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

Journal:	<i>Environmental Microbiology and Environmental Microbiology Reports</i>
Manuscript ID:	EMI-2013-1624
Manuscript Type:	EMI - Research article
Journal:	Environmental Microbiology
Date Submitted by the Author:	23-Dec-2013
Complete List of Authors:	Fritsche, Kathrin; NIOO-KNAW, Microbial Ecology van den Berg, Marlies; NIOO-KNAW, Microbial Ecology De Boer, Wietse; NIOO-KNAW, Microbial Ecology van Beek, Teris; Wageningen University, Laboratory of Phytopathology Raaijmakers, Jos; Wageningen University, Laboratory of Phytopathology van Veen, Hans; NIOO-KNAW, Microbial Ecology Leveau, Johan; University of California, Plant Pathology
Keywords:	bacteria, fungi, microbe:microbe interactions, functional diversity

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

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17 Running title: Characterization of *Collimonas* antifungal polyynes

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19 **ABSTRACT**

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

35

36 INTRODUCTION

37 The genus *Collimonas* (family Oxalobacteraceae, class Betaproteobacteria) consists of mostly
38 soil bacteria that are defined by their ability to grow at the expense of living fungal hyphae
39 under nutrient-limited conditions (de Boer *et al.*, 2004; Leveau and Preston, 2008; Leveau *et*
40 *al.*, 2010). So far, three species have been described: *C. fungivorans*, *C. pratensis*, and *C.*
41 *arenae* (Höppener-Ogawa *et al.*, 2008). All three species have representatives for which
42 antifungal activity was demonstrated (Leveau *et al.* 2010; Mela *et al.*, 2012), but the
43 metabolites involved in this activity have not yet been characterized. In a recent study by
44 Mela *et al.* (2011), it was shown that on nutrient-poor water agar plates the hyphal growth of
45 *Aspergillus niger* was inhibited by *Collimonas fungivorans* strain Ter331. This inhibition did
46 not require direct contact between the bacteria and the fungus, as it was not affected by
47 physical separation of the two organisms by an 8-kDa cut-off dialysis membrane (Fritsche *et*
48 *al.*, 2008). This suggested that the antifungal activity was due to a small, diffusible factor
49 produced by the bacterium. In confrontation with *A. niger*, several genes putatively involved
50 in the production of antifungal compound(s) were upregulated in *C. fungivorans* Ter331
51 (Mela *et al.*, 2011). Using a comparative genomic hybridization approach (Mela *et al.*, 2012),
52 the same group of genes, designated as cluster K, was found in its entirety on the genome of
53 *C. fungivorans* Ter14, only partially in *C. fungivorans* Ter6, and not at all in *C. pratensis*
54 Ter91 or *C. arenae* Ter10. This finding aligned well with the observation that *C. fungivorans*
55 Ter331 and Ter14, but not *C. fungivorans* Ter6, *C. pratensis* Ter91 and *C. arenae* Ter10,
56 inhibit growth of *A. niger* in a confrontation assay (Mela *et al.*, 2012). The objective of the
57 present study was to isolate and determine the bioactivity of the compound(s) responsible for
58 the observed antifungal activity of *C. fungivorans* Ter331 against *A. niger*, to carry out
59 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.

62

63 RESULTS

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

74 We tested 36 other *Collimonas* strains in the confrontation assay with *A. niger* (Table 1).
75 From this analysis, it became apparent that the antifungal activity was unique to members of
76 *C. fungivorans* subgroup B, which includes Ter331 and which was defined previously
77 (Höppener-Ogawa *et al.*, 2008) based on BOX-PCR fingerprint patterns of 42 *Collimonas*
78 isolates. Only two strains from this subgroup B (out of 14 strains tested) were unable to
79 inhibit *A. niger*. As we will show below, these isolates (i.e. SO 30 and SO 115) also lacked
80 one or more cluster K genes, suggesting the involvement of these genes in the fungistatic
81 phenotype. None of the *C. fungivorans* subgroup C strains (n=4), including *C. fungivorans*
82 type strain Ter6, was active against *A. niger*, and neither were any of the tested *C. arenae*
83 (n=4) or *C. pratensis* (n=15) strains. The non-collimonad control *Pseudomonas protegens* Pf-
84 5 also did not inhibit hyphal growth of *A. niger*.

85

86 **Identification and genomic characterization of *C. fungivorans* mutants with reduced**87 **antifungal activity.** We used WYA-Nag in a medium-throughput setup (see Experimental88 Procedures) to test a total of 3,300 random plasposon mutants of *C. fungivorans* Ter331 for89 reduced activity against *A. niger*. Six such mutants were identified and selected for further

90 analysis. Two (8G9 and 13E12) were tested and confirmed to be impaired in halting the

91 hyphal growth of not only *A. niger* but also all other fungi and oomycetes whose growth was92 affected negatively by wildtype *C. fungivorans* Ter331 under the same conditions (Figure S1).

