteria-induced pigmentation. Here, no pigmentation after the introduction of bacteria was observed (Fig. S5), authentic standards of relevant pigments are shown in Fig. S6.

One hypothesis that may support the more passive response of ectomycorrhizal fungi is that saprophytes associate with less bacteria than ectomycorrhizae [40], i.e. fungal lifestyle dictates the surrounding community structure, which may indicate that saprophytes are more competitive. In support of this, mycorrhizae generally support (or at least allow for) the formation of biofilms on their hyphae [41]. Hence our results, at least with respect to pigmentation, follow suit. Conversely, such microbial communities can be very specific and biotic interactions can have very specific outcomes. Examples have shown that the mushroom's identity shapes its specific bacterial community [42]; fungal–bacterial and bacterial–bacterial interactions and consequences therefrom (antagonistic, growth promoting or neutral) can be quite species-specific [43, 44]; a dissimilar fungal response by the exact same 'mycorrhiza helper' strain was possible [45]; a 'mycorrhiza helper' strain

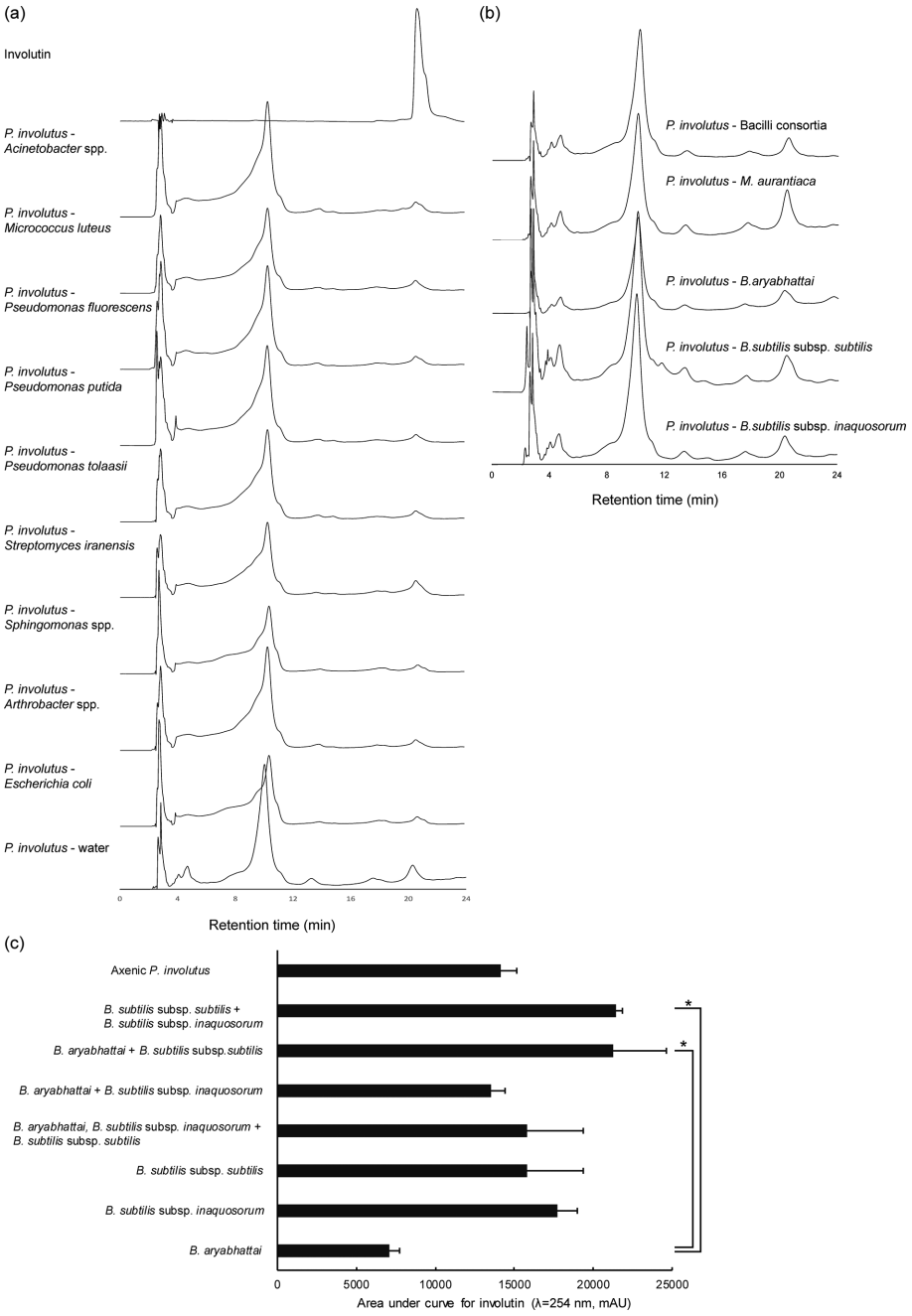

Fig. 3. (a, b) Representative HPLC chromatograms (λ=254 nm) of *P. involutus* co-cultures. Chromatograms are proportionally scaled. (a) Testing growth condition I using various bacteria from Table 1 whereby no substantial accumulation of involutin was observed. Spectroscopic data for involutin is shown in Fig. S6. Co-cultures with *B. subtilis* 3610 and other growth manipulations of *B. subtilis* are shown in Fig. S4. (b) Testing growth condition II using various soil-isolates. Again, no substantial accumulation of involutin was observed. (c) Mushroom soil-isolates, *Bacillus aryabhattai, B. subtilis* subsp. *inaquosorum*, *B. subtilis* subsp. *subtilis,* were both individually co-incubated with *P. involutus* as well as in various consortia in organic nitrogen-rich medium (condition III). The arithmetic mean of involutin titres (HPLC, λ=254 nm) from three biological replicates and standard error are shown. Statistical significance was observed between pairwise *B. aryabhattai* and two tripartite co-cultures (*P<0.01), but no statistical significance was observed between the control (axenic *P. involutus*) and the remaining samples. Representative chromatograms are shown in Fig. S4.

