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Biosynthetic genes and activity spectrum of antifungal polyynes from *Collimonas fungivorans* Ter331

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Summary

The antifungal activity of bacteria from the genus *Collimonas* has been well documented, but the chemistry and gene functions that underlie this phenotype are still poorly understood. Screening of a random plasposon insertion library of *Collimonas fungivorans* Ter331 for loss-of-function mutants revealed the importance of gene cluster K, which is annotated to code for the biosynthesis of a secondary metabolite and which features genes for fatty acid desaturases and polyketide synthases. Mutants in gene cluster K had lost the ability to inhibit hyphal growth of the fungus *Aspergillus niger* and were no longer able to produce and secrete several metabolites that after extraction and partial purification from wildtype strain Ter331 were shown to share a putative ene-triyne moiety. Some but not all of these metabolites were able to inhibit growth of *A. niger*, indicating functional variation within this group of *Collimonas*-produced polyyne-like 'collimomycins'. Polymerase chain reaction analysis of isolates representing different *Collimonas* species indicated that the possession of cluster K genes correlated positively with antifungal ability, further strengthening the notion that this cluster is involved in collimomycin production. We discuss our findings in the context of other bacterially produced polyynes and the potential use of collimomycins for the control of harmful fungi.

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Introduction

The genus *Collimonas* (family *Oxalobacteraceae*, class *Betaproteobacteria*) consists of mostly soil bacteria that are defined by their ability to grow at the expense of living fungal hyphae under nutrient-limited conditions (de Boer *et al*., 2004; Leveau and Preston, 2008; Leveau *et al*., 2010). So far, three species have been described: *Collimonas fungivorans*, *Collimonas pratensis* and *Collimonas arenae* (Höppener-Ogawa *et al*., 2008). All three species have representatives for which antifungal activity was demonstrated (Leveau *et al*., 2010; Mela *et al*., 2012), but the metabolites involved in this activity have not yet been characterized. In a recent study by Mela and colleagues (2011), it was shown that on nutrient-poor water agar plates the hyphal growth of *Aspergillus niger* was inhibited by *C. fungivorans* strain Ter331. This inhibition did not require direct contact between the bacteria and the fungus, as it was not affected by physical separation of the two organisms by an 8 kDa cut-off dialysis membrane (Fritsche *et al*., 2008). This suggested that the antifungal activity was due to a small, diffusible factor produced by the bacterium. In confrontation with *A. niger*, several genes putatively involved in the production of antifungal compound(s) were upregulated in *C. fungivorans* Ter331 (Mela *et al*., 2011). Using a comparative genomic hybridization approach (Mela *et al*., 2012), the same group of genes, designated as cluster K, was found in its entirety on the genome of *C. fungivorans* Ter14, only partially in *C. fungivorans* Ter6 and not at all in *C. pratensis* Ter91 or *C. arenae* Ter10. This finding aligned well with the observation that *C. fungivorans* Ter331 and Ter14, but not *C. fungivorans* Ter6, *C. pratensis* Ter91 and *C. arenae* Ter10, inhibit growth of *A. niger* in a confrontation assay (Mela *et al*., 2012). The objective of the present study was to isolate and determine the bioactivity of the compound(s) responsible for the observed antifungal activity of *C. fungivorans* Ter331 against *A. niger*, to carry out preliminary research towards the chemical structure of these compounds, which we

refer to here as collimomycins, and to identify the genes that underlie the antifungal phenotype of Ter331.

Results

On water–yeast agar supplemented with 2 mM *N*-acetylglucosamine (WYA-Nag), *C. fungivorans* Ter331 inhibited hyphal growth of *A. niger* and induced branching and hyphal swelling (Fig. 1). Similar antagonistic activity was observed against *Aspergillus* spp. *westerdijkiae* and *versicolor*, *Penicillium* spp. *glabrum* and *chrysogenum* (see Supporting Information Fig. S1), as well as *Verticilium dahliae* JR2 race 1 and the oomycete pathogens *Saprolegnia parasitica* and *Phytophthora megakarya* CSAKO (not shown). In contrast, growth of the phytopathogens *Sclerotium rolfsii* H001, *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *asparagi*, *F. solani*, *Botrytis cinerea* B05.10, *Gaeumannomyces graminis*, *Geotrichum candidum*, *Alternaria brassicicola*, *Penicillium expansum* and the oomycete *Pythium ultimum* was not inhibited by *C. fungivorans* Ter331 under these conditions.

We tested 36 other *Collimonas* strains in the confrontation assay with *A. niger* (Table 1). From this analysis, it became apparent that the antifungal activity was unique to members of *C. fungivorans* subgroup B, which includes Ter331 and which was defined previously (Höppener-Ogawa *et al.*, 2008) based on BOX-polymerase chain reaction (PCR) fingerprint patterns of 42 *Collimonas* isolates. Only two strains from this subgroup B (out of 14 strains tested) were unable to inhibit *A. niger*. As we will show below, these isolates (i.e. SO 30 and SO 115) also lacked one or more cluster K genes, suggesting the involvement of these genes in the fungistatic phenotype. None of the *C. fungivorans* subgroup C strains ($n = 4$), including *C. fungivorans* type strain Ter6, was active against *A. niger*, and neither were any of the tested *C. arenae* ($n = 4$) or *C. pratensis* ($n = 15$) strains. The non-collimonad control *Pseudomonas protegens* Pf-5 also did not inhibit hyphal growth of *A. niger*.

Identification and genomic characterization of C. fungivorans *mutants with reduced antifungal activity*

We used WYA-Nag in a medium-throughput setup (see *Experimental procedures*) to test a total of 3300 random plasposon mutants of *C. fungivorans* Ter331 for reduced activity against *A. niger*. Six such mutants were identified and selected for further analysis. Two (8G9 and 13E12) were tested and confirmed to be impaired in halting the hyphal growth of not only *A. niger*, but also all other fungi and oomycetes whose growth was affected negatively by wildtype *C. fungivorans* Ter331 under the same conditions (see Supporting Information Fig. S1).

