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Bacterial cell wall-degrading enzymes induce basidiomycete natural product biosynthesis

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Summary
Natural products play a vital role for intermicrobial interactions. In the basidiomycete arena an important representative is variegatic acid, a lactone natural product pigment whose ecological relevance stems from both inhibiting bacterial swarming and from indirect participation in breakdown of organic matter by brown-rotting fungi. Previous work showed that the presence of bacteria stimulates variegatic acid production. However, the actual external molecular trigger that prompts its biosynthesis in the mushroom hyphae remained unknown. Here, we report on the identification of Bacillus subtilis subtilisin E (AprE) and chitosanase (Csn) as primary inducers of pulvinic acid pigment formation. Using the established co-culture system of B. subtilis and Serpula lacrymans, we used activity-guided FPLC-based fractionation of B. subtilis culture supernatants and subsequent peptide fingerprinting to identify candidates, and their role was corroborated by means of a pigment production assay using heterologously produced chitosanase and subtilisin. B. subtilis mutants defective in either the aprE or the csn gene still triggered pigmentation, yet to a lower degree, which points to a multicausal scenario and suggests the combined activity of these cell wall polymer-attacking enzymes as true stimulus.

Introduction
The interaction of bacteria and fungi impacts life in numerous ways. For example, bacteria and fungi form microbiomes that colonize the respiratory tract of cystic fibrosis patients and thus severely compromise human health (Deveau et al., 2018). Bacterial-fungal interactions also maintain global biogeochemical cycles and nutrient availability: lignocellulose decomposing mushrooms coexist with nitrogen-fixing bacterial taxa which suggest a mutually beneficial situation of bacteria supplying nitrogen to the fungi which in return make carbon available to the bacteria (Purahong et al., 2016). Bacterial-fungal interactions frequently rely on low molecular weight compounds which supported drug discovery efforts as otherwise silent biosynthetic pathways for bioactive natural products are activated (Khalid and Keller, 2021). For example, the intimate contact between streptomycetes and Aspergillus nidulans hyphae was shown to transcriptionally activate a genetic locus for orsellinic acid biosynthesis (Schroeckh et al., 2009, Nützmann et al., 2011). In stark contrast, the inducers for basidiomycete natural product biosynthesis in response to the presence of bacteria – and, beyond that, of external stimuli altogether – are only marginally understood. Wound-induced defence is a known phenomenon. For example, when the hymenium of the crust fungus Aleurodiscus amorphous is injured, cyanide is released from the pre-accumulated cyanogenic ether aleurodisconitril (Kindler and Spiteller, 2007). However, this type of defence represents an immediate short-term response and does neither pertain to a sustained de novo biosynthesis, nor is it stimulated in response to the presence of other microorganisms.

We previously reported on variegatic acid (Fig. 1) and other pulvinic acid-type fungal pigments, which impact
upon their surrounding microbial life as they inhibit bacterial swarming and biofilm formation (Tauber et al., 2018). Furthermore, brown-rotting fungi rely on variegatic acid as a Fe$^{3+}$ reductant as hydroxyl radicals for breakdown of organic matter are formed via Fenton chemistry (Eastwood et al., 2011, Shah et al., 2020). The common precursor of pulvinic acids is atromentin, a terphenylquinone, which is biosynthesized from L-tyrosine by the successive action of the aminotransferase AMT1 and atromentin synthetase NPS3, whose genes are encoded in a locus that is widely orthologous across various basidiomycetes. In S. lacrymans, i.e., the dry rot model fungus, the genes nps3 and amt1 are strongly induced following exposure to 13 bacterial species, including B. subtilis, Pseudomonas putida and Streptomyces iranensis. Transcriptional induction of the nps3 gene peaks at a 40 to 49-fold level after 48 h of cocultivation with the bacteria mentioned above (Tauber et al., 2016). Elevated expression levels were still observed after 7 days, e.g., nps3 transcript levels were 22-fold elevated in the presence of B. subtilis. Local yellow-orange mycelial pigmentation with pulvinic acids visually reflects this intermicrobial interaction. However, it remained obscure if de novo biosynthesis of pulvinic acids in the microbial consortium is triggered by a bacterial or by a fungal enzyme and which particular agent serves as elicitor.

