Review

Endofungal bacteria as producers of mycotoxins

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Mycotoxins are compounds of fungal origin that can adversely affect human, animal and plant health through food spoilage or infection, even to the point of epidemics such as turkey X disease and ergotism. The biosynthetic pathways of various mycotoxins (such as aflatoxin and fumonisins) are generally well understood. However, two examples have recently been described where a mycotoxin is not synthesized by the fungus itself but by bacteria residing within the fungal cytosol. These discoveries have implications in various fields, such as ecology, medicine and food processing.

Mycotoxins and their impact on health and ecology

Mycotoxins are toxic metabolites isolated from fungi that occur in great number and variety. Their presence in food contaminated with moulds can elicit deleterious effects in even low concentration, such as chronic or acute toxic damage of liver and kidney, and can even cause death in livestock and humans [1–3]. The discovery of the underlying chemical mechanisms involved in causing turkey X disease and ergotism were groundbreaking discoveries in the 20th century. Apart from these epidemic intoxications, fungal toxins can also induce neuronal damage and inflammation, and even trigger cancer [4,5]. Hundreds of toxic compounds from fungi are known, including aflatoxin, fumonisin, patulin and the ergot alkaloids [1–3], just to name a few. Various ecological functions have been proposed for mycotoxins: in many cases they might serve as defensive weapons against competitors, whereas in other cases they might act mainly as virulence factors (e.g. phytotoxins) [6]. Fascinating interactions between plants and fungal endophytes have been reported for *Epichloë* spp. in tall fescue grass: alkaloids (e.g. lolitrem B or ergovaline) produced by the fungi appear to be beneficial for the plant host but they cause dangerous toxicoses in cattle [7,8]. However, in most cases the true biological roles of the toxins remain unknown.

With the advent of improved analytical methods and modern genetic tools for fungi, much attention has been focused on studying the biosynthesis of mycotoxins at genomic, biochemical and chemical levels. The pathways for the large mycotoxin families (aflatoxin, fumonisins, trichothecenes, ochratoxins, patulin) are generally well understood [9,10,11]. Knowledge on the biosynthesis of a specific toxin greatly contributes to detect toxinogenic fungi and to discriminate them from non-toxin producers.

Until recently, it was assumed that any mycotoxin isolated from a particular fungal strain was produced by such strains. However, we now know that, in certain cases, the toxin is not synthesized by the fungus but by associated bacteria. Here, we highlight and discuss recent discoveries that demonstrate that some mycotoxins isolated from fungi of the genus *Rhizopus* are actually produced by symbiotic bacteria living within the cytosol of the fungal cells.

Discovery and characterization of toxin-producing endofungal bacteria

Two toxins isolated from fungi—and, hence, known as 'mycotoxins'—have been shown to be synthesized by associated endobacteria. In the following, we introduce the two cases and recapitulate shortly how their bacterial producers were serendipitously discovered, successfully isolated from fungal mycelia, and further characterized.

Rhizoxin, the causative agent of rice seedling blight

Rhizoxin (Figure 1a), the causative agent of rice seedling blight, is an important example for a mycotoxin that is both ecologically and pharmaceutically relevant [12,13]. In the early 1980s, the compound (a polyketide macrolide) was isolated from cultures of the saprotrophic fungus *Rhizopus microsporus* (formerly known as *Rhizopus chinensis*) that grew on rice seedlings [12,13].

Rhizoxin possesses remarkable biological activities. Application of the pure compound induces an abnormal swelling of rice seedling roots, the typical symptoms of the

Glossary

Macrolide: a cyclic ester, usually with a 14- to 16-membered ring, produced by a polyketide synthase. Important macrolides are antibiotics such as erythromycin, or antimitotic compounds such as epothilone or rhizoxin.
Mutualism: a symbiotic relationship where both partners profit from each other by exchange of goods or services (e.g. food, defense mechanisms).
Non-ribosomal peptide synthetase (NRPS): an enzyme complex catalyzing the activation and condensation of amino acids to form small peptides. Typically, an NRPS consists of several modules, each of them catalyzing the coupling of one specific amino acid to the growing peptide.
Parasitism: a symbiotic association where one organism, the parasite, is taking resources from the host and reduces its fitness.
Polyketide: a secondary metabolite produced by condensation of acetyl-CoA and malonyl-CoA units in a manner similar to fatty acid biosynthesis. Various degrees of reduction, cyclization or further decoration of the polyketide chain lead to structurally diverse metabolites such as polyphenols or macrolides.
Polyketide synthase (PKS): an enzyme complex catalyzing the formation of polyketides.
Symbiosis: a general term for a close and specific interaction of two different organisms (according to Anton de Bary).