In three mutants (10E11, 14C11 and 14G4), the plasposon insertions were mapped to genes involved in amino acid biosynthesis. Mutant 10E11 carried the plasposon in gene Cf_2563 (CFU_2502), which codes for the large chain of carbamoyl-phosphate (CP) synthetase, an enzyme that provides CP as precursor for the biosynthesis of arginine and pyrimidine (Holden *et al.*, 1999). In mutant 14G4, the plasposon insertion was positioned in gene Cf_3833 (CFU_3751), which codes for 5-enolpyruvylshikimate-3-phosphate synthase, the penultimate enzyme in the shikimate pathway. The end-product

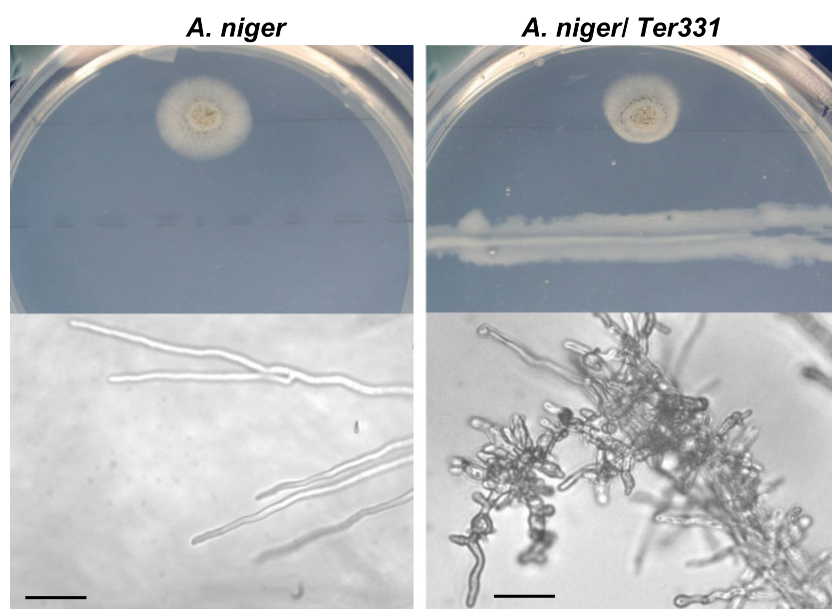

Fig. 1. Macroscopic (top) or microscopic (bottom) view of hyphal growth of *A. niger* in the absence (left) or presence (right) of *C. fungivorans* Ter331 on WYA-Nag agar. Scale bars in the bottom photographs represent approximately 20 μm.

Table 1. Inhibition of *A. niger* by *Collimonas* strains and PCR detection of cluster K genes.

Collimonas species and subgroup	Strain number		LMG[b]	Inhibition of *A. niger*	PCR 1133[a]	PCR 1138[a]	PCR 1139[a]	PCR 1141[a]
C. fungivorans subgroup B	Ter	331		+	+	+	+	+
	Ter	165		+	+	+	+	+
	Ter	228	23972	+	+	+	+	+
	Ter	266	23971	+	+	+	+	+
	Ter	299		+	+	+	+	+
	Ter	14		+	+	+	+	+
	SO	6		+	+	+	+	+
	SO	7		+	+	+	+	+
	SO	8		+	+	+	+	+
	SO	9		+	+	+	+	+
	SO	30		–	–	n.t.	n.t.	n.t.
	SO	114		+	+	+	+	+
	SO	115		–	+	–	–	–
	SO	147	23973	+	+	+	+	+
C. fungivorans subgroup C	Ter	6[T]	21973	–	+	–	–	–
	Ter	166		–	+	–	–	–
	Ter	330		–	+	–	–	–
	Ter	300		–	+	–	–	–
C. arenae	Ter	10[T]	23964	–	–	–	–	–
	Ter	146		–	–	–	–	–
	Ter	252		–	–	–	–	–
	Ter	282	23966	–	–	+/–	–	–
C. pratensis	Ter	91[T]	23965	–	–	–	–	–
	Ter	227		–	–	–	–	–
	Ter	90		–	–	–	–	–
	Ter	118		–	–	–	–	–
	Ter	113		–	–	–	–	–
	Ter	291	23970	–	–	–	–	–
	SO	31		–	–	n.t.	n.t.	n.t.
	SO	32		–	–	n.t.	n.t.	n.t.
	SO	85		–	–	n.t.	n.t.	n.t.
	SO	108		–	–	n.t.	n.t.	n.t.
	SO	110		–	–	n.t.	n.t.	n.t.
	SO	111	23968	–	–	n.t.	n.t.	n.t.
	SO	113		–	–	n.t.	n.t.	n.t.
	SO	117		–	–	n.t.	n.t.	n.t.
	SO	195		–	–	n.t.	n.t.	n.t.
P. protegens	Pf-5			–	+	–	–	–

a. +, PCR product with the same size as that of Ter331; –, no PCR product, +/–, weak PCR product. n.t., not tested.
b. Catalog number in the BCCM/LMG Bacteria Collection.

of this pathway is chorismate, which serves as substrate for the biosynthetic pathway of the aromatic amino acids phenylalanine, tyrosine and tryptophan (Herrmann, 1999). In mutant 14C11, the plasposon localized to gene Cf_752 (CFU_0701), which codes for the amidotransferase component of anthranilate synthase, an enzyme that converts chorismate to anthranilate as part of the tryptophan biosynthetic pathway.

For the other three mutants (8G9, 13E12 and 28A12), plasposon insertion sites were located in proximity to each other and within or close to the previously identified gene cluster K (Mela *et al*., 2011) on the Ter331 genome (Fig. 2). Genes in this cluster (Cf_1128–1142; see Supporting Information Table S1) were previously shown to be upregulated in confrontation of *C. fungivorans* Ter 331 with *A. niger* (Mela *et al*., 2011). The insertion in mutant 13E2 disrupted gene Cf_1135, which codes for a protein annotated as a fatty acid CoA ligase. Cf_1135 is the first gene in a predicted operon of seven, with three genes (Cf_1134, Cf_1133 and Cf_1131) coding for fatty acid desaturases. In this operon, gene Cf_1132 is predicted to code for a putative acyl carrier protein (ACP), Cf_1130 for a hydrolase and Cf_1129 for a rubredoxin-type protein.