In axenic fungal culture, pigment production was induced when commercially available cell wall-degrading enzyme blends ('lysing enzymes') or a protease were added (Tauber et al., 2018). While these results suggested that the induction cascade is initiated by an enzymatically caused lesion of the cell wall, this particular experimental setting remained artificial. Therefore, to identify the signal molecules relevant in the ecological setting, we here report on the activity-guided isolation of the pigment-inducing principle. Bacillus sp. are among the most abundant members of bacterial communities surrounding Serpula hyphae (Embacher et al., 2021). Consequently, we used the B. subtilis/S. lacrymans interaction as model and show that B. subtilis subtilisin E and chitosanase activity is required to induce the biosynthesis of pulvinic acids.

**Results**

**Identification of pigment-inducing enzymes**

During previous work on S. lacrymans, we noticed that pulvinic acid-type natural products were induced by B. subtilis and numerous other bacteria, and lytic enzymes (Tauber et al., 2016). These results prompted us to identify the natural agent that elicits the cellular signal cascade eventually inducing the biosynthesis genes. To this end, cell-free culture supernatants of B. subtilis grown in LB medium, containing the bacterial secretome, were applied onto unpigmented mycelium of axenically grown S. lacrymans. A first yellowish hue was apparent after 24 h, which then intensified over the following 2 days. LC–MS-analysis confirmed variegatic acid production (Fig. 1), while sterile LB medium, used as control, did not cause any pigmentation. Centrifugal filter devices were used to produce four fractions of the cell-free supernatant that contained molecules < 10, 10–30, 30–50 and > 50 kDa, respectively. LC–MS-based quantification...
proved that the fraction <10 kDa hardly induced variegatic acid production, whereas strongest induction was seen with the 10–30 kDa fraction (Fig. 1). Our results are suggestive of a water-soluble macromolecule, likely an enzyme, as inducer.

We used again cell-free *B. subtilis* culture supernatant to separate native enzymes chromatographically by FPLC (Fig. S1). After each chromatographic step, *in vivo* assays to detect pigment-inducing activity were performed with the fractions. Cell extracts were first subjected to anion-exchange chromatography (AIEC). Subsequently, positively assayed fractions were further separated by size-exclusion chromatography (SEC) on a Superdex 200 column. The most active SEC fraction was prepared for peptide fingerprinting, tryptically digested and subjected to LC–MS/MS-analysis. The mass fragments were searched against the UniProt *B. subtilis* strain 168 proteome database which identified 15 proteins (Table S1). Five hits, which include the serine protease subtilisin E, chitosanase, an endo-β-1,3(4)-glucanase, an endo-β-1,4-xylanase and expansin, i.e., a non-catalytic protein binding to cell walls and promotion their expansion, Table 1) represented the most plausible candidates, as (i) their molecular masses fell into the previously determined range of 10–30 kDa, and (ii) they catalysed degradation of cell wall components (or in the case of expansin, at least bind to cellulose or chitin), which is consistent with our previous observations of lytic enzymes acting as inducers.

**Identification of pigment inducers using *B. subtilis* gene deletion mutants**

The above findings are coherent in a sense that (i) plausible cell wall-related proteins/lytic enzymes were found that would explain the phenomenon and (ii) they match our previous observations with commercial enzymes (Tauber et al., 2018). However, some ambiguity remains, as we cannot resolve, which of the candidate proteins, or more than one, act(s) as trigger(s). Consequently, we followed an independent approach to identify the most likely candidate(s) and used culture supernatants of the respective *B. subtilis* mutants defective in either *aprE*, *csn*, *bglS*, *xynA* or *yoaJ* (Koo et al., 2017).

In quintuplicated assays, supernatants were applied undiluted or in diluted form (1:2; 1:4; 1:8). Variegatic acid induction in *S. lacrymans* mycelium was chromatographically compared relative to the effect of diluted or undiluted supernatants of *B. subtilis* wild type (Fig. 2). We hypothesized that a scenario with only one single enzyme as inducer will not lead to any pigmentation, regardless of the dilution, when the respective gene is inactivated. In case of multiple inducers, one inducing activity is eliminated by the gene deletion, while pigmentation is still expected to occur through a second enzymatic activity, which gradually decreases when increasingly diluted.