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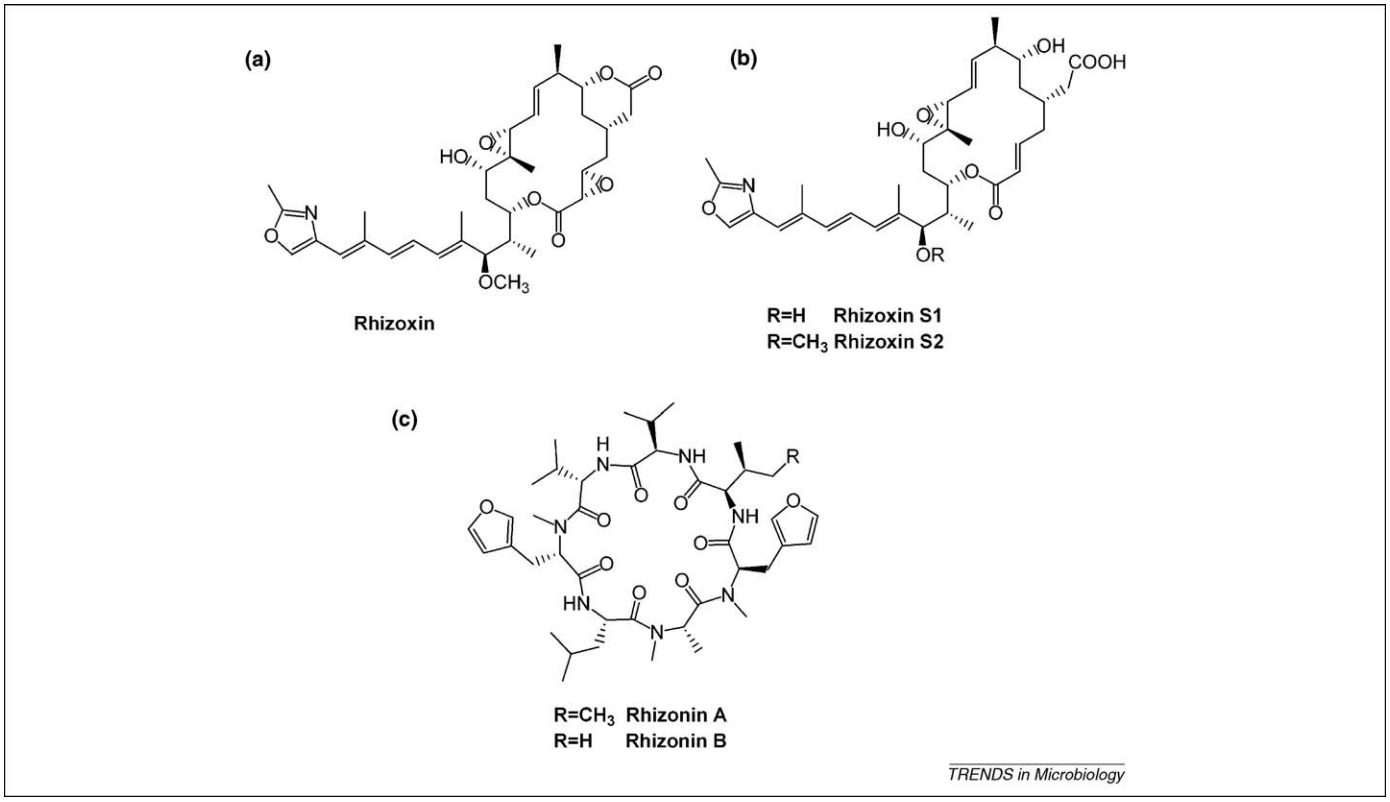

Figure 1. Structures of macrolides belonging to the rhizoxin complex **(a,b)** and of the cyclopeptides rhizonin A and rhizonin B **(c)**.

plant disease [14], at a concentration as low as 10 ng mL^{-1} [13,15]. This is achieved by efficiently binding to rice cell β-tubulin and thus blocking cell division in the roots. Owing to its remarkably strong antimitotic activity in most eukaryotic cells, including various human cancer cell lines, rhizoxin has also found considerable interest as a potential antitumor drug [16,17].