In mutant 8G9, the plasposon insertion was located upstream of Cf_1141, which encodes a 3-oxoacyl ACP synthase, and within gene Cf_1143, which codes for a LysR-type regulator and forms a predicted operon with gene Cf_1144, annotated as coding for a major facilitator superfamily (MFS) transporter. The latter showed resemblance to another MFS protein encoded by cluster K, i.e. the predicted product of gene Cf_1136. This gene was annotated as coding for a drug resistance transporter, with similarity to FarB of *Neisseria gonorrhoea*, which has been shown to confer resistance against antibacterial fatty acids (Lee and Shafer, 1999). Cf_1136 forms a predicted operon with three other genes, coding for a fatty

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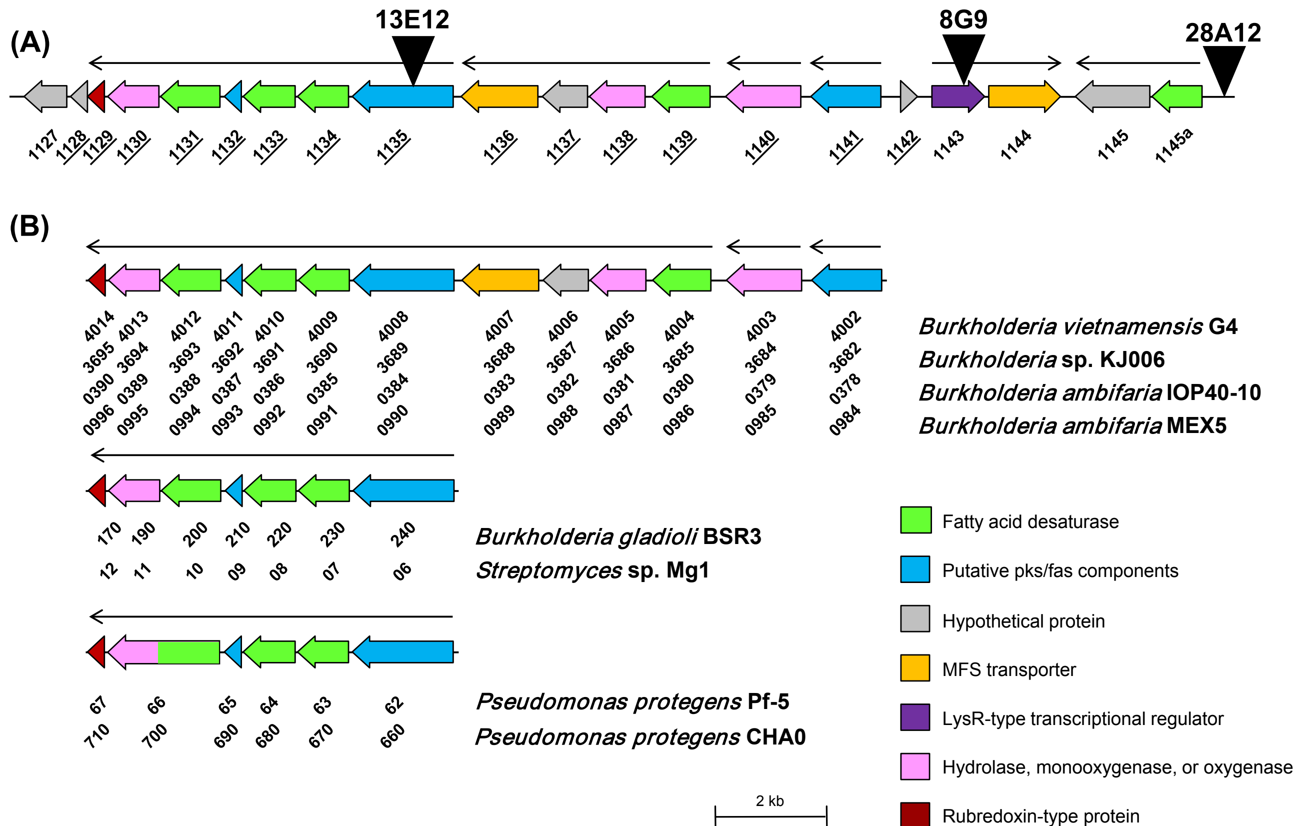

Fig. 2. A. Graphical representation of cluster K and surrounding genes on the *C. fungivorans* Ter331 genome. Genes are labelled according to the naming convention established in Mela and colleagues (2011) and Mela and colleagues (2012). For example, the gene labelled 1135 corresponds with gene Cf_1135 in these papers. Genes for which the label is underlined are part of gene cluster K: genes in this cluster were upregulated during confrontation of *C. fungivorans* Ter331 with *A. niger* (Mela *et al*., 2011). Gene colours indicate predicted function (see Supporting Information Table S1). Arrowed lines above the genes indicate operonic organization, as predicted by FgenesB (http://www.softberry.com). Black arrow heads indicate the plasposon insertion sites in mutants 13E12, 8G9 and 28A12.
B. Also shown are orthologous regions from the genomes of *B. vietnamiensis* G4 (gene accession numbers Bcep1808_. . . .), *Burkholderia* sp. KJ006 (MYA_. . . .), *B. ambifaria* IOP40-10 (BamIOP4010DRAFT _. . . .) and MEX5 (BamMEX5DRAFT_. . . .), *B. gladioli* BSR3 (bgla_1g20. . .), *Streptomyces* sp. Mg1 (SSAG_033. .), and *P. protegens* Pf-5 (PFL_026. .) and CHA0 (PFLCHA0_c02. . .).

acid desaturase (Cf_1139), a Rieske-type oxygenase (Cf_1138) and a hypothetical protein (Cf_1137) respectively. In mutant 28A12, the plasposon was located upstream of gene Cf_1145a, which codes for yet another fatty acid desaturase and forms a predicted operon with gene Cf_1145 with unknown function.

The content and synteny of gene cluster K and its flanking regions appeared to be conserved to various degrees in several other bacterial genomes (Fig. 2B). In *Burkholderia vietnamiensis* G4, *Burkholderia* sp. KJ006 and *Burkholderia ambifaria* IOP40-10 and MEX5, we found a near-complete orthologous version of cluster K. Only part of the cluster (Cf_1135-Cf_1141) was identified in the genomes of *Burkholderia gladioli* BSR3, *Streptomyces* sp. Mg1 (accession number DS570401) and *P. protegens* Pf-5 (Paulsen *et al*., 2005; Loper *et al*., 2012) and *P. protegens* CHA0. In the two *Pseudomonas* genomes, the Cf_1131 and Cf_1130 orthologues occur fused into a single gene. *Pseudomonas protegens* Pf-5 is well known for its antifungal activity (Loper and Gross, 2007). Recently (Hassan *et al*., 2010), genes PFL_0261–0268 of Pf-5 (orthologous to Cf_1135-1129) were identified as belonging to a novel orphan gene cluster for which the metabolic product, designated compound B, is yet unknown.