In fact, drastically decreased variegatic acid contents were found for the supernatants of both the *aprE* and *csn* deletion mutants (subtilisin E and chitosanase, Fig. 2). The undiluted supernatants of either mutant still induced virtually like the wild type strain, whereas the median values of 1:4 dilutions showed a 57% lower production of variegatic acid for the subtilisin E mutant (*ΔaprE*), compared to wild type, and a 43% lower production with the chitosanase mutant *Δcsn*. For the 1:8 dilutions, values as low as 86% and 83% were found for *ΔaprE* and *Δcsn*, respectively. The data analysis, i.e., the box-and-whiskers plot, of these two mutants returned definitive results (Fig. 2) through decreasing median values with increasing dilution. However, the supernatants of the other mutants (gene deletions for expansin *yoaJ*, xylanase *xynA* or endo-β-1,3(4)-glucanase *bglS*) did not produce a consistent picture, as the error range includes both positive and negative values, and median values above or below the wild type control, which indicates both increased and decreased pigment titres, compared to wild type. Notably, in the cases of *yoaJ*, *xynA* or *bglS* mutants, subtilisin E and chitosanase are expected to reach wild type levels. Collectively, these results indicate that these two enzymes contribute most to this natural product stimulation and that this bacterium/fungus interkingdom interaction is mediated by more than one trigger.

**Validation of pigment induction with recombinantly produced proteins**

Although subtilisin E and chitosanase mutants showed the most pronounced effects, we heterologously produced all

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**TABLE 1. *B. subtilis* proteins, identified as potentially pigment-inducing. The first molecular mass of subtilisin E refers to the complete pre-pro-protein. All molecular masses refer to the native, untagged proteins.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Encoding gene</th>
<th>Verified function</th>
<th>Molecular mass (in kDa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilisin E</td>
<td><em>aprE</em></td>
<td>Proteolysis</td>
<td>39.4 (pro-pre-subtilisin)</td>
<td>Henner et al., 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>27.7 (subtilisin)</td>
<td></td>
</tr>
<tr>
<td>Chitosanase</td>
<td><em>csn</em></td>
<td>Chitosan degradation</td>
<td>31.3</td>
<td>Rivas et al., 2000</td>
</tr>
<tr>
<td>Endo-β-1,3(4)</td>
<td><em>bglS</em></td>
<td>Glucan/lichenan-degradation</td>
<td>27.1</td>
<td>Murphy et al., 1984</td>
</tr>
<tr>
<td>Endo-β-1,4-xylanase</td>
<td><em>xynA</em></td>
<td>Xylan degradation</td>
<td>23.2</td>
<td>Lindner et al., 1994</td>
</tr>
<tr>
<td>Expansin</td>
<td><em>yoaJ</em></td>
<td>Cellulose/chitin-binding</td>
<td>25.5</td>
<td>Kerff et al., 2008</td>
</tr>
</tbody>
</table>

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five proteins in E. coli as C-terminally hexahistidine-tagged fusion proteins and purified them via Nickel affinity chromatography. Subtilisin was heterologously produced as a leader peptide-less polypeptide which then autocatalytically removed the propeptide domain to yield the mature 27.7 kDa proteolytically active enzyme. The purification was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and yielded proteins of the expected size (Fig. S2, Table 1). Subtilisin E was visualised by SDS-PAGE and identified due to its proteolytic activity, as described (Bjerga et al., 2016). LC–MS/MS identified four subtilisin E peptide fragments which supports heterologous production of the correct enzyme. Following elution, we first verified the authenticity of the other four produced proteins by MALDI-TOF. The proteins were tested for enzymatic activity in vitro (Fig. S3). Chitosanase was incubated with chitosan, endo-β-1,4-xylanase with beechwood xylan, and endo-β-1,3(4)-glucanase with lichenan (to test for endo-β-1,3(4)-glucanase activity) or laminarin, respectively, assaying specifically for endo-β-1,3-glucanase activity. The polysaccharide-depolymerizing activity was quantified by p-aminobenzoic acid in a colorimetric assay which yields a yellow colour in the presence of reducing sugar monomers (Lever, 1972). For all enzymes, degrading activity was found (Fig. S3). The enzymatic activity of subtilisin was confirmed by the milk agar assay, which indicates proteolytic activity by a halo-like cleared zone that develops around the site of enzyme application in the otherwise turbid agar. This assay was positive, indicative of active recombinant enzyme, yet the halo was absent in controls with protein extract of E. coli transformed with the empty expression vector. In parallel, subtilisin was incubated with 2 mM...
phenylmethylsulfonylfluorid (PMSF), an established serine protease inhibitor, which decreased subtilisin's protease activity to 35%. Full inhibition of the proteolytic activity was not observed, which may be due to partial PMSF inactivation in aqueous preparations (James, 1978). Expansin does not possess enzymatic activity, yet shows a characteristic binding to cellulose, a property that was used for the assay. Expansin was therefore mixed with cellulose, and protein bound to cellulose was removed from the solution by centrifugation, which was verified by SDS-PAGE (Fig. S3). Once all recombinant proteins were confirmed active in the respective assays, we exposed *S. lacrymans* mycelium, grown on agar plates for 3 days, to equal amounts of the recombinant proteins to investigate, which of the candidates induces variegatic acid formation. Expansin did not trigger any pigment development (Fig. 3), and not even a faint hue was visually noticeable. Both endo-β-1,4-xylanase (XynA) and endo-β-1,3(4)-glucanase (BglS) caused very moderate, statistically insignificant pigment induction (approximately 2 μg mg⁻¹ fresh biomass). Consistent with the outcome of the tests with *B. subtilis* mutants, the most pronounced pigment induction was found for subtilisin E (AprE) and chitosanase, which is reflected by seven- and fivefold, respectively, higher variegatic acid titres. A control with heat-treated chitosanase was not catalytically active and, consequently, did not induce pigmentation (Fig. S3). We therefore identified both a polysaccharide-degrading and an extracellular protein-degrading enzyme as inducers, and found the protease subtilisin E as the strongest inducer.