93 In three mutants (10E11, 14C11, and 14G4), the plasposon insertions were mapped to genes

94 involved in amino acid biosynthesis. Mutant 10E11 carried the plasposon in gene Cf_2563

95 (CFU_2502) which codes for the large chain of carbamoyl-phosphate (CP) synthetase, an

96 enzyme that provides CP as precursor for the biosynthesis of arginine and pyrimidine (Holden

97 *et al.*, 1999). In mutant 14G4, the plasposon insertion was positioned in gene Cf_3833

98 (CFU_3751) which codes for 5-enolpyruvylshikimate-3-phosphate synthase, the penultimate

99 enzyme in the shikimate pathway. The end-product of this pathway is chorismate, which

100 serves as substrate for the biosynthetic pathway of the aromatic amino acids phenylalanine,

101 tyrosine and tryptophan (Herrmann, 1999). In mutant 14C11, the plasposon localized to gene

102 Cf_752 (CFU_0701) which codes for the amidotransferase component of anthranilate

103 synthase, an enzyme that converts chorismate to anthranilate as part of the tryptophan

104 biosynthetic pathway.

105 For the other three mutants (8G9, 13E12, 28A12), plasposon insertion sites were located in

106 proximity to each other and within or close to the previously identified gene cluster K (Mela

107 *et al.*, 2011) on the Ter331 genome (Figure 2). Genes in this cluster (Cf_1128-1142; Table108 S1) were previously shown to be upregulated in confrontation of *C. fungivorans* Ter 331 with109 *A. niger* (Mela *et al.*, 2011). The insertion in mutant 13E2 disrupted gene Cf_1135, which

110 codes for a protein annotated as a fatty acid CoA ligase. Cf_1135 is the first gene in a
 111 predicted operon of seven, with three genes (Cf_1134, Cf_1133, and Cf_1131) coding for
 112 fatty acid desaturases. In this operon, gene Cf_1132 is predicted to code for a putative acyl
 113 carrier protein (ACP), Cf_1130 for a hydrolase, and Cf_1129 for a rubredoxin-type protein.
 114 In mutant 8G9, the plasposon insertion was located upstream of Cf_1141, which encodes a 3-
 115 oxoacyl ACP synthase, and within gene Cf_1143, which codes for a LysR-type regulator and
 116 forms a predicted operon with gene Cf_1144, annotated as coding for a Major Facilitator
 117 Superfamily (MFS) transporter. The latter showed resemblance to another MFS protein
 118 encoded by cluster K, i.e. the predicted product of gene Cf_1136. This gene was annotated as
 119 coding for a drug resistance transporter, with similarity to FarB of *Neisseria gonorrhoea*,
 120 which has been shown to confer resistance against antibacterial fatty acids (Lee and Shafer,
 121 1999). Cf_1136 forms a predicted operon with three other genes, coding for a fatty acid
 122 desaturase (Cf_1139), a Rieske-type oxygenase (Cf_1138), and a hypothetical protein
 123 (Cf_1137), respectively. In mutant 28A12, the plasposon was located upstream of gene
 124 Cf_1145a, which codes for yet another fatty acid desaturase and forms a predicted operon
 125 with gene Cf_1145 with unknown function.
 126 The content and synteny of gene cluster K and its flanking regions appeared to be conserved
 127 to various degrees in several other bacterial genomes (Figure 2B). In *Burkholderia*
 128 *vietnamiensis* G4, *Burkholderia* sp. KJ006, and *Burkholderia ambifaria* IOP40-10 and
 129 MEX5, we found a near-complete orthologous version of cluster K. Only part of the cluster
 130 (Cf_1135-Cf_1141) was identified in the genomes of *Burkholderia gladioli* BSR3,
 131 *Streptomyces* sp. Mg1 (accession number DS570401) and *Pseudomonas protegens* Pf-5
 132 (Paulsen *et al.*, 2005; Loper *et al.*, 2012) and *P. protegens* CHA0. In the two *Pseudomonas*
 133 genomes, the Cf_1131 and Cf_1130 orthologs occur fused into a single gene. *P. protegens* Pf-
 134 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 orthologs 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 expected from its genomic context (Figure 2), the non-collimonad control strain *P. protegens* Pf-5 carried an ortholog 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 (Figure 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 (Figure 4A) or mutant 28A12 (Figure 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 (Figure 4B). By adopting a different elution gradient, a higher resolution of peaks was obtained (Figure 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 (λ 327/328, 306/307, 287/288, 271, 240/241, 229/230 nm) closely resembled those of compounds that contain an ene-triynone moiety ($-\text{C}=\text{C}-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{C}=\text{C}-$), according to Bohlmann *et al.* (1973) (i.e. λ 328, 307, 288, 271, 242, and 231 nm). While all five fractions showed this UV spectrum (Figure 5B), only two were shown to be active against *A. niger*, namely fraction 3 (Figure 5B, marked with *, includes peaks c and d) and fraction 5 (Figure 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-triynone-based moiety, i.e. featuring multiple conjugated $\text{C}\equiv\text{C}$ 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, 2007; Abraham, 2010). Many