The structure of the toxin, together with isotope labeling experiments, clearly indicated that rhizoxin is derived from a polyketide metabolic pathway. Genes encoding polyketide synthases (PKS), in particular their ketosynthase (KS) domains, can be targeted in the genome of bacteria and fungi by PCR using specific degenerate primers [18,19]. Interestingly, because fungal and bacterial PKS genes are similar but clearly distinct, their sequences can be readily assigned to either one or the other group of microbes. It was remarkable that no fungal PKS genes could be detected in the genome of rhizoxin producer *R. microsporus*. However, fragments of bacterial PKS genes were amplified from total DNA of the fungus using degenerate primers. Further detection of 16S rRNA genes in all rhizoxin-positive *Rhizopus* strains pointed towards a bacterial association rather than horizontal gene transmission of bacterial genes into the fungal genome.

An external association of bacteria and fungi was excluded by light microscopic analyses, which suggested that the fungus might harbor bacterial endosymbionts (Box 1). In fact, bacteria were detected within the fungal cytosol of a growing *R. microsporus* culture through confocal laser scanning microscopy. Live/dead staining revealed the presence of a high number of endobacteria as green fluorescent, rod-shaped spots, indicating that their cell wall was intact and thus they were alive. The endosymbionts were also detected and analyzed by freeze–fraction electron microscopy [20] and transmission electron microscopy [21] (Figure 2).

The crucial role of endofungal bacteria (Box 2) in rhizoxin production was tested by treating the fungus with the antibiotic ciprofloxacin, which is active against

Box 1. Symbionts as producers of toxins and other natural products

In many cases, it has been suspected that natural products isolated from various eukaryotes (e.g. tunicates, sponges or insects) are actually biosynthesized by intimately associated bacterial symbionts [51,52]. However, these endobacteria are typically recalcitrant to cultivation, and in most cases only indirect genomic evidence supports the ''endosymbiont hypothesis'' for the origin of some natural products.

Biosynthesis genes of bacterial origin could be detected and isolated from several eukaryotic producers. Both marine sponges as well as beetles of the genus *Paederus*, for instance contain antimitotic compounds of the pederin group that appear to be synthesized by endobacterial symbionts [53,54]. Other examples of secondary metabolites produced by symbionts in marine ecosystems are the antitumor agent bryostatin (from '*Candidatus* Endobugula sertula', the symbiont of the bryozoan *Bugula neritina*) and patellamides (from *Prochloron didemni*, the cyanobacterial symbiont of the ascidian *Lissoclinum patella*) [55]. Last but not least, the wide-ranging group of microbial endophytes, or plant endosymbionts, should be mentioned as a rich source of diverse natural products. A plethora of bacteria and fungi have been isolated from plant tissue, many of them producing secondary metabolites with potential medicinal or agricultural application, among them strong antifungal compounds such as cryptocandin [67] from *Cryptosporiopsis* cf. *quercina*. It seems that secondary metabolite production by symbionts is a common theme in nature and it is surprising that only two examples of bacteria producing ''mycotoxins'' are known to date.

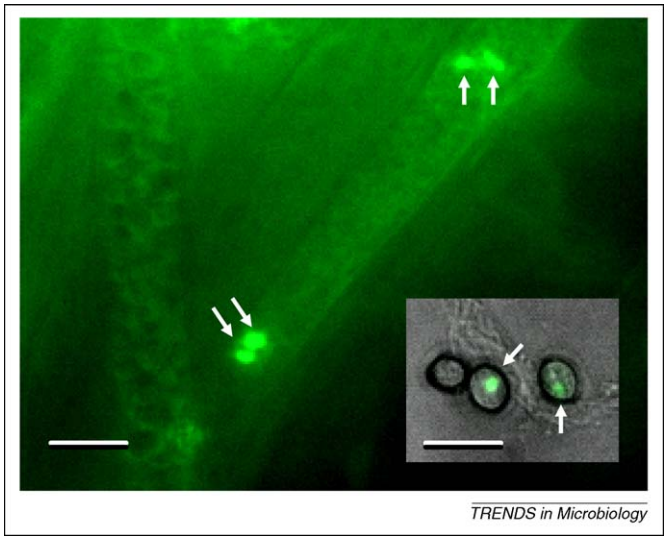

Figure 2. Confocal laser scanning micrographs of rhizoxin-producing bacterial endosymbionts (*Burkholderia rhizoxinica*, labeled with GFP) in the mycelium of *Rhizopus microsporus* (main image) and in spores (inset). Bars: 10 μm (main image), 5 μm (inset).

bacteria but not against fungi. Remarkably, no rhizoxin was detected in the resulting fungal strain [22]. The final proof was given by the successful isolation of a rhizoxin-producing bacterial strain from the fungus; the bacterium could be grown in pure culture [23]. The entire 16S rRNA gene sequence was amplified and sequenced, and database searches revealed that the bacteria belong to the genus *Burkholderia*. The isolated strain was described as a new species called *Burkholderia rhizoxinica* reflecting its biosynthetic potential [20]. Notably, analysis of an upscaled fermentation of *B. rhizoxinica* revealed over 20 antimitotic derivatives (two examples are shown in Figure 1b), with rhizoxin being a minor component. Some derivatives exhibit 1000 to 10,000 higher cytotoxic or antiproliferative activities, as compared to rhizoxin, and rank among the most potent antimitotic agents known to date [23].