**Pigment induction by lytic enzymes of other microorganisms**

The above results may be viewed as representative of a natural microbial environment, as microorganisms typically secrete multiple lytic enzymes simultaneously. To investigate whether lytic enzymes of other microorganisms lead to the same response in *S. lacrymans*, we tested lytic enzymes of different species, i.e., commercially available *Streptomyces coelicolor* chitosanase (SCO0677), *Tritiachium album* proteinase K (ProK) and a *Thermotoga neapolitana* endo-β-1,3-glucanase (laminarinase A, LamA). Consistent with the findings made with *Bacillus* enzymes, variegatic acid production was found with both ProK and the chitosanase SCO0677. Comparable amounts of the pigment were observed (14.1 vs 8.1 μg mg⁻¹ fresh *Serpula* biomass, Fig. 4). As observed with the *Bacillus* enzymes, the endo-β-1,3-glucanase LamA only caused a minor pigmentation response.

**Pigment induction by a fungus-fungus interaction**

To reflect the presence of fungi in more complex microbial consortia, we also tested if pigment formation is induced by *Beauveria bassiana* a filamentous fungus well...
known for secreting proteases (Joshi et al., 1995). We also included the yeast Debaryomyces hansenii, i.e., a fungus which is known not to secrete proteases (Ahearn et al., 1968). During axenic growth, these fungi were individually tested for secretion of proteases (milk agar test) and chitinolytic enzymes (chitin agar). While neither activity was detected for D. hansenii, B. bassiana displayed secretion of proteases, but not extracellular chitinase/chitosanase activity. These two fungi were co-incubated with S. lacrymans, and variegatic acid production was analysed by LC–MS (Fig. 5). In fact, B. bassiana induced production of variegatic acid in S. lacrymans. Since no secretion of chitin-degrading enzymes was detected, the protease activity seemed sufficient to stimulate pigment production. As expected, D. hansenii did not induce any pigmentation in S. lacrymans. To exclude that induction results from mechanical damage, the mycelium was physically wounded. However, pigment formation was not observed (Fig. S4).

**Pigment induction by low molecular weight compounds**

In plants, oligosaccharides trigger defence reactions and small molecule production, e.g., jasmonic acid and abscisic acid (Hadwiger, 2013). Therefore, we could not discount the possibility that a small molecule, released from cell wall by enzymatic activity, contributes to fungal pigment induction as well. Oligopeptides or monomeric amino acids appeared unlikely as inducers as they were present in the fungal medium (yeast extract) also used for negative controls. To determine if enzymatically released cell wall fragments such as chitoooligosaccharides induce variegatic acid production, partially hydrolysed chitosan samples with an average degree of oligomerization of 6.9, 3.6 and 1.4, were tested, i.e., a range of chitoooligomers that was previously established to induce responses in plants most strongly (Aziz et al., 2006, Winkler et al., 2017). For comparison, monomeric D-glucosamine and untreated polymeric chitosan was included as well. Only marginal pigmentation was found for D-glucosamine, chitosan and all chitoooligomer fraction, regardless their molecular weight (Fig. S5). This finding excluded oligomeric chitosan as a major inducer.