possesses both the capacity to promote and suppress fungal growth [46]; and the presence of the symbiotic plant partner affects the secretion of fungal bioactive compounds [47]. Thus, we cannot definitively exclude that other bacteria could have stimulated pigmentation in *P. involutus*. Similarly, there may have been other fungal responses that

were missed because we only looked for pigmentation in order to draw comparisons with *S. lacrymans*. Still, the conditions that induced and altered pigmentation in *S. lacrymans* and *O. olearius* do not parallel the observations from *P. involutus* and *S. bovinus*. The dissimilar pigment response was further investigated bioinformatically and biochemically to help explain the discrepancy.

Dissimilar genetic regulation of atromentin synthetase genes

Our initial observation between orthologous atromentin biosynthetic gene clusters of 12 basidiomycetes was a common promoter motif shared in the clustered atromentin synthetase (*NPS*) and aminotransferase genes (*AMT*) [9]. We now expanded our search to a total of 23 atromentin-producing basidiomycetes [17]. Applying MEME software [21, 22] to discover ungapped motifs in upstream sequences of the clustered genes to the new, extended set of fungi genomes, we again observed the aforementioned core promoter motif, termed motif 1, for *NPS* and *AMT* which was absent for most clustered alcohol dehydrogenase genes (Fig. 4a; Table S3). In addition, we not only found remarkable conservation of the atromentin clusters and respective promoter motifs, but also of the whole orthologous *NPS* promoter regions, suggesting that promoters co-evolved with their respective genes (Fig. 4b; combined tree: Fig. S7). The high degree of conservation gave rise to our initial hypothesis that multiple basidiomycetes would have a similar pigmentation response to bacteria.

As only *S. lacrymans*, but not *P. involutus*, was stimulated for pigment production by bacteria, we re-inspected the upstream regions of the atromentin clusters to look for variations in transcriptional regulatory elements. Aiming at the differences between ectomycorrhizal and non-mycorrhizal/brown-rot fungi, we confronted these two groups using representative species. For a more focused search, we used subgroups from the larger groups as shown in Fig. 4(a). Within the ectomycorrhizal group, the first subgroup was termed the 'larger *Paxillus* group', denoted with *, and therein also '*Paxillaceae*', denoted with **. From the non-mycorrhizal/brown-rot group, the subgroup was termed the '*Serpula* group', denoted with *.