Therefore, the phytotoxin rhizoxin has been erroneously known as a fungal metabolite for over two decades. In fact, the fungus—incapable of producing polyketides—harbors bacterial endosymbionts for the production of the antimitotic agent, which weakens and even kills the plant. The bacteria in return apparently benefit from the 'safe' niche and from nutrients provided by the fungus [22].

Rhizonin, a food-spoiling, hepatotoxic cyclopeptide

The second example of a mycotoxin that is actually produced by endobacteria relates to the cyclopeptides rhizonin A and rhizonin B (Figure 1c). The source of the toxins was a *R. microsporus* strain recovered from contaminated Mozambican peanuts [24,25]. Ironically, although these compounds were termed the 'first mycotoxins from Zygomycota' around two decades ago, they are actually bacterial metabolites. Both cyclopeptides exhibit severe nonspecific hepatotoxicity and induce acute and chronic failure of the liver in laboratory animals. A wide range of hepatic lesions and 100% lethality was observed in rats [26].

The structure of rhizonin, in particular the presence of nonproteinogenic amino acids, suggests that its biosynthesis is probably carried out by a nonribosomal peptide synthetase (NRPS). Fungal NRPSs are involved in biosynthesis of penicillin [27] and cyclopeptides such as the immunosuppressive cyclosporin [28–30]. NRPSs typically consist of giant multidomain enzymes with a modular architecture. A minimal set of adenylation (A) and condensation (C) domains, and a peptidyl carrier protein is present in each module. The A and C domains are highly conserved and allow the design of specific degenerate primers for targeting NRPS gene fragments by PCR [31,32]. In analogy to previous findings regarding rhizoxin biosynthesis, fungal NRPS genes could not be detected by PCR in DNA extracted from *R. microsporus*, whereas fragments of bacterial NRPS genes were amplified [21].

The presence of endobacteria in the rhizonin-positive *Rhizopus* strain was unequivocally proven by transmission electron microscopy and the amplification of bacterial 16S rRNA gene sequences. The rod-shaped bacterial symbionts within the mycelium could be isolated and grown in pure culture. It turned out that the rhizonin producer is a close relative of *B. rhizoxinica* and is also capable of producing rhizoxin. Despite high similarity on the 16S rRNA sequence level, genome–genome hybridization experiments suggest defining a new species named *Burkholderia endofungorum* [20].

The same line of evidence as described for elucidating the true rhizoxin producer was repeated. First, the fungal culture was treated with ciprofloxacin to generate a symbiont-free, rhizonin-negative fungal strain. The lack of rhizonin production in the cured strain revealed that the

Box 2. Endofungal bacteria

Endofungal bacteria are bacterial symbionts of fungi residing within the fungal mycelium. Bacteria are known for their potential to occupy remarkably diverse ecological niches and to form tight mutualistic associations with other organisms. In many cases, bacteria complete their life cycles within their host and some are even transmitted vertically in analogy to mitochondria. Endosymbiotic bacteria have long been known to reside in insects, worms, marine animals (sponges, tunicates), as well as plants (e.g. root nodules, leaf galls). However, until recently only few cases have been found where fungi can harbor bacterial endosymbionts. Schüßler *et al.* investigated an endosymbiotic association of the fungus *Geosiphon pyriforme* and cyanobacteria, which is known since the 19th century [56,57]. In the early 1970s 'bacterium-like organelles (BLOs)' were detected in the cytoplasm of arbuscular–mycorrhizal fungi by electron microscopy [58,59]. However, for over a decade the identity of these BLOs remained obscure, as they could not be cultured in cell-free media [60]. With the advent of molecular techniques (e.g. fluorescence *in situ* hybridization, FISH) it became feasible to detect and characterize even 'non-culturable' endosymbionts, which led to a series of discoveries. Bianciotto *et al.* reported that the obligately endosymbiotic mycorrhizal fungus *Gigaspora margarita* itself harbors obligately intracellular bacteria [61] and found that almost all species of this family are associated with endosymbionts [62]; Barbieri *et al.* succeeded in the *in situ* detection of *Cytophaga–Flexibacter–Bacteroides* in *Tuber borchii* (truffles) [63], some of which are possibly living within the fungal hyphae; more recently, Bertaux *et al.* succeeded in the *in situ* detection of intracellular bacteria related to *Paenibacillus* spp. as well as α-proteobacteria in the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* [64,65]. The toxinogenic *Burkholderia–Rhizopus* symbiosis [22] is the first example where both microbial partners can be cultured independently. A recent publication reports the detection of intimately associated bacteria in members of the fungal order *Sebacinales* [66].

presence of bacteria is essential for mycotoxin production. Second, bacterial symbionts were grown in pure culture for secondary metabolite production [22,23]. Analyses of their metabolic profile with detection of rhizonin finally disclosed that the bacterial symbionts, and not the fungi, are the true producers of the 'mycotoxin'.