**Cell wall analysis**

Although a broad survey on cell wall composition in basidiomycetes exists (O’Brien and Ralph, 1966), data on cell wall composition is still scarce for species of the order Boletales, which S. lacrymans taxonomically belongs to. To verify that the substrates are present in the fungal cell wall, for which bacterial lytic enzymes had been detected, we analysed the composition of the Serpula cell wall. The fractionation was based on boiling and alkali treatment, followed an established procedure (Henry et al., 2016) and led to three fractions (Fig. S5). The first fraction (referred to as SDS–βM) used sodium dodecyl sulfate and β-mercaptoethanol to solubilize, wash off and collect proteins embedded in, yet not covalently linked to, the fungal cell wall, as well as non-covalently bound saccharides. Subsequently, the remaining fungal wall material was boiled in alkaline solution. The alkaline soluble fraction (AS) primarily contains hexoses, while chitin, and glucans associated with chitin, remained in the alkaline insoluble matter (AI). The SDS–βM, AS and AI fractions represented 42%, 22% and 36%, respectively, of the total cell wall biomass.

The fraction SDS–βM was the major fraction and consisted of about 60% protein (Fig. S6), as estimated by bicinechinonic acid (BCA) assays (Smith et al., 1985). Gas chromatographic saccharide analysis identified four monosaccharides, i.e., mannose, glucose, galactose and (N-acetyl)glucosamine, with galactose being quantitatively the most abundant sugar monomer and virtually absent from the AI and AS fractions. The AI fraction contains two major monosaccharides, glucose and (N-acetyl) glucosamine in similar amounts. However, this chromatographic step does not distinguish N-acetylg glucosamine and non-acetylated glucosamine. Therefore, the 3-methyl-2-benzothiazolone hydrazone (MBTH) colorimetric assay (Gressler et al., 2019) was subsequently used to quantify the amount of primary amino groups, i.e., the specific amount of glucosamine. This analysis revealed that 95% of the glucosamine residues were N-acetylated (if the AI and AS fractions are considered) which translates into about 17% chitin in the Serpula cell wall, calculated from the total mass of the AS and AI.
fractions. In the AS fraction, the degree of N-acetylation of chitin was 78%, i.e., lower than in the AI fraction (95.5%). The AS fraction was also characterised by the presence of 77% hexoses, absence of hexosamines and 22% of protein. The presence of proteins which were not solubilized by the prior SDS/β-mercaptoethanol treatment suggests that they are potentially covalently linked to insoluble polysaccharides, as described in yeast (Orlean, 2012). Furthermore, xylose was only found in the AS, but not in the other fractions. Our analysis of S. lacrymans cell walls identified a high chitin and protein content, compared to model fungi such as Aspergilli, yet comparable to other basidiomycetes (O’Brien and Ralph, 1966). Therefore, the serine protease subtilisin E and the Csn chitosanase, identified from Bacillus supernatants and verified as strongest pigment inducing factors, do have ample and plausible substrates in the Serpula cell wall.

Discussion

Bacterium/fungus interkingdom interactions maintain numerous ecosystem functions, impact plant, animal and
human health, and have thus been the subject of intensive previous research, reviewed e.g., by Deveau and colleagues (2018), Scherlach and Hertweck (2020), Steffan and colleagues (2020) and Khalid and Keller (2021). Investigations pertaining to the role of small molecule natural products showed that Aspergillus nidulans or A. fumigatus natural product pathways were triggered by the presence of and direct physical contact with Streptomyces rapamycinicus (Schroeckh et al., 2009, König et al., 2013). Likewise, a small Ralstonia solanacearum lipooligopeptide, ralsolamycin, elicited bikaverin production in F. fujikuroi (Spraker et al., 2018). Another case of a small molecule-mediated interaction between a pro- and a eukaryote pertains to the Pseudomonas protegens cyclic lipopeptide orfamide A (Aiyar et al., 2017) as this compound induces Ca²⁺ signalling and rapid deflagellation in the unicellular green alga Chlamydomonas reinhardtii, i.e., the prokaryote paralyses the eukaryote.

While the above interactions involved partner of various phylogenetic groups, they all reflect specialised, species- or genus-specific crosstalk between organisms. Our results add new aspects to the question how intermicrobial interactions impact on natural product biosynthesis. We show that primarily two B. subtilis polymer-degrading enzymes, the serine protease subtilisin E (AprE) and chitosanase (Csn), elicit natural product biosynthesis in S. lacrymans. The results were repeatable with proteases and chitosanases of other microorganisms and with the protease-secreting fungus B. bassiana, which points to a general, unspecialized chemical response, dissimilar from the above-mentioned interactions. The unspecialized character, presented here, is supported by previous work on S. lacrymans and other basidiomycetes, e.g., the wood-rotting variegate acid producer Omphalotus olearius, which responded to the presence of both Gram-negative and Gram-positive bacteria with pigment production (Tauber et al., 2018). Taken together, our results reflect a general principle that is not restricted to a particular species or genus, but appears broadly distributed among various basidiomycete genera. Unlike the above Aspergillus and Fusarium-related work, the Bacillus/Serpula interaction did not require direct contact between the bacterium and fungus to induce natural products, nor was a small molecule the mediating agent. Furthermore, variegate acid, i.e., the fungal compound is a motility inhibitor (Tauber et al., 2018), which again contrasts the Chlamydomonas/Pseudomonas interaction, in which the prokaryotic partner releases a natural product to immobilize the eukaryote.