185 have bioactive properties, such as antibacterial, cytotoxic, herbicidal and/or antifungal
186 activities (Abraham, 2010; Shi Shun and Tykwinski, 2006). The best studied bacterial
187 polyynes so far are the ene-diyne antitumor antibiotics from actinomycetes, e.g. calicheamicin
188 from *Micromonospora echinospora* sp. *calichensis* (Ahlert *et al.*, 2002; Belecki *et al.*, 2009).
189 Other examples of bacterially produced polyynes are cepacin A and B from *Burkholderia*
190 *cepacia* (Parker *et al.*, 1984) and caryoynencin A-C from *Burkholderia caryophylli* (Kusumi *et*
191 *al.*, 1987; Yamaguchi *et al.*, 1995). The predicted UV maxima for cepacins (226, 236, 294,
192 309 nm) and caryoynencins (273, 290, 311, 331, 356, 382 nm), based on Bohlmann (1973),
193 are different from those that we observed for collimomycins, suggesting that the latter
194 represent a structure that is different from that of previously described bacterial polyynes.
195 Initial attempts at purifying individual ene-triynes by means of semi-preparative HPLC failed
196 due to the extreme instability of these metabolites. This behavior has also been observed by
197 others (Parish *et al.*, 2004). However by means of LC-MS it was possible to derive a
198 molecular weight for compounds **b** and **d** (Fig. 5) of 286 and 274 Da, respectively. The MS
199 data of the other peaks did not allow for an unambiguous determination of the molecular
200 weight, most likely due to decomposition, poor ionization and/or in-source fragmentation.
201 The mode of action of polyacetylenes is still poorly understood. It has been suggested
202 (Dembitsky, 2006) that their cytotoxicity is related to membrane damage. This would be in
203 line with the observation that *A. niger* responds to the presence of *C. fungivorans* Ter331 by
204 upregulation of several genes involved in membrane fluidity (Mela *et al.*, 2011). It would also
205 be consistent with the observation (Figure 1) that *Collimonas* induced *Aspergillus*
206 hyperbranching, which has been linked to membrane integrity (Lin and Momany, 2004). For
207 the actinomycete-produced antifungal polyyne L-660,631, the mode of action in *Candida*
208 *albicans* was demonstrated to be inhibition of ergosterol biosynthesis (Onishi *et al.*, 2006).
209 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 (Figure S1), which are not known to feature ergosterol in their membranes.

The data in Figure 5 seem to suggest that the putative polyynes 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 *et al.* (1995) demonstrated that triyne and diyne analogs of diene-tetraynic caryophenols were less active as antifungals compared to the tetrayne analog. However, the type of accessory groups at one or both sides of the polyynes backbone also contributes to bioactivity. For example, antimycobacterial activity of faltarindiol 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 which 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 LC-NMR 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

235 production of collimomycins by *C. fungivorans* Ter331. This cluster K features an
236 overrepresentation of genes coding for putative fatty acid desaturases (Table S1, Figure 2).
237 Such enzymes are often implicated in the polyene biosynthesis. For example, in soldier
238 beetles, monounsaturated oleic acid is converted in five steps by three desaturases into the
239 immediate precursor of the polyacetylenic compound dihydromatricaria acid (Haritos *et al.*,
240 2012). One of the genes in cluster K (i.e. Cf_1132) is predicted to code for a
241 phosphopantetheine-binding protein of the type that is often found associated as a module
242 within polyketide synthase (PKS) gene clusters, and so perhaps this protein is involved in the
243 biosynthesis of the putative ene-triene core (Minto and Blacklock, 2008), or in the process of
244 decorating one or both ends of the putative ene-triene backbone of collimomycins.
245 Elucidation of the structure of the various collimomycins will allow a more precise mapping
246 of cluster K genes onto its biosynthetic pathway. We hypothesize that this pathway also
247 features carbamoyl-phosphate or intermediates/products of the shikimate pathway, based on
248 the observed inability of *C. fungivorans* Ter331 mutants 10E11, 14C11, and 14G4 to produce
249 collimomycins. The putative transporter genes found in cluster K (Cf_1136 and Cf_1144)
250 may represent an adaptation by *C. fungivorans* Ter331 to transport collimomycins outside of
251 the cell, possibly as part of a resistance mechanism.