Molecular basis for production of rhizoxin

The biosynthesis gene cluster responsible for rhizoxin biosynthesis is located in the genome of the endosymbiont *B. rhizoxinica*. The 81-kb gene cluster consists of ten open reading frames (ORFs designated *rhiA–rhiJ*), which are flanked by two transposase genes. The involvement of the gene cluster in rhizoxin biosynthesis was proven by a targeted gene inactivation in the genome of the cultured symbiont, which resulted in non-producing strains [33,34].

The gene products of *rhiA–rhiF* represent a giant processing line (a modular PKS–NRPS) that assembles the carbon backbone of the macrolide (Figure 3). The backbone chain is released by a thioesterase with cyclization to yield the macrolide. Tailoring reactions such as epoxidation and *O*-methylation furnish the final product [33,35].

It is remarkable that the rhizoxin biosynthesis genes are most similar to orthologs identified as putative NRPS–PKS in the genome of *Pseudomonas fluorescens* Pf-5 (a plant commensal), where they are similarly organized in a single gene locus [36]. Although this organism was not known as a producer of rhizoxin, closer investigations showed that *P. fluorescens* Pf-5 is indeed able to produce a similar blend of rhizoxin derivatives as the fungal endosymbionts [37]. In *B. rhizoxinica* the genome region responsible for rhizoxin biosynthesis is flanked by transposase genes, indicating that it might be a mobile genetic element. Although in *P. fluorescens* Pf-5 the gene cluster is not described as a putative foreign or mobile element, it is remarkable that the closely related strain *P. fluorescens* Pf-1 does not harbor the rhizoxin gene locus.

Without further genomic studies, the origin and evolution of rhizoxin biosynthesis genes remains a mystery. However, the presence of this gene cluster in both β-proteobacteria (*Burkholderia*) and γ-proteobacteria (*Pseudomonas*) represents a striking example for horizontal transfer of a giant genomic island coding for secondary metabolite biosynthesis. Furthermore, it should be emphasized that the ecological role of the produced compound appears to be surprisingly different in the two phyla. Whereas rhizoxin from fungal endosymbionts serves as an "offensive weapon" employed by a phytopathogenic