Chitinolytic enzymes have been recognised to help the bacterium Burkholderia rhizoxinica enter the hyphae of its partner fungus, the plant pathogenic zygomycete Rhizopus microsporus, and thus play a key role in establishing an endosymbiotic partnership. The bacterial endosymbiont then confers the capacity on the fungus to produce the antimitotic and phytotoxic agent rhizoxin (Moebius et al., 2014). In this work, a chitosanase was identified as one major factor that stimulates natural product biosynthesis in a non-symbiotic system in which the fungus is the actual producer. Chitosan fragments, released through the action of chitinolytic enzymes, elicits plant defence reactions both locally and systemically, also including the stimulating small molecule production, such as jasmatic acid and abscisic acid (Hodwig, 2013). However, our results for the bacterium/ basidiomycete system point away from a signalling function as oligomeric chitosan did not lead to a pronounced natural product stimulation.

We therefore assume a starkly dissimilar signalling mechanism in the case of the basidiomycetes. We also assume that is the enzymatic activity as such, not the released saccharide fragments, that initiates a signalling cascade, probably as a consequence of the fungal cell wall and its intrinsic proteins that become impacted by enzymatic activities. While our work sheds light onto the inducing mechanism (summarized in Fig. 6), follow-up work is warranted to identify elements of the signalling pathway and how the information is relayed from the exterior to the nucleus. Knowledge on such pathways, e.g., the cell wall integrity signalling cascade (Valiante et al., 2015, Levin, 2005) or the high osmolarity glycerol (HOG) pathway (Roman et al., 2020, Hohmann, 2009) is primarily based on ascomycete model organisms, such as Aspergillus fumigatus, Saccharomyces cerevisiae or Candida albicans. Signalling and regulation of natural product production is an active area of research, and it is expected that further insights will be gained in the future.

![Fig. 6. Schematic summary of basidiomycete natural product biosynthesis induced by lytic enzymes.](wileyonlinelibrary.com)
product genes in basidiomycetes is generally poorly understood, and even more so in response to environmental cues. Our results established the first element in the cascade and how a model basidiomycete relies on degrading enzymes, produced by bacteria surrounding its hyphae, to induce the production of bioactive natural products, which underscores the relevance of intermicrobial interactions for chemical ecology and natural product research.

**Experimental procedures**

**Axenic microbial cultivation and co-cultivation**

*S. lacrymans* S7 (Eastwood et al., 2011) was grown on MEP agar (malt extract 30 g l⁻¹, peptone 3 g l⁻¹, agar 18 g l⁻¹, pH 5.6) for 10 d at room temperature in the dark. *B. subtilis* 168 (Branda et al., 2001) wild type and mutants (aprE, *B. subtilis* BKE10300; csn: *B. subtilis* BKE26890; bglS: *B. subtilis* BKE39070; xynA: BKE18840; yoaJ: *B. subtilis* BKE18630, Bacillus Genetics Stock Center, Columbus, OH) were cultured in 50 ml LB medium (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, 10 g l⁻¹ NaCl, pH 7). The medium was inoculated with one colony and incubated overnight at 37°C and 180 rpm. To collect extracellular enzymes for activity tests, the culture was centrifuged at 13,500 × g, sterilized by filtration, and subsequently fractionated by Amicon centrifugal filter device (molecular weight cut-off 10 kDa, 30 kDa and 50 kDa). *Debaryomyces hansenii* SF013940 and *Beauveria bassiana* SF013941 were obtained from the Jena Microbial Resource Collection (JMRC) and were routinely cultivated on LB and MEP agar, respectively, at room temperature.

**In vivo activity assays**

*S. lacrymans* was grown on MEP agar plates (25 ml per plate) for 10 d. *B. subtilis* cell-free supernatant and fractions thereof (in triplicate) for pigment induction by applying 100 μl of supernatant or of the fractions (FPLC or size exclusion filtration) directly onto the mycelium and incubation for another 3 d in the dark. Non-inoculated LB medium (100 μl) served as control. Variegatic acid produced by the fungus was quantified as described in the Supporting Information. Inducing activity of supernatants of *B. subtilis* mutants (inactivated genes: aprE, csn, bgIS, xynA or yoaJ) was done identically, but additionally applying a dilution series (1:2, 1:4, 1:8, v/v, diluted with sterile LB medium).