To further substantiate the claim that cluster K genes are involved in the antifungal activity of *Collimonas*, we designed and used degenerate primers to amplify and detect orthologues of genes Cf_1133, Cf_1138, Cf_1139 and Cf_1141 in the genomes of our *Collimonas* culture collection. The results of the PCR analysis are presented in Table 1. The possession of all four genes correlated perfectly with the ability of *Collimonas* strains to inhibit *A. niger* in a confrontation assay. Conversely, the absence of one or more of these four genes (i.e. in *C. fungivorans* subgroup B strains SO 30 and 115, in all *C. fungivorans* subgroup C strains, and in all *C. arenae* and *C. pratensis* strains) correlated with the inability to inhibit *A. niger*. As

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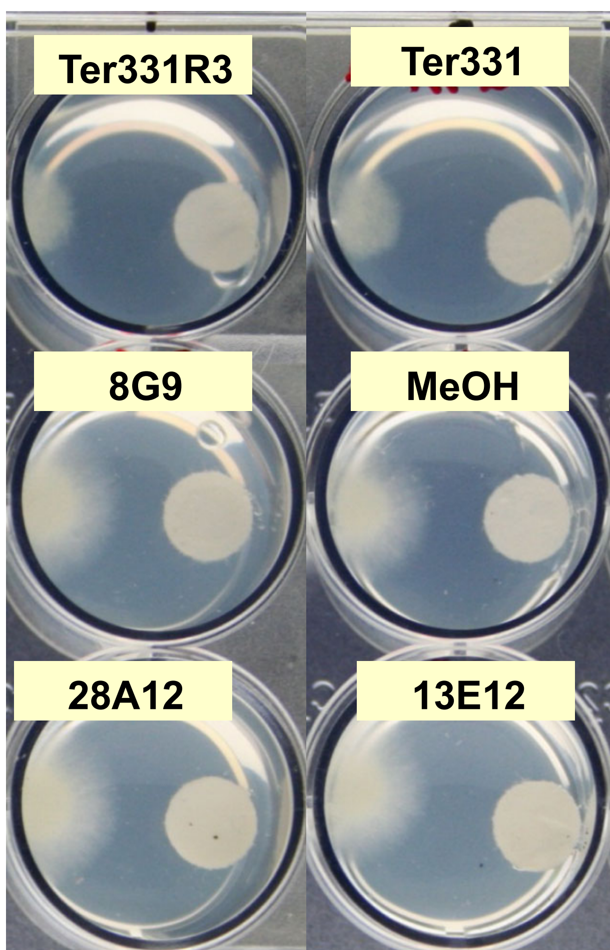

Fig. 3. Antifungal activity screening of extracts from *C. fungivorans* Ter331, its Rif-resistant derivative Ter331R3 and mutants 8G9, 13E12 and 28A12. Details are described in the section *Experimental procedures*, under the heading 'Extraction of antifungal compound(s) from *C. fungivorans* Ter331 and activity profiling'. In short, each well contained 1 ml of WYA-Nag agar, on which was deposited 5 μl of extract on a filter paper disc (right) opposite of a spore suspension of *A. niger* (left). Inhibition of fungal outgrowth was observed only with Ter331 and Ter331R, not with any one of the mutants or with the methanol (MeOH) control.

expected from its genomic context (Fig. 2), the non-collimonad control strain *P. protegens* Pf-5 carried an orthologue of Cf_1133, but not Cf_1138, Cf_1139 or Cf_1141. It was also not inhibitory towards *A. niger* (Table 1). In these respects, Pf-5 mimicked strains of *C. fungivorans* subgroup C, further suggesting the importance of Cf_1138, Cf_1139 and/or Cf_1141 for antifungal activity of Ter331 and other *C. fungivorans* subgroup B strains.

Extraction of collimomycin(s)

Extracts from WYA-Nag agar on which *C. fungivorans* Ter331 was grown were inhibitory to hyphal growth of *A. niger*, whereas extracts from mutants 8G9, 13E12 and 28A12 grown under the same conditions were not (Fig. 3). Analysis by reversed-phase high-performance liquid chromatography (RP-HPLC) with diode array detection revealed the presence of specific peaks in the extracts from *C. fungivorans* Ter331, which were absent in the extracts obtained from mutant 13E12 (Fig. 4A) or mutant 28A12 (see Supporting Information Fig. S2). RP-HPLC fractions of wildtype Ter331 and mutant 13E12 extracts were collected, concentrated and tested for activity against *A. niger*. None of the 13E12 fractions showed antifungal activity, whereas two out of seven fractions of Ter331 did (Fig. 4B). By adopting a different elution gradient, a higher resolution of peaks was obtained (Fig. 5), and all five fractions containing one or more of the major peaks (a–h) showed a highly characteristic sawtooth-shaped UV spectrum. With the mutant extracts, no such spectral patterns were ever found (not shown). This type of pattern is characteristic for polyynes, which are polyacetylenic compounds with alternating triple and single carbon–carbon bonds (Bohlmann *et al.*, 1973). The observed UV spectral peaks (λ327/328, 306/307, 287/288, 271, 240/241 and 229/230 nm) closely resembled those of compounds that contain an ene-triyne moiety (–C=C–C≡C–C≡C–C≡C–), according to Bohlmann and colleagues (1973) (i.e. λ328, 307, 288, 271, 242 and 231 nm). While all five fractions showed this UV spectrum (Fig. 5B), only two were shown to be active against *A. niger*, namely fraction 3 (Fig. 5B, marked with *, includes peaks c and d) and fraction 5 (Fig. 5B, marked with *, includes peaks f, g and h).