As mentioned, promoter sequences followed the same evolutionary paths as their cognate genes. A MEME search for promoters of all 23 species confirmed that there were no further motifs other than the core motif 1 between species within the 'larger *Paxillus* group' and '*Serpula* group'. The promoters of the '*Paxillaceae* group' were highly conserved and this conservation was unusually high for fungal promoters [on average ~80 % similarity with up to 100 % coverage (Table 2)]. *Hydnomerulius pinastri* had the least amount of coverage when compared to the other species, likely due to divergence within the *Paxillaceae*. The overall conservation of the promoter sequences suggested the possible occurrence of other motifs shared within. To find the motifs that were specific to either group, we ran a discriminative MEME search confronting the 'larger *Paxillus*' and '*Serpula*' groups. No significant motifs were predicted for the '*Serpula* group', possibly because of higher phylogenetic divergence within the group [6, 39]. Promoters of the 'larger *Paxillus* group', in contrast, showed several interesting features that were not present in the '*Serpula* group'. First, we found highly conserved palindromic sequences around the core motif 1 of the *NPS* in *Paxillaceae*, and in two *Suillus* spp. (*S. brevipes* and *S. luteus*), as shown underlined in Fig. 4(a). Interestingly, a separate search thereafter showed the observed palindromic sequence in *Pisolithus microcarpus* (family: *Sclerodermataceae*). Secondly, we found two further statistically significant motifs (motif 2: consensus DYRSDCABSBBB, *E-value* 1.3e-004; and motif 3: consensus YGARYCRRNBM, *E-value* 1.2e-003) in the promoters of the 'ectomycorrhizal group' that were absent in *S. lacrymans* and other representatives of non-mycorrhizal/brown-rot fungi (Fig. 4a: motifs 2 and 3). Motif 2 seemed as prevalent as motif 1 for all examined ectomycorrhiza fungi, even in the distantly related *Thelephora ganbajun*. With one exception, *Hydnomerulius pinastri*, a brown-rotter that is monophyletic within the ectomycorrhizal '*Paxillaceae* group', contained the additional observed motifs. The motif search results and their respective position to the 'start' site and *P*-values are listed in Table S3.

To summarize, we found significant differences in the promoter structure of *NPS* in *S. lacrymans* and *P. involutus*, with the latter possessing a highly conserved pattern of three motifs. Even though the core motif 1 was present in the majority of the atromentin-producing fungi and thus the transcription factor in question could recognize each binding site similarly, the fact that there exists a palindromic sequence around the core motif as well as a co-occurrence of two additional motifs (possible composite elements) for *P. involutus* indicated that there may be a different regulatory mechanism, e.g. involving homodimer-binding and co-transcription factors. Many species considered to be ectomycorrhiza-forming also share brown-rot mechanisms, and many of the species in question formed a paraphyletic group [39]. It is possible that the additional genetic regulation involved in the biosynthesis of atromentin was the result of a divergence in lifestyle from brown-rot to symbiosis, especially with the formation of the *Paxillaceae* clade. We question how and why the distantly related *T. ganbajun* has the same motifs, the inconsistencies in the promoter regions between *S. luteus*, *S. brevipes* and the remaining *Suillus* spp., and which (co-)transcription factors are supposedly widely shared.