The pigment-inducing activity of enzymes (subtilisin E, chitosanase, endo-β-1,3(4)-glucanase, endo-β-1,4-xylanase, expansin, heterologously produced in *E. coli* strains) was tested by pipetting 100 μl of solution of the respective enzyme directly onto the *S. lacrymans* mycelium, grown as described above and incubated for 3 d in the dark. *Bacillus* enzymes/proteins (AprE, Csn, BglS, XynA and YoaJ), LamA and SCO0677 (Zvetlov et al., 1997, Heggset et al., 2010) and ProK (Sigma-Aldrich) were added to the reactions at 1.6–3.2 nmol. As controls, protein extracts of *E. coli* transformed with insertless expression vectors (pET28 or pET28aH6TEV, see below) and LB medium were applied. Hydrolysed chitosan was prepared and used as described in the Supporting Information. To assay if *B. bassiana* and *D. hansenii* (and *B. subtilis* for comparison), have variegatic acid-inducing, proteolytic or chitosan–/chitin-degrading activity, an aliquot of an overnight LB culture of *D. hansenii* or *B. subtilis* or a MEP agar culture of *B. bassiana*, were used to inoculate a MEP agar plate. One half of the plate was replaced (i) with *S. lacrymans*, pre-grown on MEP agar, (ii) non-inoculated milk agar (per 100 ml H₂O: 1 g milk powder, 1% agarose) or (iii) with chitin agar (acid-swollen chitin 10 g l⁻¹, (NH₄)₂SO₄ 3 g l⁻¹, KH₂PO₄ 2 g l⁻¹, MgSO₄ × 7 H₂O 0.3 g l⁻¹, Calcofluor White 1% (v/v), pH 6.5). The plates were incubated for 3 d and then analysed (Supporting Information).

**Purification and identification of native Bacillus proteins**

Native proteins were purified by sequential anion exchange (AEIC) and size exclusion chromatography (SEC) on a GE Healthcare Äktapure 25 Fast Protein Liquid Chromatography (FPLC) System. Three biological and three technical replicates were run. Fractions were screened for activity by the assay described above. The proteins were purified from the centrifuged (30 min, 4°C, 13,750 × g) and filter-sterilized supernatant of a 50 ml *B. subtilis* overnight culture (LB medium, OD₆₀₀ = 5, filter pore size 0.22 μm). Buffer A was: 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 50 mM NaCl, pH 6.5, buffer B was buffer A with 1.5 M NaCl; pH 6.5. For AEIC, 50 ml of this supernatant were loaded on a HiTrap Capto ImpRes column (5 ml bed volume) which had been equilibrated before with 5 column volumes (CV) of buffer A. After loading, the column was washed with 5 CV buffer B. For elution, a step gradient at a flow rate of 5 ml min⁻¹ was applied: initially 5% buffer B; increase to 19% B in increments of 2%, then to 30% and in 10% steps to 50%, then to 100% B. Each step included 7 CV of mobile phase. During elution, 12 fractions were collected and individually tested for pigment-inducing activity (above). The most inducing fraction was reproduced identically from a fresh *B. subtilis* culture and concentrated on a MerckMillipore Amicon centrifugal filter (10 kDa cut off). From the concentrate, 2 ml were loaded on the SEC column (Superdex 200 increase 10/300 Gl column, 24 ml bed volume, equilibrated in buffer A), fitted to the above FPLC instrument. For elution, buffer A at a flow of
0.5 ml min⁻¹ was applied. Subsequently, the proteins of inducing FPLC fractions were analysed by LC–MS/MS-based peptide fingerprinting. Fractions were solubilized in 50 mM triethylammonium bicarbonate (TEAB) in 50% (v/v) trifluoroethanol. Proteins were reduced and alkylated for 30 min at 70°C with 10 mM TCEP (tris(2-carboxyethyl)phosphine) and 12.5 mM chloroacetamide (final concentration). Subsequently, the samples were further purified, following the precipitation protocol of Wessel and Flugge (1984). Protein precipitates were resolubilized in 100 mM TEAB and digested overnight (18 h) with a trypsin + LysC mixture (Promega) at a protein:protease ratio of 25:1. Samples were evaporated in a vacuum centrifuge, solubilized in 25 μl of 0.05% TFA in water:ACN 98:2 (v/v) and filtered prior to injection. LC–MS/MS analysis was carried out on an Ultimate 3000 nano RSLC system coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) as described (Nossmann et al. 2019). To identify the proteins in the FPLC fractions, tandem mass spectra were searched against the UniProt reference Bacillus subtilis strain 168 proteome database (2018/09/13; https://www.uniprot.org/proteomes/UP000001570), using Proteome Discoverer (PD) 2.2 (Thermo) and the algorithms of Sequest HT (version of PD2.2) and MS Amanda 2.0, applying previously described settings and validation strategies (Nossmann et al., 2019).