Discussion

The only antimicrobial compound described to date for the genus *Collimonas* is violacein (Hakvag *et al.*, 2009). Production of this purple pigment has been demonstrated for several other members of the *Oxalobacteraceae* and is believed to provide protection against protozoan predation (Matz *et al.*, 2004). Here, we report the isolation, separation and partial characterization of a class of metabolites from *C. fungivorans* Ter331, some of which show antifungal activity. These metabolites, which we collectively refer to as collimomycins, shared a characteristically shaped UV spectrum suggesting an ene-triyne-based moiety, i.e. featuring multiple conjugated C≡C bonds. Such acetylenic compounds occur in a wide structural variety in nature, and their production has been described for plants, fungi, marine sponges and bacteria (Dembitsky, 2006; Minto and Blacklock, 2008; Abraham, 2010). Many have bioactive properties, such as antibacterial, cytotoxic, herbicidal and/or antifungal activities (Shi Shun and Tykwinski, 2006; Abraham, 2010). The best studied bacterial polyynes so far are the ene-diyne

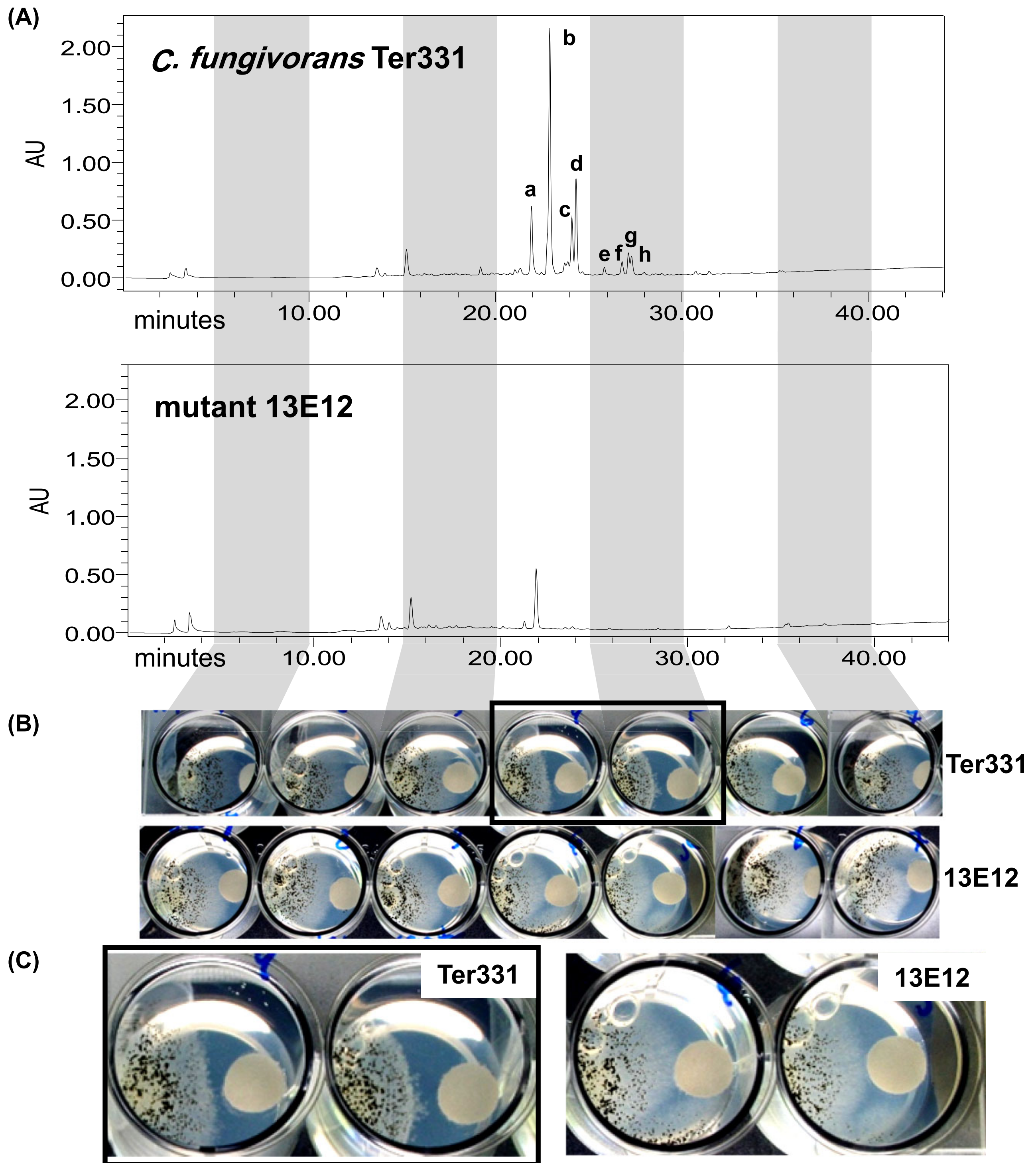

Fig. 4. A. Aligned HPLC profiles of methanol extracts from *C. fungivorans* Ter331 and mutant 13E12. Gradient used was 0–100% acetonitrile over a period of 30 min. Peak labels are a through h, and correspond to peak labels in Fig. 5.
B. Antifungal activity screening of seven 5 min fractions collected for both *C. fungivorans* Ter331 (top row) and mutant 13E12 (bottom row) extracts. Assay was done in the same way as shown in Fig. 3. Antifungal activity was observed only in the two fractions that are boxed by the black rectangle.
C. Blow-up of the two wildtype wells that are shown boxed in panel B, as well as of the two corresponding wells for mutant 13E12 extract showing loss of activity.