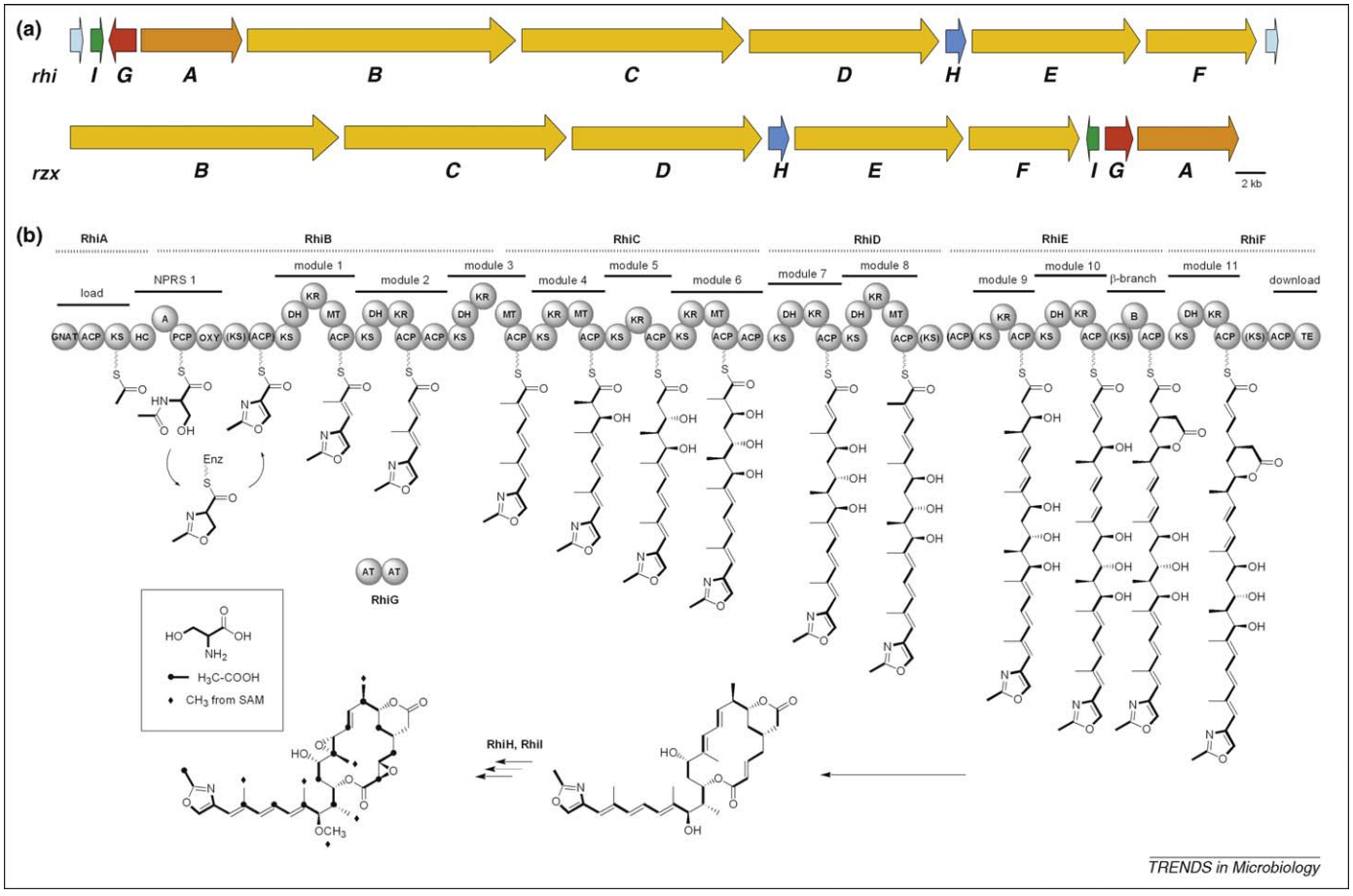

Figure 3. Molecular basis for rhizoxin biosynthesis. **(a)** Organization of the *rhi* and *rzx* biosynthesis gene clusters from endosymbiotic *Burkholderia rhizoxinica* and the plant commensal *Pseudomonas fluorescens* Pf-5, respectively. **(b)** Molecular processing line deduced from *rhiA–rhiF*. Protein functions and abbreviations: RhiG, acyltransferase (AT); RhiH, cytochrome P-450 monooxygenase; RhiI, *O*-methyltransferase; RhiJ, putative oxygenase; GNAT, *N*-acetyltransferase; KS, ketosynthase; AT, acyl transferase; ACP, acyl carrier protein; HC, condensation/heterocyclization; A, adenylation; PCP, peptidyl carrier protein; OXY, oxygenase; KR, ketoreductase; DH, dehydratase; MT, *C*-methyltransferase; TE, thioesterase; B, domain involved in β-branching; SAM, S-adenosylmethionine. Rhizoxin biosynthesis deviates from typical NRPS–PKS systems in several aspects: parts of modules are encoded on different genes, the PKS utilizes an AT *in trans* [34], and a β-branch is introduced by a Michael-type addition of the malonyl building block [35].

fungus to attack the plant, the same compound in the plant commensal might be acting as a "defensive weapon" to protect the host plant from other phytopathogenic fungi [37].

Ecology and evolution of the *Burkholderia–Rhizopus* symbiosis

How has the fungus acquired its rhizoxin-producing endobacteria and gained self-resistance? During the initial studies on the true biogenetic source of rhizoxin, it was found that a symbiont-free strain of *R. microsporus* can be reinfected with cultured endosymbionts by cocultivation [22]. Despite a rather low rate of bacterial invasion into the fungal cells, this observation provided strong evidence for a horizontal mode of transmission. Thus, it is probable that the mutual relationship observed today evolved via a parasitism-to-mutualism shift.

