A cryptic PKS–NRPS gene locus in the plant commensal *Pseudomonas fluorescens* Pf-5 codes for the biosynthesis of an antimitotic rhizoxin complex†

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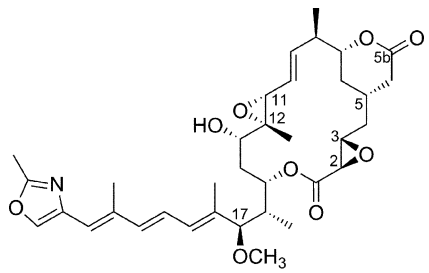
Targeted gene inactivation and metabolic profiling revealed that the cryptic PKS–NRPS gene cluster in the genome of the plant commensal *Pseudomonas fluorescens* Pf-5 codes for the biosynthesis of antiproliferative and antifungal rhizoxin derivatives.

Rhizoxin (**1**, Fig. 1) is a potent antiproliferative agent that was first isolated from the plant pathogenic fungus *Rhizopus microsporus*. It has been shown that the 16-membered polyketide macrolide represents the causative agent of rice seedling blight, a plant disease that results in severe losses in agriculture.[1] Rhizoxin exerts its destructive effect by binding to the β-tubulin subunit and thus blocking mitosis.[2] Due to its excellent antiproliferative activities not only in rice plants but also in most eukaryotic cells **1** has attracted considerable interest as an antifungal and antitumoral agent.[3–6]



Rhizoxin (**1**)

Fig. 1 Structure of rhizoxin, the causal agent of rice seedling blight.

During our studies on the molecular basis of rhizoxin biosynthesis in *R. microsporus* we made an unexpected observation. We found that **1** is in fact not produced by the fungus, but by bacteria that live within the fungal cytosol.[7,8] By curing the fungus from endosymbiotic bacteria and re-infecting an aposymbiotic strain we could clearly correlate rhizoxin biosynthesis to the endosymbionts, which belong to the genus *Burkholderia*.[7,9] The ultimate proof was provided by the cultivation of the endofungal bacteria in the absence of the fungal host and by the analysis and characterization of a complex of highly active rhizoxin derivatives.[10] Cloning, sequencing and inactivation of the rhizoxin (*rhi*) biosynthesis gene cluster in the cultured bacterial symbionts finally revealed that the macrolide is assembled by a giant modular polyketide synthase–non-ribosomal peptide synthetase (PKS–NRPS) hybrid.[11] Database searches showed that the rhizoxin biosynthesis genes are most similar to orthologues that have been identified in the genome of the plant commensal *Pseudomonas fluorescens* Pf-5.[12] All genes related to the *rhi* genes from the *Burkholderia* symbiont fall into a single gene locus, which has remained cryptic to date.[12] The gene organization clearly deviates from the *rhi* gene cluster, and out of 17 ORFs (PFL2980-PFL2997, Table S1†) only nine are related to the *rhi* cluster. However, due to the high sequence similarities it was tempting to speculate that this organism would be capable of producing complex polyketides related to rhizoxin.[11] We first searched for conserved motifs in the deduced gene products of the PKS–NRPS gene locus. The sequence was translated into a loading module, an NRPS module and eleven functional PKS modules that are terminated by a thioesterase domain. The architecture of the thiotemplate system strongly resembled the rhizoxin NRPS–PKS found in the fungal endosymbionts. In addition, a tandem acyl transferase (AT), and the two tailoring enzymes, a cytochrome P-450 monooxygenase and an *O*-methyltransferase (*O*-MT) were encoded by the *Pseudomonas* gene cluster. This finding strongly suggested that *P. fluorescens* Pf-5 has the potential to produce members of the antimitotic rhizoxin complex. To test this hypothesis we monitored the metabolic profile of *P. fluorescens* Pf-5 under various growth conditions. Using authentic references and an optimized analytical set-up (HPLC-DAD/MS)‡ we succeeded in detecting low amounts of rhizoxin derivatives (in total 2 mg L^{-1}) in a crude extract of a *P. fluorescens* Pf-5 culture grown in nutrient medium (Fig. 2). Seven rhizoxin derivatives could be identified. The most abundant metabolite proved to be identical with rhizoxin S2 (**2**, 1 mg L^{-1}). Significantly lower amounts (μg L^{-1}) of the *Z*-isomer of **2**, rhizoxin Z1 (**5**),[10] as well as the didesepoxy derivatives, rhizoxin D1 (**6**) and D3 (**4**),[10] were produced. Rhizoxin S1 (**3**), WF1360 F (**7**),[13] and rhizoxin D (**8**)[10,13] were detected in trace amounts only. These compounds are also produced by the cultured endosymbionts of *R. microsporus*, albeit at a slightly altered ratio.[10] Also, the production is much lower in *Pseudomonas*.