For basidiomycetes there is a scarcity of sustenance on regulatory mechanisms regarding natural products. For example, *in vitro* combined with *in vivo* evidence for basidiomycete transcription factors in natural product regulation is mostly undescribed. As a rare example, a putative transcriptional gene in *Ustilago maydis* was identified [48]. Deletion of said gene caused constitutive production of a siderophore under suppressed conditions, and the gene was speculated to encode for a zinc factor-like transcription

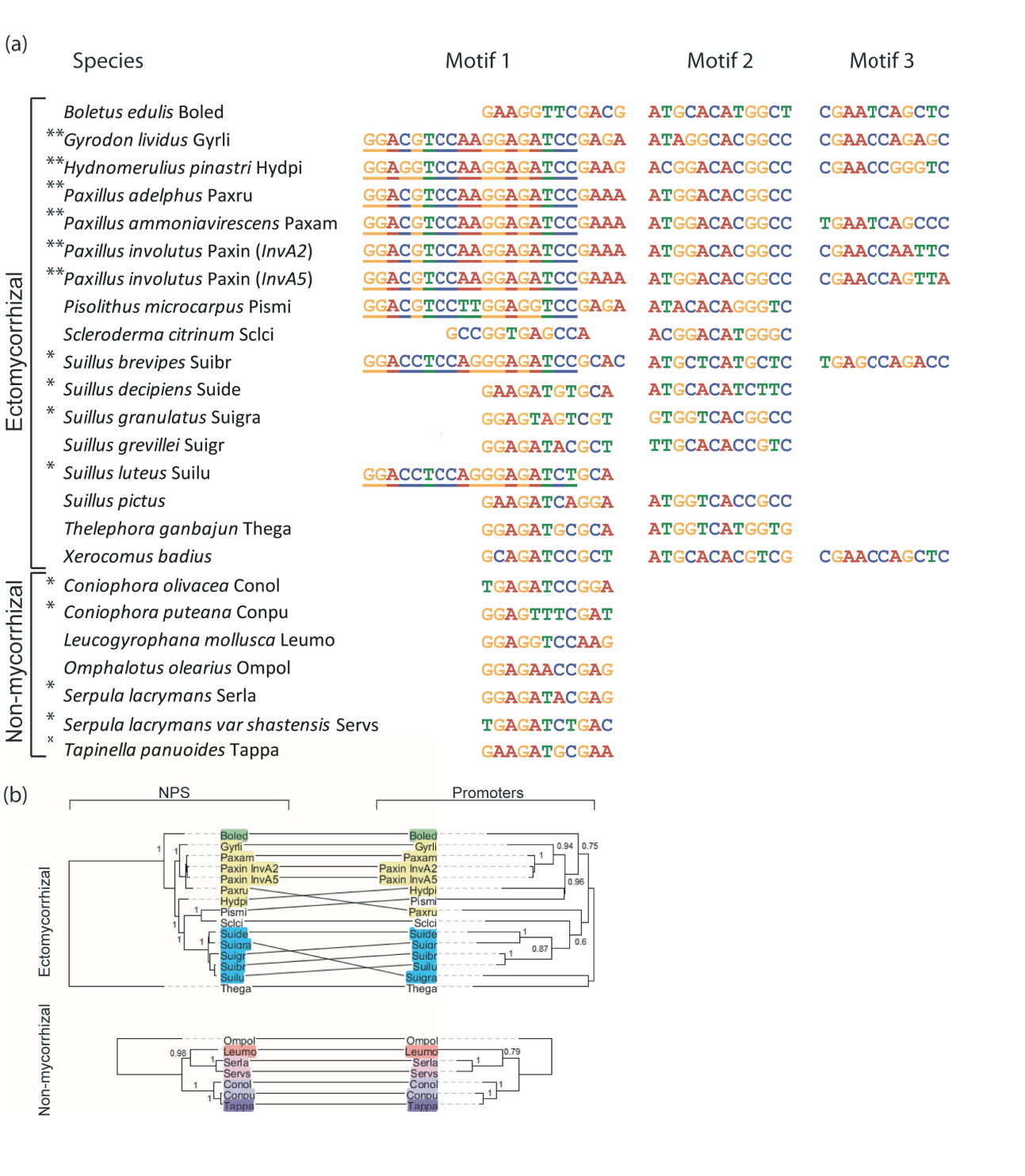

Fig. 4. *In silico* analyses of the upstream regions of annotated or characterized atromentin synthetase genes (*NPSs*) from various atromentin-producing basidiomycetes. Annotated genomes were accessed via the JGI MycoCosm portal, except for *T. panuoides* and *S. grevillei*. (a) A total of 23 atromentin-producing basidiomycetes, including abbreviation of species' names used in the tanglegram (below), that are grouped into ectomycorrhizal fungi and non-mycorrhizal/brown-rot fungi, and then further subgrouped. Within the ectomycorrhizal group, the first subgroup was termed the 'larger *Paxillus* group' (*), and therein '*Paxillaceae*' (**). Within the non-mycorrhizal/brown-rot group, the '*Serpula* group' (*). Motif 1 was shared by all fungi, whereas motifs 2 and 3 were only found in the ectomycorrhizal fungi group and in the brown-rotter *Hydnomerulius pinastri*. The palindromic sequence around motif 1, shared mostly in '*Paxillaceae*', is underlined. All motifs with their respective position to the 'start' and *P*-value are listed in Table S3. For *P. involutus*, two

atromentin synthetases (*InvA2* and *InvA5*) were used. (b) A tanglegram comparing trees'architectures that represent congruent evolutionary histories of the promoter region (nucleotide) and respective NPS (amino acids). The trees were built separately for the '*Paxillaceae*' and '*Serpula*' groups, and rooted by *T. ganbajun* and *O. olearius*, respectively. Only *NPSs* that were part of gene clusters were used for the tree reconstruction.

factor. The three largest families of fungal transcription factors are 'C_6 Zn cluster', 'C_2H_2 Zn finger' and 'HD-like' [49]. The Zn cluster family has progressively increased its distribution in the genomes from chytrids, to zygomycetes, basidiomycetes, and finally to ascomycetes (where it has the largest distribution), and is considered the most common family of transcription factors that regulate fungal gene clusters [49–51]. While we can get an idea of what families of transcription factors are associated with different fungal groups, their associated motifs are rarely