Reciprocally beneficial associations can evolve mechanisms of vertical transmission, which is a more efficient way to ensure stability of symbiosis [38]. Similarly to other fungal endosymbionts (Box 2), *B. rhizoxinica* is vertically transmitted through spores of its fungal host [39] (Figure 4). This was assessed by reinfection of fungal mycelia (using laser-mediated microinjection or cocultivation) with green fluorescent protein (GFP)-producing strains of *B. rhizoxinica*; labeled bacteria were visible within vegetative spores [39]. Unexpectedly, fungal spores are only formed when symbionts are present and, therefore, vegetative reproduction of the fungal host is strictly dependent on the endosymbionts. Apparently, during evolution the fungus might have lost its ability to produce endogenous sporulation factors and became reliant on endobacteria for reproduction, which provides an elegant mechanism to maintain the bacterial–fungal association [39]. To further estimate the extent of horizontal and vertical transmission during evolution of the alliance, phylogenetic analyses of bacterial endosymbionts and their corresponding hosts were conducted [40]. Eight strains of *R. microsporus* harboring endobacteria, originating from all five continents, were identified from a strain collection. Both the endobacteria and the fungal hosts were characterized by multilocus sequence typing, i.e. several conserved gene loci were sequenced and subjected to phylogenetic analyses. The results indicated that the

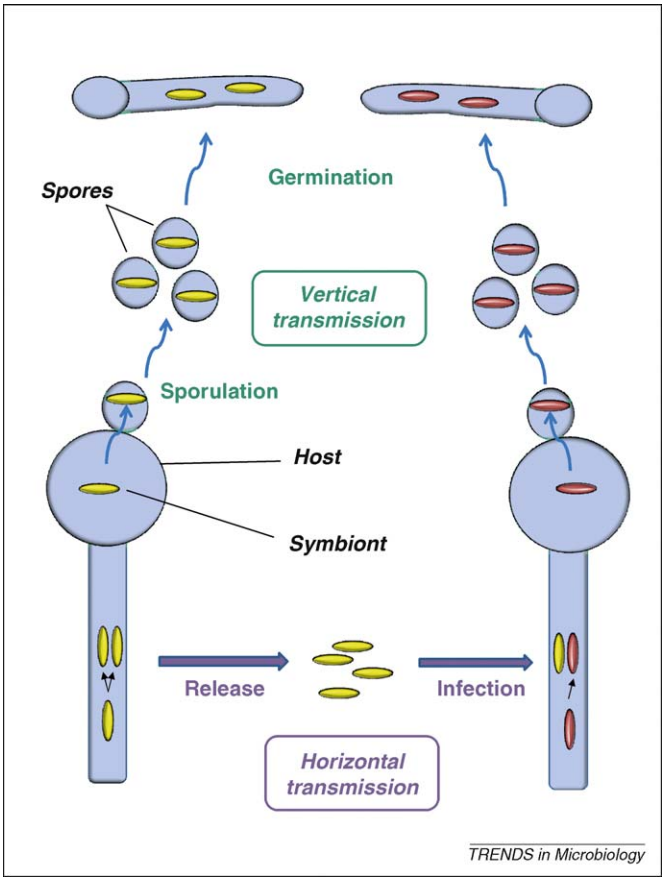

Figure 4. Hypothetical lifecycle of endofungal bacteria and their hosts. Endobacteria replicate within the fungal mycelium. During vertical transmission they enter vegetative spores and are transferred to the next generation. Horizontal transmission involves release from the host cell and infection of another compatible host organism.