252 In summary, the results presented here indicate that *C. fungivorans* Ter331 produces
253 antifungal compounds (collimomycins) that likely belong to the class of the polyenes. By a
254 combinatorial approach using mutant library screening for loss-of-function mutants and RP-
255 HPLC analysis of agar extracts from wild type and mutants, the production of collimomycins
256 could be linked to a cluster of genes involved in the biosynthesis of these compounds. We
257 found a positive correlation between the possession of these genes and ability to inhibit the
258 fungus *Aspergillus niger*. The polyenes 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. *C. 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-resistant derivative of Ter331 that was previously used (Leveau *et al.*, 2006) to generate a genome-wide random mutant library with plasposon pTnMod-KmOlaCZ (Dennis and Zylstra, 1998). Ter331R3 was grown on King's B agar (KB) containing 40 µg rifampicin (Rif) per ml, while plasposon mutants were selected and grown on KB Rif supplemented with kanamycin (50 µg per ml). 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 *Aspergillus niger* N402 (Bos *et al.*, 1988; ATCC 64974), *A. westerdijkiae* CBS 112803, *A. versicolor* CBS 117.34, *Penicillium 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 water yeast agar (WYA) (Mela *et al.*, 2011) supplemented with 2 mM *N*-acetylglucosamine. *Collimonas* was line-inoculated in the middle of the plate (Figure 1). Ten microliters of a suspension of 10⁴ *A. niger* spores per ml 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, NL). 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 μ l WYA containing 2 mM *N*-acetylglucosamine and 20 μ g of the pH-indicator bromocresol purple per ml. After removal of the sealing mat, the agar in each well was inoculated from the bottom with one of 3,300 *Collimonas* mutants, while the top side was inoculated with 3 μ l 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 color 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-colored 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, 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 (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 containing 2 mM *N*-acetylglucosamine. Agar pieces of approximately 1 cm² (surface area) were cut out, suspended in 80% acetone and shaken for 1h at room temperature. After centrifugation at 4,800 rpm for 15 min, the liquid phase was transferred and acetone was evaporated by nitrogen flow. The remaining aqueous phase was acidified with trifluoroacetic acid (final concentration 0.1% v/v) and extracted with 2 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 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 supplemented with 2 mM *N*-acetylglucosamine. *A. niger* was inoculated (5 µl of a suspension of 10⁶ spores per ml in 0.9% NaCl) in each well and away from the center 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 analyzed 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. The solvent was water/acetonitrile containing 0.1% trifluoroacetic acid (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 2 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: Finnigan Surveyor Autosampler, MS Pump Plus HPLC, PDA Plus detector, and Finnigan LXQ mass spectrometer with ESI-interface with positive ionization. Data were processed with XCalibur software. 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 orthologs of Cf_1133, _1138, _1139, and _1141, based on alignment of these genes from *C. fungivorans* Ter331 with those found on the genomes of *Burkholderia vietnamiensis* G4, *Burkholderia 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, AAIGCRTGRTGRTTTRTTYTG; 1138B_f1, TGGTAYCAYGGIYTNMGITAYGA; 1138B_r1, GCIACRTCYTTRTCYTCCAT; 1139B_f2, ATGACNCAYTGGAARCA YCA; 1139B_r1, CCRAARAANARIGCNGTRCA; 1141B_f1, GARYTIGTNTGGCARMG; 1141B_r1, ACCATIGCCATYTG DATRAA. Bacterial DNA (1 µl of 10 ng/µl) was added to a 25 µl (final volume) mixture containing 0.8 µM of each primer, 200 µM dNTP's, 2.5 µl of 10x buffer (Promega, Leiden, The Netherlands), 2.5 mM MgCl₂ 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 sec. The denaturing step was 30 sec at 94 °C and the elongation step was 50 sec at 72 °C. Cycling was completed by a final step of 5 min at 72 °C.

ACKNOWLEDGMENTS

The authors thank Arthur Ram for providing *A. niger* N402, Rob Samson for access to other *Aspergillus* and *Penicillium* species, Saskia Gerards for maintenance of the *C. fungivorans* Ter331 plasposon library, Menno van der Voort for analytical assays on the agar extracts and Elbert van der Klift and Frank Claassen for their help with HPLC. This research was supported in part by funds from the Netherlands Genomics Initiative/Ecogenomics cluster and by startup funds to JHJL at UC Davis. This is NIOO-KNAW publication number XXXX.