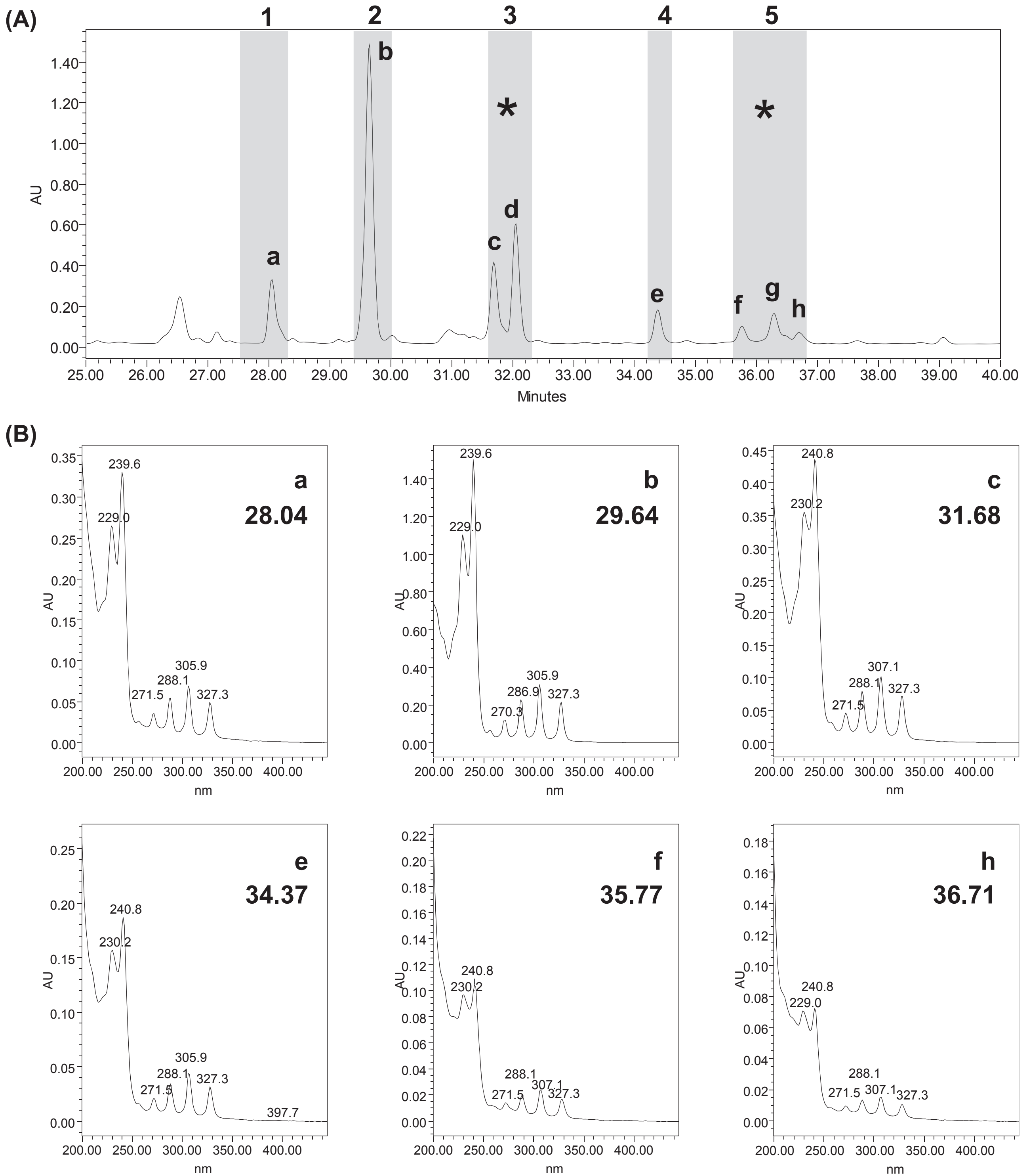

Fig. 5. A. Improved-resolution HPLC profile of methanol extract from *C. fungivorans* Ter331. Gradient used was 0–100% acetonitrile over a period of 50 min. Five fractions were collected (shaded areas), and asterisks mark those fractions that retained antifungal activity against *A. niger*. Peak labels are a through h, and correspond to peak labels in Fig. 4.
B. UV spectra of peaks in fractions collected from HPLC profile in panel A. The UV spectra of peaks d (32.04) and g (36.27) are not shown; they were very similar to those of c and f respectively.

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antitumour antibiotics from actinomycetes, e.g. calicheamicin from *Micromonospora echinospora* sp. *calichensis* (Ahlert *et al.*, 2002; Belecki *et al.*, 2009). Other examples of bacterially produced polyynes are cepacin A and B from *Burkholderia cepacia* (Parker *et al.*, 1984) and caryoynencin A-C from *Burkholderia caryophylli* (Kusumi *et al.*, 1987; Yamaguchi *et al.*, 1995). The predicted UV maxima for cepacins (226, 236, 294 and 309 nm) and caryoynencins (273, 290, 311, 331, 356 and 382 nm), based on Bohlmann and colleagues (1973), are different from those that we observed for collimomycins, suggesting that the latter represent a structure that is different from that of previously described bacterial polyynes. Initial attempts at purifying individual ene-triynes by means of semi-preparative HPLC failed because of the extreme instability of these metabolites. This behaviour has also been observed by others (Parish *et al.*, 2004). However, by means of liquid chromatography-mass spectrometry (LC-MS) it was possible to derive a molecular weight for compounds b and d (Fig. 5) of 286 and 274 Da respectively. The MS data of the other peaks did not allow for an unambiguous determination of the molecular weight most likely because of decomposition, poor ionization and/or in-source fragmentation.

The mode of action of polyacetylenes is still poorly understood. It has been suggested (Dembitsky, 2006) that their cytotoxicity is related to membrane damage. This would be in line with the observation that *A. niger* responds to the presence of *C. fungivorans* Ter331 by upregulation of several genes involved in membrane fluidity (Mela *et al.*, 2011). It would also be consistent with the observation (Fig. 1) that *Collimonas* induced *Aspergillus* hyperbranching, which has been linked to membrane integrity (Lin and Momany, 2004). For the actinomycete-produced antifungal polyyne L-660,631, the mode of action in *Candida albicans* was demonstrated to be inhibition of ergosterol biosynthesis (Onishi *et al.*, 2006). Ergosterol is an essential component of fungal membranes, and its biosynthesis represents an attractive and selective target for the development of novel fungicides. In confrontation with *C. fungivorans* Ter331, one of the genes upregulated in *A. niger* was An03g00580 (Mela *et al.*, 2011), which codes for a key enzyme in the biosynthesis of ergosterol (van den Brink *et al.*, 1998). This might suggest that in *A. niger*, the membrane is a direct or indirect target of collimomycins produced by *C. fungivorans* Ter331. However, we cannot rule out other or additional targets, especially because we observed activity against oomycetes (see Supporting Information Fig. S1), which are not known to feature ergosterol in their membranes.

The data in Fig. 5 seem to suggest that the putative polyyne moiety alone is not sufficient for collimomycin activity, as several fractions featuring the same characteristic UV spectrum did not inhibit the growth of *A. niger*. The bioactivity of polyynes has been attributed in part to the reactivity of the triple bonds that these compounds possess (Abraham, 2010). Indeed, Yamaguchi and colleagues (1995) demonstrated that triyne and diyne analogues of dienetetraynic caryoynencins were less active as antifungals compared with the tetrayne analogue. However, the type of accessory groups at one or both sides of the polyyne backbone also contributes to bioactivity. For example, antimycobacterial activity of falcarindiol varied with alterations to one or both terminal groups while leaving the diyne backbone intact (Deng *et al.*, 2008). We hypothesize that *C. fungivorans* Ter331 produces several different compounds that share a putative ene-triyne feature but differ in the remaining part of the molecule. Future efforts aimed at elucidating the structure of active as well as inactive collimomycins have to overcome the inherent instability of these ene-triynes (Parish *et al.*, 2004). An adapted isolation and purification protocol, avoiding light, oxygen and evaporation as much as possible, followed by LC-MS and liquid chromatography-nuclear magnetic resonance spectroscopy will unravel the chemistry that is important for their activity.