To correlate the formation of the rhizoxin derivatives with the expression of the putative PKS–NRPS gene cluster in the genome of *Pseudomonas fluorescens* Pf-5 we aimed at disrupting the AT gene (ORF PFL2996), which codes for an essential component of the hybrid synthase (Fig. 3). For this purpose, a pK19-based suicide plasmid bearing a 0.58 kb AT gene fragment and an

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† Electronic supplementary information (ESI) available: Fig. S1 Gel photograph showing results of PCR targeting the *kan* resistance cassette, Fig. S2 Mass spectra of rhizoxin derivatives produced by *P. fluorescens* Pf-5 and Table S1 Deduced gene functions encoded by the *rzx* gene cluster. See DOI: 10.1039/b707762a

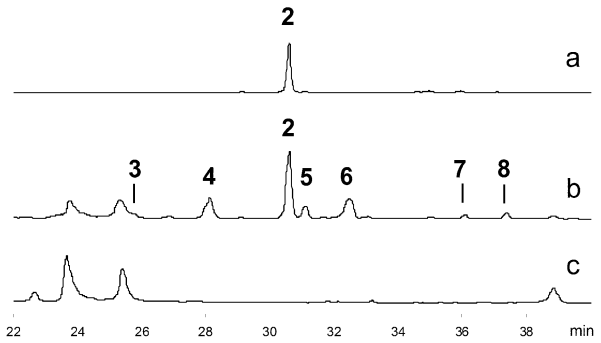

Fig. 2 Chromatographic profile of extracts from *P. fluorescens* Pf-5 (b) and the mutant lacking an intact AT gene (c), and an authentic reference of **2** (a).

additional kanamycin resistance marker was used.[11] Plasmid DNA was introduced into electrocompetent cells of *P. fluorescens* Pf-5 by electroporation.[14] Transformed cells were recovered in TSB and eventually plated onto nutrient agar supplemented with kanamycin.§ Integration of the plasmid into the genome was verified by PCR using primers targeting the resistance cassette (Fig. S1†). Positive colonies were selected and cultivated in liquid media to analyze the metabolic profiles by HPLC. As shown in Fig. 2c, the selected mutant is not capable of producing any rhizoxin derivatives, thus confirming the postulated role of the cryptic PKS–NRPS (*rzx*) gene cluster in *P. fluorescens* Pf-5.

The plant commensal is known to inhabit the plant rhizosphere and to function as a biocontrol agent in agricultural soils. In particular, the bacterium has been implicated in protecting plants from fungal infections. For several well-characterized antibiotics, such as phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, lipopeptides, and hydrogen cyanide, biocontrol properties have been demonstrated experimentally.[15,16] The finding that the plant associated bacterium is capable of producing members of the rhizoxin family of antibiotics is certainly an important addition to the currently available metabolic data. Agar diffusion assays revealed that the main product, rhizoxin S2 (**2**) significantly inhibits growth of the plant pathogenic fungus *Fusarium oxysporum*.¶ The potent rhizoxin derivatives produced under laboratory conditions thus contribute to the battery of antifungal agents produced by *P. fluorescens* Pf-5. Thus, the ecological role of rhizoxin may be context-dependent: in alliance with a resistant fungal host like *Rhizopus* sp. sensitive plants (rice seedlings) may be attacked. Conversely, if a rhizoxin-tolerant plant provides the rhizosphere niche for *P. fluorescens*, rhizoxin may serve as a biocontrol agent against sensitive fungal plant pathogens.