REFERENCES

- Abraham, W.-R. (2010) Biosynthetic oils, fats, terpenes, sterols, waxes: analytical methods, diversity, characteristics. In: Handbook of hydrocarbon and lipid microbiology, K. N. Timmis (ed.), pages 79-95.
- Ahlert, J., Shepard, E., Lomovskaya, N., Zazopoulos, E., Staffa, A., Bachmann, B.O., *et al.* (2002) The calicheamicin gene cluster and its iterative type I enediyne PKS. *Science* 297: 1173-1176.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* 215: 403-410.
- Belecki, K., Crawford, J.M., and Townsend, C.A. (2009) Production of octaketide polyenes by the calicheamicin polyketide synthase CalE8: implications for the biosynthesis of enediyne core structures. *J Amer Chem Soc* 131: 12564-12566.
- Bohlmann, F., Burkhardt, T., and Zdero, C. (1973) Naturally occurring acetylenes. Academic Press, London.
- Bos, C.J., Debets, A.J., Swart, K., Huybers, A., Kobus, G., and Slakhorst, S.M. (1988) Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Curr Genet* 14: 437-443.
- De Boer, W., Leveau, J.H.J., Kowalchuk, G.A., Klein Gunnewiek, P.J.A., Abeln, E.C.A., Figge, M.J., *et al.* (2004) *Collimonas fungivorans* gen. nov., sp nov., a chitinolytic soil bacterium with the ability to grow on living fungal hyphae. *Int J Syst Evol Microbiol* 54: 857-864.
- Dembitsky, V.M. (2006) Anticancer activity of natural and synthetic acetylenic lipids. *Lipids* 41: 883-924.
- Deng, S., Wang, Y., Inui, T., Chen, S.-N., Farnsworth, N.R., Cho, S., *et al.* (2008) Anti-TB polyynes from the roots of *Angelica sinensis*. *Phytoter Res* 22: 878-882.

- 402 Dennis, J.J., and Zylstra, G.J. (1998) Plasposons: modular self-cloning minitransposon
403 derivatives for rapid genetic analysis of gram-negative bacterial genomes. *Appl Environ*
404 *Microbiol* 64: 2710–2715.
- 405 Fritsche, K., de Boer, W., Gerards, S., van den Berg, M., van Veen, J.A., and Leveau, J.H.J.
406 (2008) Identification and characterization of genes underlying chitinolysis in *Collimonas*
407 *fungivorans* Ter331. *FEMS Microbiol Ecol* 66: 123-135.
- 408 Hakvag, S., Fjaervik, E., Klinkenberg, G., Borgos, S.E.F., Josefsen, K.D., Ellingsen, T.E., and
409 Zotchev, S.B. (2009) Violacein-producing *Collimonas* sp. from the sea surface microlayer
410 of coastal waters in Trondelag, Norway. *Marine Drugs* 7: 576-588.
- 411 Haritos, V.S., Horne, I., Damcevski, K., Glover, K., Gibb, N., Okada, S., and Hamberg, M.
412 (2012) The convergent evolution of defensive polyacetylenic fatty acid biosynthesis genes
413 in soldier beetles. *Nature Communications* 3: 1150.
- 414 Hassan, K.A., Johnson, A., Shaffer, B.T., Ren, Q.H., Kidarsa, T.A., Elbourne, L.D.H., *et al.*
415 (2010) Inactivation of the GacA response regulator in *Pseudomonas fluorescens* Pf-5 has
416 far-reaching transcriptomic consequences. *Environ Microbiol* 12: 899-915.
- 417 Herrmann, K.M. (1999) The shikimate pathway. *Annu Rev Plant Physiol Plant Mol Biol* 50:
418 473-503.
- 419 Holden, H.M., Thoden, J.B., and Raushel, F.M. (1999): Carbamoyl phosphate synthetase: an
420 amazing biochemical odyssey from substrate to product. *Cell Mol Life Sci* 56: 507-522.
- 421 Höppener-Ogawa, S., de Boer, W., Leveau, J.H.J., van Veen, J.A., de Brandt, E., Vanlaere,
422 E., *et al.* (2008) *Collimonas arenae* sp nov and *Collimonas pratensis* sp nov., isolated from
423 (semi-)natural grassland soils. *Int J Syst Evol Microbiol* 58: 414-419.
- 424 Kusumi, T., Ohtani, I., Nishiyama, K., and Kakisawa, H. (1987) Caryoynencins, potent
425 antibiotics from a plant pathogen *Pseudomonas caryophylli*. *Tetrahedron Lett* 28: 3981-
426 3984.