experimentally verified for basidiomycetes (e.g. reviewed for *U. maydis* [52]). We preliminary searched our motif 1 from the ectomycorrhizal group against a motif database (JASPAR CORE (2016) fungi) in the tool TomTom [21]. Top hits were motifs associated with the Zn coordinating class of transcription factors, but no definitive conclusions could be made from such a search. With this knowledge though, it may not be overly zealous to presume that the transcription factor in question that regulates atromentin biosynthesis may fall into this class. Similarly, to our knowledge, there is no *in vivo* evidence describing a global regulator like LaeA in basidiomycetes, although, for example, Velvet domain-containing protein homologues that would associate with LaeA were noted in *Coprinopsis cinerea* as well as in most of the fungal kingdom [53–55]. Compounding our research for regulatory elements of the atromentin gene cluster was the fact that the gene cluster has no adjacent annotated regulatory genes. Therefore, we relied on motif searches for a first insight into possible regulatory mechanisms of the atromentin gene cluster. In conclusion, our approach suggested, although not yet experimentally proven, that additional regulatory requirements are involved in atromentin regulation for ectomycorrhizal fungi.

Bioactivity of pulvinic acid-type pigments

We investigated the bioactivity of the pigments to determine if the pigment response may have a specific role during co-incubation. We chose our mode *S. lacrymans* – *B. subtilis* co-incubation system as a model for further investigation. We first tested whether a growing colony of *B. subtilis* 3610 exposed to compounds freely diffusing from a filter disc would show phenotypic changes, mainly focusing on the formation of wrinkled colony biofilms [28, 56]. When *B. subtilis* was exposed to methanol as a control, atromentic acid, involutin, or atromentin, *B. subtilis* developed wrinkled colony biofilms that showed an opaque surface (i.e. no effect). However, upon exposure to variegatic or xerocomic acid, colonies developed flat or only slightly wrinkled colonies that showed decreased expansion on the agar plates (Fig. 5a). We first assumed that these effects might have been due to antimicrobial activity of the tested compounds. Therefore, we monitored the growth of *B. subtilis* in liquid cultures exposed to each pigment. After 18 h of incubation, we did not observe growth differences between cultures exposed to methanol (2.5 % v/v) or the pigments (0.25 mg ml^{-1}) (Fig. 5b). This was congruent with our previous results that the pulvinic acid-type pigments do not inhibit microbial growth [9].