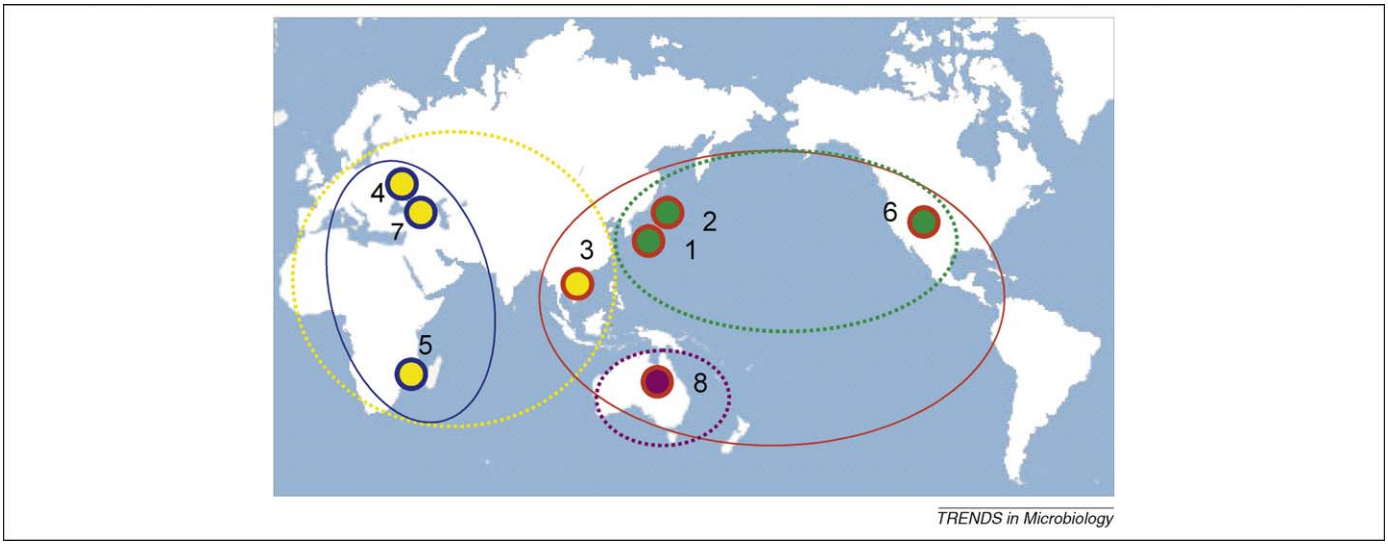

Figure 5. Global distribution and evolution of the *Rhizopus–Burkholderia* symbiosis. Eight isolates of various geographical origins were phylogenetically analyzed (buttons 1 to 8). Inner colors of buttons represent endosymbiont clades, outer colors represent host clades. Color code: green, Pacific group of endosymbionts; yellow, Eurasian–African group of endosymbionts; violet, Australian clade of endosymbionts; red, extended Pacific group of hosts; blue, Eurasian–African group of hosts. Host switching events are suggested for strains 3 and 8.

endobacteria have a common ancestor and possess the gene cluster coding for rhizoxin biosynthesis, thus belonging to the *Burkholderia rhizoxinica* complex [40]. Some of the isolates form clades that correlate with their geographical origin (Figure 5). Comparison of host and symbiont phylogeny demonstrated that both partners formed clades with a similar topology, which is indicative of cospeciation as a result of vertical transmission of associated symbionts. However, a few topological differences existed between the phylogenetic trees, suggesting that host switching events have taken place during evolution of the symbiosis. Even though molecular data shed light on some aspects of *Burkholderia–Rhizopus* coevolution, many open questions remain.

One interesting question arising within this context is how rhizoxin resistance was developed by the fungal host, as the compound is toxic to many other fungi. It was known that an asparagine residue in position 100 of β-tubulin of rice plants and *Aspergillus nidulans* is correlated to rhizoxin sensitivity [41], and altering this amino acid in *A. nidulans* conferred resistance to the toxin [42]. Consequently, conserved β-tubulin sequences from species throughout the fungal kingdom were analyzed to infer development of rhizoxin resistance across a phylogeny of different fungal phyla. The results clearly suggested that a hypothetical common ancestor of the fungal kingdom was rhizoxin-sensitive. However, it seems that a rhizoxin-insensitive fungal host was invaded by the rhizoxin-producing bacteria. In other words, the evolution of self-resistance probably preceded the establishment of the symbiosis [43].

Concluding remarks and future perspectives

Mycotoxins are relevant natural products that can cause severe damage to human, animal and plant health. Currently, more than 300 mycotoxins are known from more than 10,000 producer strains, and their role in causing disease cannot be neglected. The term mycotoxin originates from the fact that these toxic compounds were originally isolated from fungi, in particular from the Ascomycota. The finding that some mycotoxins (i.e. rhizoxin and rhizonin) are actually produced by endofungal bacteria has implications in various aspects. One could consider that otherwise non-toxinogenic fungi might be transformed into "toxin producers" through the infection of bacteria. By contrast, it is a bidding approach to treat health-threatening fungi with antibacterial compounds to subdue the true producers of virulence factors. Yet there is no evidence that bacteria contribute to the virulence of fungi causing zygomycoses in humans [44]. Interestingly, in the case of rhizoxin, the isolated bacteria are superior to the entire fungal–bacterial symbiosis in producing antitumoral and antifungal agents that might be developed into therapeutic agents.

The finding of toxinogenic endobacteria is also of interest in the area of food processing, not only because of the danger of intoxification through fungal food spoilage but also because *Rhizopus* spp. are frequently used as enzymatic sources for the production of fermented foods, such as tempe, sufu and sorghum [45–50]. Knowledge of the true toxin producer will enable advanced analytical methods to detect contaminations and to identify unsuitable strains for fermentation in food biotechnology.

Finally, from an ecological point of view, the occurrence of toxin-producing bacterial–fungal alliances is fascinating. The *Rhizopus–Burkholderia* symbiosis represents an excellent model system to study the evolution of microbial interactions and the molecular basis of mutualism vs. pathogenicity. In light of this, it will be intriguing to learn the factors governing these symbioses and how many more endofungal bacteria are synthesizers of "mycotoxins".

Acknowledgements

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