- 427 Lee, E.H., and Shafer, W.M. (1999) The *farAB*-encoded efflux pump mediates resistance of
428 gonococci to long-chained antibacterial fatty acids. *Mol Microbiol* 33: 839-845.
- 429 Leveau, J.H.J., Gerards, S., Fritsche, K., Zondag, G., and van Veen, J.A. (2006) Genomic
430 flank-sequencing of plasmid insertion sites for rapid identification of functional genes. *J*
431 *Microbiol Methods* 66: 276-285.
- 432 Leveau, J.H.J., and Preston, G.M. (2008) Bacterial mycophagy: definition and diagnosis of a
433 unique bacterial-fungal interaction. *New Phytologist* 177: 859-876.
- 434 Leveau, J.H.J., Uroz, S., and de Boer, W. (2010) The bacterial genus *Collimonas*:
435 mycophagy, weathering and other adaptive solutions to life in oligotrophic soil
436 environments. *Environ Microbiol* 12: 281-292.
- 437 Lin, X., and Momany, M. (2004) Identification and complementation of abnormal hyphal
438 branch mutants *ahbA1* and *ahbB1* in *Aspergillus nidulans*. *Fungal Genet Biol* 41: 998-1006.
- 439 Loper, J.E., and Gross, H. (2007) Genomic analysis of antifungal metabolite production by
440 *Pseudomonas fluorescens* Pf-5. *Eur J Plant Pathol* 119: 265-278.
- 441 Loper, J.E., Hassan, K.A., Mavrodi, D.V., Davis II, E.W., Lim, C.K., Shaffer, B.T., *et al.*
442 (2012) Comparative genomics of plant-associated *Pseudomonas* spp: insights into diversity
443 and inheritance of traits involved in multitrophic interactions. *PLoS Genet* 8:e1002784
- 444 Matz, C., Deines, P., Boenigk, J., Arndt, H., Eberl, L., Kjelleberg, S., and Jürgens, K. (2004)
445 Impact of violacein-producing bacteria on survival and feeding of bacterivorous
446 nanoflagellates. *Appl Environ Microbiol* 70: 1593-1599.
- 447 Mela, F., Fritsche, K., de Boer, W., van Veen, J.A., de Graaff, L.H., van den Berg, M., and
448 Leveau, J.H.J. (2011) Dual transcriptional profiling of a bacterial/fungal confrontation:
449 *Collimonas fungivorans* versus *Aspergillus niger*. *ISME J* 5: 1494-1504.
- 450 Mela, F., Fritsche, K., de Boer, W., van den Berg, M., van Veen, J.A., Maharaj, N.N., and
451 Leveau, J.H.J. (2012) Comparative genomics of bacteria from the genus *Collimonas*:

- linking (dis)similarities in gene content to phenotypic variation and conservation. *Environ Microbiol Reports* 4: 424-432.
- Minto, R.E., and Blacklock, B.J. (2008) Biosynthesis and function of polyacetylenes and allied natural products. *Prog Lipid Res* 47: 233-306.
- Myers, A.G., Cohen, S.B., and Kwon, B.M. (1994) A study of the reaction of calicheamicin Y1 with glutathione in the presence of double-stranded DNA. *J Am Chem Soc* 116:1255-1291 1255.
- Onishi, J.C., Abruzzo, G.K., Fromtling, R.A., Garrity, G.M., Milligan, J.A., Pelak, B.A., and Rozdilsky, W. (2006) Mode of action of L-660,631 in *Candida albicans*. *Ann New York Acad Sci* 544: 229.
- Parish, C.A., Huber, J., Baxter, J., Gonzalez, A., Collado, J., Platas, G., *et al.* (2004) A new ene-triene antibiotic from the fungus *Baeospora myosura*. *J Natural Products* 67: 1900-1902.
- Parker, W.L., Rathnum, M.L., Seiner, V., Trejo, W.H., Principe, P.A., and Sykes, R.B. (1984) Cepacin-A and cepacin-B, 2 new antibiotics produced by *Pseudomonas cepacia*. *J Antibiotics* 37: 431-440.
- Paulsen, I.T., Press, C.M., Ravel, J., Kobayashi, D.Y., Myers, G.S.A., Mavrodi, D.V., *et al.* (2005) Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nature Biotechnology* 23: 873-878.
- Shi Shun, A.L.K., and Tykwinski, R.R. (2006) Synthesis of naturally occurring polyynes. *Angew Chem Int Ed* 45: 1034-1057.
- Van den Brink, H.J.M., Van Gorcom, R.F.M., Van den Hondel, C., and Punt, P.J. (1998) Cytochrome P450 enzyme systems in fungi. *Fungal Genet Biol* 23: 1-17.
- Yamaguchi, M., Park, H.J., Ishizuka, S., Omata, K., and Hiram, M. (1995) Chemistry and antimicrobial activity of caryophenolins analogs. *J Med Chem* 38: 5015-5022.

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

<i>Collimonas</i> species and subgroup	strain number	LMG	Inhibition of <i>A. niger</i>	PCR 1133*	PCR 1138*	PCR 1139*	PCR 1141*
<i>C. fungivorans</i> 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	+	+	+	+	+
<i>C. fungivorans</i> subgroup C	Ter 6 ^T	21973	-	+	-	-	-
	Ter 166		-	+	-	-	-
	Ter 330		-	+	-	-	-
	Ter 300		-	+	-	-	-
<i>C. arenae</i>	Ter 10 ^T	23964	-	-	-	-	-
	Ter 146		-	-	-	-	-
	Ter 252		-	-	-	-	-
	Ter 282	23966	-	-	+/-	-	-
<i>C. pratensis</i>	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.
<i>Pseudomonas protegens</i>	Pf-5		-	+	-	-	-

*: +, PCR product with the same size as that of Ter331; -, no PCR product, +/-, weak PCR product; n.t., not tested.