Next, we examined the ability of variegatic and xerocomic acid to affect the motility behaviour of *B. subtilis*. Biofilm colonies of *B. subtilis* exposed to these compounds showed decreased colony expansion. We therefore monitored the swarming motility of *B. subtilis* colonies as they swarm across an agar plate whereby they would be challenged with an area infused with variegatic or xerocomic acid [29]. *B. subtilis* showed constant motility and swarming over an area infused with methanol (control) at a similar rate as on areas distant from the methanol deposition spot. In

Table 2. The promoter regions (−1000/+2 bp) of the five species from *Paxillaceae* showing the percentage of region similarity (% identity)

For *P. involutus*, two characterized *NPSs* were included. The grey shades show the percentage of coverage. Corresponding JGI protein IDs are listed in Table S3.

% identity	***P. involutus* (*InvA2*)**	***P. involutus* (*InvA5*)**	***Paxillus ammoniavirescens***	***Paxillus adelphus***	***Hydnomerulius pinastri***	***Gyrodon lividus***	
P. involutus (*InvA2*)	–	87 %	87 %	84 %	72 %	74 %	
P. involutus (*InvA5*)	87 %	–	85 %	83 %	82 %	72 %	90–100 %
Paxillus ammoniavirescens	87 %	85 %	–	83 %	92 %	73 %	70–90 %
Paxillus adelphus	84 %	83 %	83 %	–	80 %	72 %	50–70 %
Hydnomerulius pinastri	72 %	82 %	92 %	81 %	–	77 %	30–50 %
Gyrodon lividus	74 %	72 %	73 %	72 %	76 %	–	<30 %