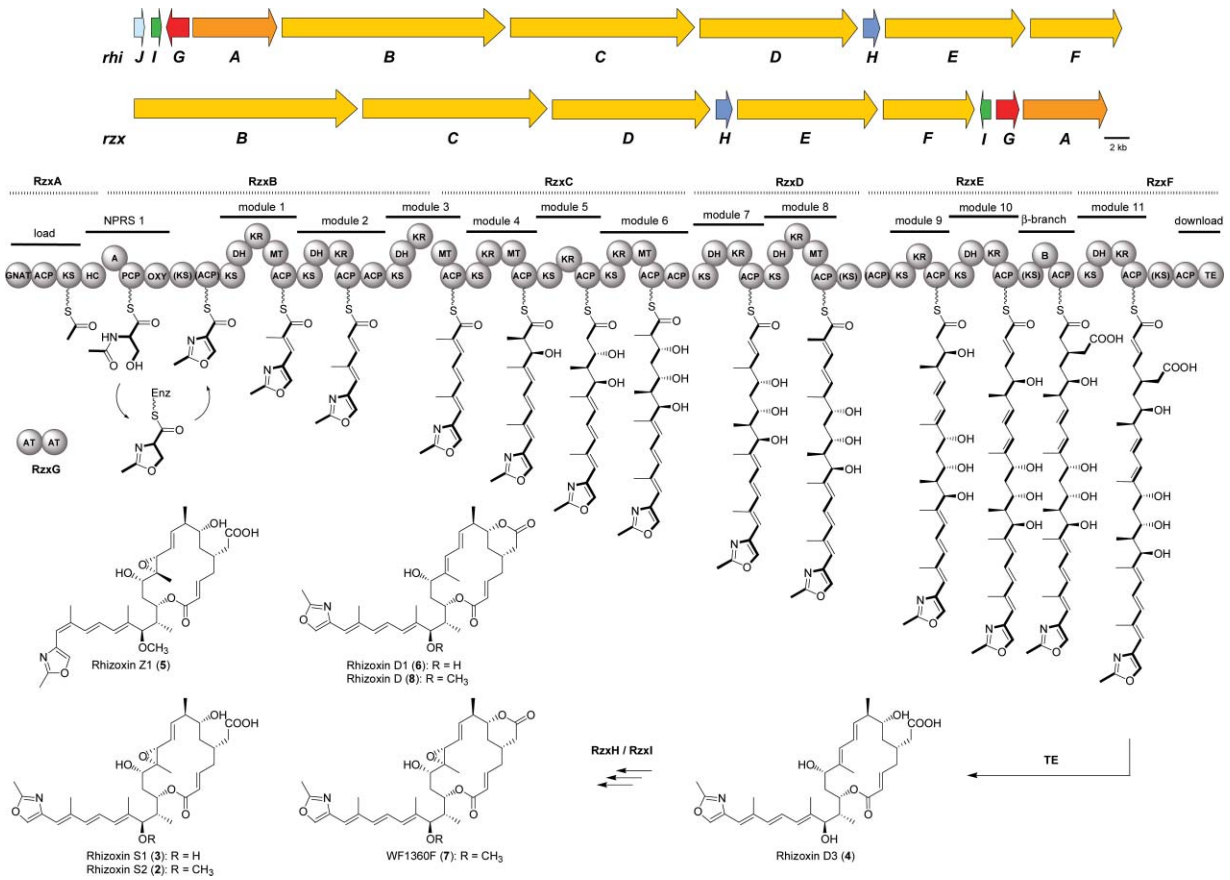

Fig. 3 Organization of the *rhi* and *rzx* biosynthesis gene clusters from endosymbiotic *Burkholderia* sp. B1 and the plant commensal *P. fluorescens* Pf-5, respectively (top). Molecular processing line deduced from *rzxA-F* and structures of **2–8**. RzxA–F: modular PKS–NRPS; RzxG: AT, acyltransferase, RzxH: cytochrome P-450 monooxygenase, RzxI: *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 possibly involved in β-branching (bottom).

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Notes and references

‡ Analytical HPLC was performed on a Shimadzu HPLC system consisting of an autosampler, high-pressure pumps, column oven, and DAD. HPLC conditions: C18 column (Grom Sil 100 ODS 0AB, 3 μm, 250 × 4.6 mm) and gradient elution (MeCN–0.1% TFA (H_2O) 25 : 75, 5 min, in 35 min to MeCN–0.1% TFA (H_2O) 80 : 20, in 5 min to 100% MeCN), flow rate 1 mL min^{-1}.

§ The pK19-based suicide plasmid bearing the 0.58 kb AT gene fragment was introduced into electrocompetent cells of *P. fluorescens* Pf-5. Transformed cells were suspended in 1 mL TSB and cultivated overnight at 30 °C at 110 rpm. Cells were then plated onto nutrient agar supplemented with kanamycin (10 μg mL^{-1}, 25 μg mL^{-1}).

¶ Antifungal activity was determined by agar plate diffusion. Inhibition diameter: 1.8 cm at $c = 10$ μg mL^{-1} (MeOH).

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