Table S1: Genes and gene predictions for cluster K on the Ter331 genome (Mela et al., 2011)						
Gene Cf_XXXX (CFU_XXXX)	Length/Mw aa/kDa	Features ¹	Most similar protein, Organism, Accession Number (gene ID genome sequence)	Length of alignment in aa %identity/ %similarity	Best experimental hit (organism) Accession No., reference ²	Length of alignment in aa %identity/ %similarity
1127 (1076)	265/27.9		hypothetical protein Predicted hydrolases or acyltransferases <i>Sorangium cellulosum</i> 'So ce 56' CAN93372, Schneiker <i>et al.</i> , 2007	241 38/53	lactone-specific esterase <i>Pseudomonas fluorescens</i> AAC36352, Hildebrandt <i>et al.</i> , 2002	143 30/49
1128	39/4.2		Hypothetical, no hit		-	
1129 (1078)	61/6.9		Rubredoxin-type Fe(Cys) ₄ protein <i>Burkholderia vietnamiensis</i> G4 ABO56995 (GENE ID: 4950229 Bcep1808_4014)	61 67/77	Rubredoxin <i>Acinetobacter</i> sp. M-1 BAB33291; Tani <i>et al.</i> , 2001	52 65/78
1130 (1079)	317/33.5		Predicted hydrolase <i>Burkholderia ambifaria</i> IOP40- 10EDT06087	287 49/65	FMP <i>Ralstonia eutropha</i> H16 AAA21950; Priefert <i>et al.</i> , 1991	192 27/42
1131 (1080)	366/42.1	5 TMH	fatty acid desaturase <i>Burkholderia ambifaria</i> MEX-5 EDT43257	362 68/82	delta-12 desaturase <i>Synechococcus</i> sp. PCC 6714 BAA02921; Sakamoto <i>et al.</i> , 1994	321 34/54
1132 (1081)	110/12.1		phosphopantetheine-binding <i>Burkholderia ambifaria</i> IOP40-10 EDT06085	109 68/80	polyketide synthase (phosphopante- theine attachment site <i>Chondromyces crocatus</i> CAJ46689; Rachid <i>et al.</i> , 2006	72 40/65
1133 (1082)	322/35.1	5 TMH	delta-9 acyl-phospholipid desaturase <i>Burkholderia ambifaria</i> IOP40-10 EDT06084	298 78/86	fatty acid desaturase JamB <i>Lyngbya majuscula</i> AAS98775; Edwards <i>et al.</i> , 2004	283 40/58
1134 (1083)	315/34.6	4 TMH	Stearoyl-CoA 9-desaturase <i>Burkholderia ambifaria</i> MEX-5 EDT43254	304 58/70	fatty acid desaturase JamB <i>Lyngbya majuscula</i> AAS98775; Edwards <i>et al.</i> , 2004	305 36/55
1135 (1084)	617/65.8		AMP-dependent synthetase and ligase ABO56989 (GENE ID: 4950224 Bcep1808_4008)	582 54/67	nonribosomal peptide synthetase <i>Streptomyces atroolivaceus</i> AAN85512; Cheng <i>et al.</i> , 2003	570 40/54
1136 (1085)	472/49.2	14 TMH	major facilitator superfamily MFS_1 <i>Burkholderia ambifaria</i> MEX-5 EDT43252	472 85/90	ChaT1 protein <i>Streptomyces chartreusis</i> CAH10178; Xu <i>et al.</i> , 2005	411 31/47
1137	281/30.6		hypothetical protein <i>Burkholderia ambifaria</i> IOP40-10	277 80/87	-	

			EDT06080			
1138 (1086)	344/38.9		Rieske (2Fe-2S) domain protein <i>Burkholderia ambifaria</i> IOP40-10 EDT06079	333 88/95	alpha-subunit oxygenase VanA <i>Streptomyces</i> sp. NL15-2K BAF33363; Nishimura <i>et al.</i> , 2006	338 34/49
1139 (1087)	360/40.5	5 TMH	fatty acid desaturase <i>Burkholderia ambifaria</i> IOP40-10 EDT06078	360 75/85	carotene ketolase CrtW <i>Algoriphagus</i> sp. KK10202C ABB88952; Tao <i>et al.</i> , 2006	261 22/38
1140 (1088)	461/50.5		monooxygenase, FAD-binding <i>Burkholderia ambifaria</i> MEX-5 EDT43248	461 80/88	Halogenase, <i>Actinosynnema pretiosum</i> subsp. <i>auranticum</i> AAM54090; Yu <i>et al.</i> , 2002	347 29/40
1141 (1089)	430/44.4		3-oxoacyl-acyl carrier protein synthase II <i>Burkholderia ambifaria</i> MEX-5 EDT43247	421 84/92	<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i> CAI36078; Pitman <i>et al.</i> , 2005	421 63/78
1142	57/6.4	1 TMH	similar to CG3523-PA <i>Tribolium castaneum</i> XP_974066	39 43/61	-	
1143	326/36.4	1 HTH	transcriptional regulator, LysR family <i>Burkholderia vietnamiensis</i> G4 ABO54218 (<u>GENE ID: 4952917 Bcep1808_1207</u>)	309 56/76	Chain A, Crystal Structure Of The Effector Binding Domain Of A Catm Variant, Catm(V158m), <i>Acinetobacter</i> sp. 2H98_A, Ezezika <i>et al.</i> , 2007	298 24/47
1144 (1091)	440/47.4	11 TMH	major facilitator family transporter <i>Burkholderia pseudomallei</i> 668 ZP_01764941	413 72/83	MopB <i>Burkholderia cepacia</i> AAB41509; Saint and Romas, 1996	423 37/56
1145 (1092)	457/48.1	SP 12 TMH	conserved hypothetical protein <i>Burkholderia phymatum</i> STM815 ACC76384	406 48/66	-	
1145a	310/35.7	4 TMH	fatty acid desaturase <i>Burkholderia</i> <i>phymatum</i> STM815 ACC76383	307 44/62	RtxC <i>Bradyrhizobium elkanii</i> BAB55901; Okazaki <i>et al.</i> , 2004	310 24/43
(1093)	26		transposase, <i>Burkholderia thailandensis</i>	26 81/88	-	
(1094)	42		transposase, <i>Burkholderia glumae</i>	42 62/73	-	