Production and purification of recombinant Bacillus proteins

To heterologously produce C-terminally hexahistidine-tagged fusion proteins of chitosanase (Csn), endo-β-1,3 (4)-glucanase (BglS), endo-β-1,4-xylanase (XynA) and expansin (YoaJ), E. coli SoluBL21 was transformed with expression plasmids pSHb05, pSHb06, pSHb12 and pSHb10, respectively (see Supporting Information for plasmid construction). C-terminally hexahistidine-tagged subtilisin E (AprE) was produced in E. coli BL21 (DE3) transformed with pSHb18. For protein production, E. coli seed cultures were grown in 5 ml liquid LB medium, supplemented with kanamycin, overnight at 37°C and 180 rpm and used to inoculate 400 ml LB-kanamycin medium as main culture, incubated under the same conditions. Upon reaching an OD₆₀₀ = 0.6, transgene expression was induced by adding 200 μM IPTG, and the temperature was decreased to 16°C. The incubation continued overnight. Subsequently, the biomass was harvested by centrifugation (30 min, 4°C, 13,751 × g) and the cell paste was resuspended in 4 ml lysis buffer (50 mM phosphate buffer, pH 6.5, amended with 5 mM imidazole). Cells were sonicated, and the debris was removed by another centrifugation step (30 min, 4°C, 13,751 × g). The supernatant was subjected to Nickel affinity chromatography, using a 2 ml bed volume Ni-NTA column (Protino, Macherey&Nagel). The loaded column was washed during a step gradient (5 CV each concentration) of 5 mM, 10 mM, 15 mM, 20 mM, 25 mM and 35 mM imidazole in phosphate buffer. Proteins were eluted with 500 mM imidazole in phosphate buffer. Imidazole was removed and the proteins were rebuffed in 50 mM phosphate buffer pH 6.5, using 10 kDa cut off Amicon centrifugal filters. The proteins were analysed on SDS polyacrylamide gels and quantified using Bradford’s method (Bradford, 1976). Peptide fingerprinting (Supporting Information) confirmed the identity of heterologously produced proteins. Analysis of the S. lacrymans cell wall was carried out as described in the Supporting Information.

In vitro activity assays

The enzymatic activity of sugar polymer degrading enzymes (B. subtilis endo-β-1,3(4)-glucanase, B. subtilis endo-β-1,4-xylanase, B. subtilis and Streptomyces coelicolor chitosanase) (SCO0677, Heggset et al., 2010) or Thermotoga neapolitana laminarinase A (=endo-β-1,3-glucanase, Zverlov et al., 1997), each at a concentration of 0.5 nmol, was tested in overnight reactions at room temperature and assayed photometrically with 1% (w/v) p-aminobenzoyl acid (pABA) in 50 mM phosphate buffer (pH 6.5) in a total volume of 1 ml, following a published protocol (Lever, 1972). As substrate, 20 mg of chitosan (for chitosanase), 40 mg of lichenan (for endo-β-1,3-glucanase), 40 mg of laminarin (for endo-β-1,3-glucanase) or 20 mg beechwood xylan (for endo-β-1,4-xylanase XynA) were added along with 0.5 nmol of the respective enzyme. For control, buffer was added instead of enzyme. The enzymatic activity of proteases (B. subtilis subtilisin E and Tritirachium album proteinase K) was tested by directly pipetting 0.5 nmol of the purified enzyme in phosphate buffer in cavities, punched in milk agar and incubation for 1 day at room temperature. The clearing zone around the protease served as measure for proteolytic activity. In a parallel reaction, AprE was mixed with the protease inhibitor PMSF (2 mM final concentration) and incubated as above. Expansin binding to cellulose was assayed by a described pulldown assay and subsequent polyacrylamide gel electrophoresis (Artzi et al., 2016).

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Conflict of interest

The authors declare no conflict of interest.

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Appendix S1:** Supporting Information.

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