For many polyynes, the genetics underlying their biosynthesis are unknown. Here, we presented strong experimental evidence for the involvement of cluster K genes in the production of collimomycins by *C. fungivorans* Ter331. This cluster K features an over-representation of genes coding for putative fatty acid desaturases (see Supporting Information Table S1, Fig. 2). Such enzymes are often implicated in the polyyne biosynthesis. For example, in soldier beetles, monounsaturated oleic acid is converted in five steps by three desaturases into the immediate precursor of the polyacetylenic compound dihydromatricaria acid (Haritos *et al.*, 2012). One of the genes in cluster K (i.e. Cf_1132) is predicted to code for a phosphopantetheine-binding protein of the type that is often found associated as a module within polyketide synthase gene clusters, and so perhaps this protein is involved in the biosynthesis of the putative ene-triyne core (Minto and Blacklock, 2008) or in the process of decorating one or both ends of the putative ene-triyne backbone of collimomycins. Elucidation of the structure of the various collimomycins will allow a more precise mapping of cluster K genes onto its biosynthetic pathway. We hypothesize that this pathway also features CP or intermediates/products of the shikimate pathway based on the observed inability of *C. fungivorans* Ter331 mutants 10E11, 14C11 and 14G4 to produce collimomycins. The putative transporter genes found in cluster K (Cf_1136 and Cf_1144) may represent an adaptation by *C. fungivorans* Ter331 to transport collimomycins outside of the cell, possibly as part of a resistance mechanism.

In summary, the results presented here indicate that *C. fungivorans* Ter331 produces antifungal compounds (collimomycins) that likely belong to the class of the polyynes. By a combinatorial approach using mutant library screening for loss-of-function mutants and RP-HPLC analysis of agar extracts from wild type and mutants, the production of collimomycins could be linked to a cluster of genes involved in the biosynthesis of these compounds. We found a positive correlation between the possession of these genes and ability to inhibit the fungus *A. niger*. The polyynes released by *C. fungivorans* Ter331 inhibited the growth of several food spoiling and plant pathogenic fungi, and are also active against oomycetes harmful to fish or plants.

Experimental procedures

Microorganisms and culture conditions

Collimonas fungivorans Ter331 (de Boer *et al*., 2004) was originally isolated from a coastal dune site on the island of Terschelling, the Netherlands. Strain Ter331R3 is a spontaneous rifampicin (Rif)-resistant derivative of Ter331 that was previously used (Leveau *et al*., 2006) to generate a genome-wide random mutant library with plasposon pTn*Mod*-KmO*lac*Z (Dennis and Zylstra, 1998). Ter331R3 was grown on King's B (KB) agar containing 40 μg Rif ml^{-1}, while plasposon mutants were selected and grown on KB Rif supplemented with kanamycin (50 μg ml^{-1}). Other collimonads used in this study have been described previously (Höppener-Ogawa *et al*., 2008). *Pseudomonas protegens* Pf-5 (Loper *et al*., 2012) was used as a control in some experiments. The fungi used in this study included *A. niger* N402 (Bos *et al*., 1988; ATCC 64974), *A. westerdijkiae* CBS 112803, *A. versicolor* CBS 117.34, *P. chrysogenum* CBS 306.48, *P. glabrum* CBS 328.48 and *P. expansum* CBS 112450. Fungal spores were produced by culturing the fungi on half-strength potato dextrose agar (PDA; Oxoid, Basingstoke, UK) for 4 days at 30°C. Spores were collected as described previously (Mela *et al*., 2011).

Agar plate confrontation assays

Confrontation assays between *C. fungivorans* Ter331 (or other *Collimonas* strains) and *A. niger* (or other fungi) were performed on WYA (Mela *et al*., 2011) supplemented with 2 mM *N*-acetylglucosamine. *Collimonas* was line-inoculated in the middle of the plate (Fig. 1). Ten microlitres of a suspension of 10^4 *A. niger* spores per millilitre in 0.9% NaCl were spot-inoculated at a distance of 2 cm from this *Collimonas* line. In a variation on this protocol, we substituted spores with agar plugs from fungal cultures on half-strength PDA to inoculate the confrontation plates.

Screening for Collimonas *mutants with reduced antifungal activity*

Screening of the *C. fungivorans* Ter331 plasposon library (Leveau *et al*., 2006) for mutants with diminished antifungal activity towards *A. niger* was performed in bottomless 96-well microtiter plates (Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands). To set up the assay, the bottom of the plates was sealed temporarily with sealing mats (Greiner Bio-One B.V.) to allow each well to be filled with 190 μl WYA-Nag and 20 μg of the pH-indicator bromocresol purple per millilitre. After removal of the sealing mat, the agar in each well was inoculated from the bottom with one of 3300 *Collimonas* mutants, while the top side was inoculated with 3 μl of a 10^5 ml^{-1} spore suspension of *A. niger*. The plates were incubated at 20°C for 5 days. The pH-indicator in the agar facilitated the detection of non-inhibiting *Collimonas* mutants, as a colour change from purple to yellow corresponded with the release of organic acids which typifies normal, i.e. uninhibited growth of *A. niger*. We used an inverse microscope (Leica DM IRB, Wetzlar, Germany) to confirm *Collimonas*-induced deformation of *A. niger* hyphae in yellow-coloured wells. The loss of antifungal activity by mutants was validated by retesting the same mutants in a standard agar plate confrontation assay (as described above). The plasposon insertion sites of selected mutants were determined by genomic flank sequencing (Leveau *et al*., 2006), mapped onto the genome sequence of *C. fungivorans* Ter331 (NCBI accession number CP002745) and compared with published sequences using NCBI Blast (Altschul *et al*., 1990) and the Integrated Microbial Genomes site of the Joint Genome Institute (http://img.jgi.doe.gov). The organization of genes into putative operons was assessed using FgenesB (http://www.softberry.com). Southern blotting was used to confirm that each of the selected mutants harbored a single plasposon insertion (results not shown).