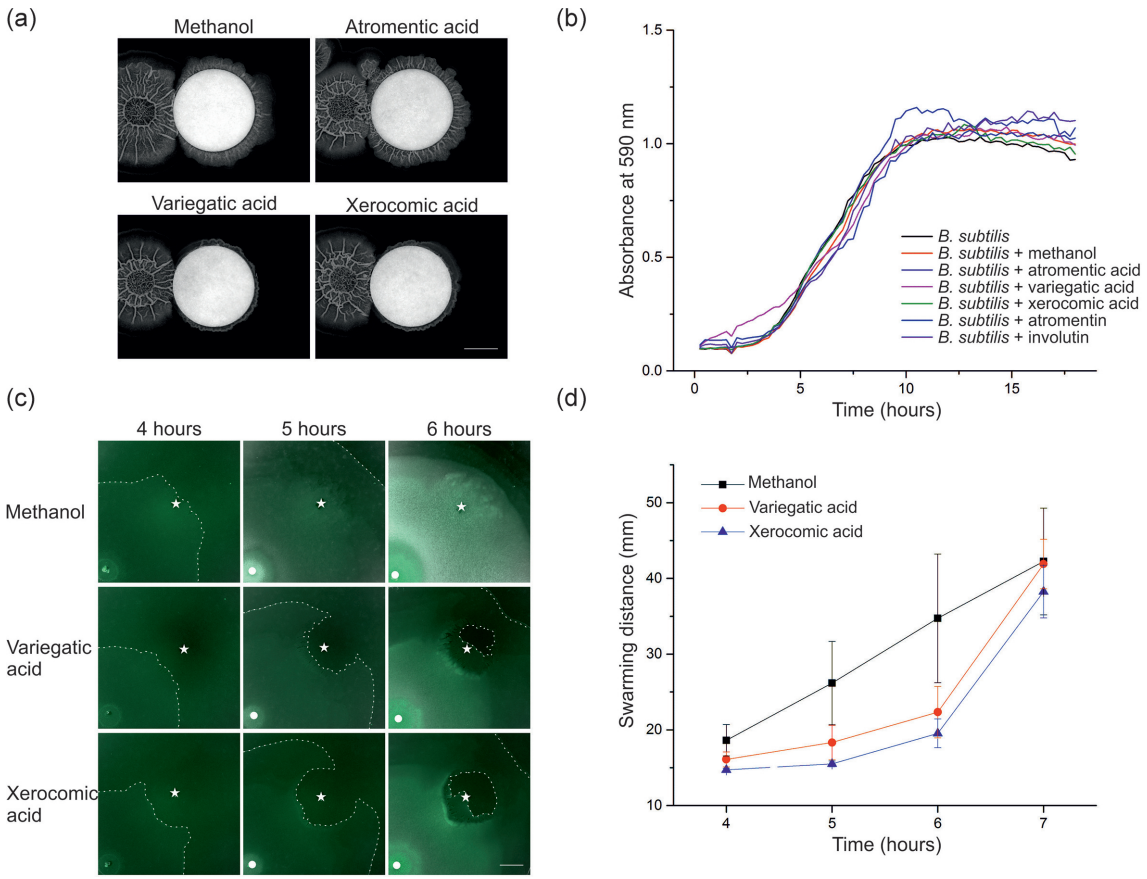

Fig. 5. Effect of diverse compounds on swarming colony expansion and spread of biofilm formation of *B. subtilis* 3610. (a) Effect of the pigments on colony biofilm development of *B. subtilis* 3610. Bright-field images of colonies are shown after 72 h of incubation. The scale bar represents 5 mm. (b) No antibiotic activity by the pigments was observed when accessed by OD_{590} kinetics of liquid cultures of *B. subtilis* 3610. (c) Delay in swarming colony expansion caused by variegatic and xerocomic acid. Composite and artificially marked images of green fluorescence and bright-field are shown. White circles mark the bacterial inoculation spot. White stars mark the deposition spot for tested compounds. Dashed white lines mark the edge of the expanding colonies. The scale bar represents 5 mm. The figure without markings is shown as Fig. S8. (d) Colony expansion after 4, 5, 6 and 7 h on 0.7 % LB agar when exposed to methanol, variegatic acid or xerocomic acid. Each data point represents the average of five independent colonies. Error bars represent SD.

contrast, swarming colonies of *B. subtilis* showed a delay in motility when exposed to variegatic or xerocomic acid (20 µg; Figs 5c, d, S8). Upon reaching the compound deposition spot (~4 h of incubation), the front of the swarming colony showed a delay in growth atop of the natural product-infused area, while *B. subtilis* continued to grow around the deposition area. After 7 h of incubation, the colony covered the natural product-infused area and continued to swarm, covering similar distances over the agar plate as colonies challenged with methanol.

Taken together, these results suggested that variegatic acid and xerocomic acid affected biofilm colony morphology of *B. subtilis* not as a consequence of antimicrobial activity, but rather by inhibiting the ability of the colonies to expand upon surfaces. It remains to be determined whether this nontoxic effect is because bacteria are able to extrude polyphenols and thus are resistant to these compounds [57], or the fungus is simply modulating the bacterium in its favour. Although *B. subtilis* utilizes different molecules for quorum sensing, compared to Gram-negative bacteria, we speculate that basidiomycetes have resources to modulate bacterial communications, given the example of lactonases from *Coprinopsis cinerea* that can cleave quorum sensing *N*-acyl-homoserine lactones [58]. Here, pulvinic acid-type pigments would be responsible for such influences. Alternatively, the significant reduction in pigmentation due to protease inhibition could be that external proteases are involved in swarming motility of *B. subtilis* [34]. The inhibition of proteases could have severely limited swarming and biofilm spreading of *B. subtilis* and thus *S. lacrymans* may not have required such a strong response to modulate *B. subtilis*. Conversely and noted beforehand, lack of released peptides from proteases may be the cause. Future research is warranted to study the influence of said compounds on the other bacteria

tested in our co-incubations and whether or not the pigments have overlapping functional roles.

Conclusions

Although the atromentin biosynthetic gene cluster appeared well conserved in basidiomycetes, our work revealed dissimilar, lifestyle-dependent pigment stimulation. We suggest that this phenomenon, at least in part, can be explained by the bioactivity of these pigments and by additional putative composite elements of the atromentin synthetase gene promoters. Hence, our results set the stage for further research to understand basidiomycete natural product regulation.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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