¹TMH, transmembrane helix; HTH, helix turn helix motif; SP, signal peptide

²Schneiker *et al.*, 2007 Nature Biotech 25: 1281-1289; Hildebrandt *et al.*, 2002 Appl Microbiol Biotechnol 59: 483-487; Tani *et al.*, 2001 J Bacteriol 183: 1819-1823; Priefert *et al.*, 1991 J. Bacteriol. 173: 4056-4071; Sakamoto *et al.*, 1994 Plant Mol Biol 24: 643-650; Rachid *et al.*, 2006 Chem Biol 13: 667-681; Edwards *et al.*, 2004 Chem Biol 11: 817-833; Cheng *et al.*, 2003 Proc Natl Acad Sci USA 100: 3149-3154; Xu *et al.*, 2005 Chem Biol 12: 579-588; Nishimura *et al.*, 2006 Biosci Biotechnol Biochem 70: 2316-2319; Tao *et al.*, 2006 Mol Genet Genomics 276: 79-86; Yu *et al.*, 2002 Proc Natl Acad Sci USA 99: 7968-7973; Pitman *et al.*, 2005 Curr Biol 15: 2230-2235; Saint and Romas, 1996 Microbiology 142: 2407-2418; Okazaki *et al.*, 2004 Appl Environ Microbiol 70: 535-541.

FIGURE LEGENDS

Figure 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 micrometers.

Figure 2. A) Graphical representation of cluster K and surrounding genes on the *C. fungivorans* Ter331 genome. Genes are labeled according to the naming convention established in Mela *et al.* (2011) and Mela *et al.* (2012). For example, the gene labeled 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 colors indicate predicted function (also see Table S1). Arrowed lines above the genes indicate operonic organization, as predicted by FgenesB (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 *Burkholderia vietnamiensis* G4 (gene accession numbers Bcep1808_....), *Burkholderia* sp. KJ006 (MYA_....), *Burkholderia ambifaria* IOP40-10 (BamIOP4010DRAFT_....) and MEX5 (BamMEX5DRAFT_....), *Burkholderia gladioli* BSR3 (bgla_1g20...), *Streptomyces* sp. Mg1 (SSAG_033..), and *Pseudomonas protegens* Pf-5 (PFL_026..) and CHA0 (PFLCHA0_c02...).

Figure 3. Antifungal activity screening of extracts from *C. fungivorans* Ter331, its rifampicin-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.

Figure 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 minutes. Peak labels are a through h, and correspond to peak labels in Figure 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 Figure 3. Antifungal activity was observed only in the two fractions that are boxed by the black rectangle. C) Blowup 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.

Figure 5. A) Improved-resolution HPLC profile of methanol extract from *C. fungivorans* Ter331. Gradient used was 0-100% acetonitrile over a period of 50 minutes. 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 Figure 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.

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

536 **Figure S2.** HPLC trace for extract from mutant 28A12 (top), compared to those of Ter331 wt
537 (middle) and mutant 13E12 (bottom). HPLC conditions were different from those shown in
538 Figures 4 and 5: Dionex HPLC with photodiode array detector; detection at 240nm; 4.6mm,
539 5µm, 250mm C18RP column (Grace/Alltech, Breda, NL); flow: 1ml/min; solvent:
540 acetonitrile/water with 0.1% (v/v) TFA; gradient: 5 min 30 to 35% acetonitrile, 5 min 35%, 15
541 min 35 to 41%, 1 min 41 to 43%, 25 min 43 to 45%, 6 min 45 to 49%, 7 min 49 to 100%, 10 min
542 100%.

Figure 1

Figure 2

Figure 3

Figure 4

Figure S1

Figure S2