Extraction of antifungal compound(s) from C. fungivorans *Ter331 and activity profiling*

In a set of preliminary experiments, we discovered that extracts obtained from confrontation plates with both *A. niger* and *C. fungivorans* Ter331 and from plates with strain Ter331 alone (i.e. in the absence of the fungus) showed comparable levels of inhibition of hyphal growth. Extracts from plates with the fungus alone did not inhibit hyphal growth (not shown). These results indicated that the antifungal compound(s) were produced by *Collimonas* in the absence of *A. niger*. Therefore, subsequent analyses were performed with extracts obtained from agar plates with *Collimonas* only, thereby preventing the co-isolation of fungal metabolites.

For the extraction of antifungal compounds from *C. fungivorans* Ter331 and its plasposon mutants, strains were grown on WYA-Nag. Agar pieces of approximately 1 cm^2 (surface area) were cut out, suspended in 80% acetone and shaken for 1 h at room temperature. After centrifugation at 4800 r.p.m. for 15 min, the liquid phase was transferred and acetone was evaporated by nitrogen flow. The remaining aqueous phase was acidified with trifluoroacetic acid (TFA; final concentration 0.1% v/v) and extracted with two volumes of ethyl acetate. After phase separation, the ethyl acetate was transferred to a new tube, evaporated by nitrogen flow and the dried extract was dissolved in 100% HPLC-pure methanol. To normalize the volumes between the different samples, we used 4 μl of methanol for every

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10 g of agar material that entered the extraction protocol. Aliquots of the extracts were immediately tested in confrontation assays with *A. niger* or stored at −20 C. The inhibition assays were performed in 24-well plates containing in each well 1 ml WYA-Nag. *Aspergillus niger* was inoculated (5 μl of a suspension of 10^6 spores per ml in 0.9% NaCl) in each well and away from the centre of the well, grown o/n at 30°C, at which point 5 μl of an extract in methanol or methanol only (control) was deposited on a Whatman filter paper disc (5 mm diameter) that was placed on the agar surface opposite of the expanding *A. niger* colony. Plates were incubated at 30°C, and inhibition of hyphal growth was scored after overnight incubation with an inverted microscope.

RP-HPLC analysis and fractionation

Agar plate extracts of cultures of wildtype strain Ter331 and mutant 13E12 were analysed by RP-HPLC (Waters Chromatography B.V., Etten-Leur, the Netherlands) equipped with a Waters 996 photodiode array detector. The separations were performed on a Waters Symmetry C18RP column (5 μm, 3.9 × 150 mm) with a flow of 0.5 ml min^{-1}. The solvent was water/acetonitrile containing 0.1% TFA. A linear gradient from 0% to 100% acetonitrile with 0.1% TFA was applied over 30 min or 50 min for improved resolution of peaks. UV detection took place at 240 nm. Fractions were collected and acetonitrile was evaporated under nitrogen flow. After addition of 0.1% (v/v) TFA to the aqueous phase, the fractions were extracted with two volumes of ethyl acetate. The ethyl acetate phase was transferred to a new tube, evaporated under nitrogen flow and the dried fractions were dissolved in MeOH, and used in 24-well inhibition assays as described above. The LC-UV-MS measurements were done on a Thermo Finnigan system (all components from Thermo Fisher Scientific, San Jose, CA, USA): Finnigan Surveyor Autosampler, MS Pump Plus HPLC, PDA Plus detector and Finnigan LXQ mass spectrometer with electrospray ionization-interface with positive ionization. Data were processed with XCALIBUR software (Thermoquest, Breda, The Netherlands). Column and solvents were the same as described above.

PCR-based detection of cluster K genes

Four pairs of degenerated primers were designed to amplify orthologues of Cf_1133, _1138, _1139 and _1141, based on alignment of these genes from *C. fungivorans* Ter331 with those found on the genomes of *B. vietnamiensis* G4, *B. ambifaria* Mex5, *B. ambifaria* IOP 40-10, *P. protegens* Pf-5 and *Streptomyces* sp. Mg-1. Primer sequences were as follows: 1133A_f1, GCICAYATGCCNTGGATG; 1133A_r1, AAIGCRTGRTGRTTRTTYTG; 1138B_f1, TGGTAYCAYGG IYTNMGITAYGA; 1138B_r1, GCIACRTCYTTRTCYTCCAT; 1139B_f2, ATGACNCAYTGGAARCAYCA; 1139B_r1, CCRA ARAANARIGCNGTRCA; 1141B_f1, GARYTIGTNTGGCAR MG; 1141B_r1, ACCATIGCCATYTGDATRAA. Bacterial DNA (1 μl of 10 ng μl^{-1}) was added to a 25 μl (final volume) mixture containing 0.8 μM of each primer, 200 μM dNTPs, 2.5 μl of 10× buffer (Promega, Leiden, the Netherlands), 2.5 mM $MgCl_2$ and 0.04 U *Taq* polymerase. Thermal cycling included the initial denaturation step of 95°C for 3 min, followed by a touchdown program in which the annealing temperature decreased from 63°C by 1°C per cycle during the first 12 cycles, followed by 13 cycli at 52°C for 30 s. The denaturing step was 30 s at 94°C, and the elongation step was 50 s at 72°C. Cycling was completed by a final step of 5 min at 72°C.

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Supporting information

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

Fig. S1. Hyphal growth of *A. niger*, *A. versicolor*, *A. westerdijkiae*, *P. chrysogenum* and *P. glabrum* in the absence and presence of *C. fungivorans* Ter331 or mutants 13E12 and 8G9.

Fig. S2. HPLC trace for extract from mutant 28A12 (top) compared with those of Ter331 wt (middle) and mutant 13E12 (bottom). HPLC conditions were different from those shown

in Figs 4 and 5: Dionex HPLC with photodiode array detector; detection at 240 nm; 4.6 mm, 5 um, 250 mm C18RP column (Grace/Alltech, Breda, the Netherlands); flow: 1 ml min^{-1}; solvent: acetonitrile/water with 0.1% (v/v) TFA; gradient: 5 min 30–35% acetonitrile, 5 min 35%, 15 min 35–41%, 1 min 41–43%, 25 min 43–45%, 6 min 45–49%, 7 min 49–100%, 10 min 100%.

Table S1. Genes and gene predictions for cluster K on the Ter331 genome (Mela *et al*., 2011).

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