

# Methylcitrate cycle activation during adaptation of *Fusarium solani* and *Fusarium verticillioides* to propionyl-CoA-generating carbon sources

Nicole Domin,<sup>1</sup> Duncan Wilson<sup>2</sup> and Matthias Brock<sup>1</sup>

## Correspondence

Matthias Brock

Matthias.Brock@hki-jena.de

<sup>1</sup>Microbial Biochemistry and Physiology, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knoell Institute, Beutenbergstr. 11a, 07745 Jena, Germany

<sup>2</sup>Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knoell Institute, Beutenbergstr. 11a, 07745 Jena, Germany

Propionyl-CoA is an inhibitor of both primary and secondary metabolism in *Aspergillus* species and a functional methylcitrate cycle is essential for the efficient removal of this potentially toxic metabolite. Although the genomes of most sequenced fungal species appear to contain genes coding for enzymes of the methylcitrate cycle, experimental confirmation of pathway activity in filamentous fungi has only been provided for *Aspergillus nidulans* and *Aspergillus fumigatus*. In this study we demonstrate that pathogenic *Fusarium* species also possess a functional methylcitrate cycle. *Fusarium solani* appears highly adapted to saprophytic growth as it utilized propionate with high efficiency, whereas *Fusarium verticillioides* grew poorly on this carbon source. In order to elucidate the mechanisms of propionyl-CoA detoxification, we first identified the genes coding for methylcitrate synthase from both species. Despite sharing 96% amino acid sequence identity, analysis of the two purified enzymes demonstrated that their biochemical properties differed in several respects. Both methylcitrate synthases exhibited low  $K_m$  values for propionyl-CoA, but that of *F. verticillioides* displayed significantly higher citrate synthase activity and greater thermal stability. Activity determinations from cell-free extracts of *F. solani* revealed a strong methylcitrate synthase activity during growth on propionate and to a lesser extent on Casamino acids, whereas activity by *F. verticillioides* was highest on Casamino acids. Further phenotypic analysis confirmed that these biochemical differences were reflected in the different growth behaviour of the two species on propionyl-CoA-generating carbon sources.

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## INTRODUCTION

Most, if not all, aerobic micro-organisms have to cope with the formation of propionyl-CoA. This metabolite can be formed from the direct activation of propionate (a common carbon source found in soil), or from the degradation of either odd-chain fatty acids or amino acids such as isoleucine, valine and methionine (Brock *et al.*, 2000; Voet & Voet, 2004; Maerker *et al.*, 2005). Accumulation of propionyl-CoA exerts toxic effects by inhibiting several enzymes of primary metabolism, such as the pyruvate dehydrogenase complex, succinyl-CoA synthase and ATP:citrate lyase (Brock & Buckel, 2004). To avoid this accumulation, several bacteria and mammals utilize the so-called methylmalonyl-CoA pathway for converting propionyl-CoA into the citric acid cycle intermediate succinyl-CoA (Martens *et al.*, 2002). However, the key enzyme of this pathway, the coenzyme B<sub>12</sub>-dependent methylmalonyl-CoA mutase, is absent in fungi (Ledley *et al.*, 1991). Most fungi instead use the coenzyme B<sub>12</sub>-independent methylcitrate cycle for the

efficient degradation of propionyl-CoA. This pathway is also present in several bacteria and has been studied mainly in the bacteria *Escherichia coli* (Brock *et al.*, 2002; Textor *et al.*, 1997) and *Salmonella typhimurium* (Horswill & Escalante-Semerena, 2001) and the fungi *Yarrowia lipolytica* (Uchiyama *et al.*, 1982), *Saccharomyces cerevisiae* (Luttik *et al.*, 2000), *Aspergillus nidulans* (Brock *et al.*, 2000, 2001; Brock & Buckel, 2004; Brock, 2005; Zhang *et al.*, 2004) and *Aspergillus fumigatus* (Ibrahim-Granet *et al.*, 2008; Maerker *et al.*, 2005). The methylcitrate pathway is characterized by the initial condensation of propionyl-CoA with oxaloacetate via methylcitrate synthase, forming the first cycle-specific intermediate, methylcitrate. Methylcitrate undergoes an isomerization event, forming methylisocitrate, which is finally cleaved into pyruvate and succinate by a cycle-specific methylisocitrate lyase. This cleavage completes the  $\alpha$ -oxidation of propionate to pyruvate, which can then be used for energy and biomass formation by standard enzymes of primary metabolism (Brock *et al.*, 2000).

Deletion of the methylcitrate synthase gene from *A. nidulans* and *A. fumigatus* leads to an accumulation of propionyl-CoA in the presence of propionyl-CoA-generating carbon sources and during starvation. Interestingly, the accumulation of propionyl-CoA not only reduces the growth rate of these fungi due to the inhibition of primary metabolism under *in vitro* conditions, as outlined above (Brock & Buckel, 2004), it also leads to the attenuation in virulence of *A. fumigatus* in a wax moth larvae model (Maerker *et al.*, 2005) and in immunosuppressed mice (Ibrahim-Granet *et al.*, 2008). These results imply that propionyl-CoA-generating nutrients are consumed during pathogenesis. Furthermore, accumulation of propionyl-CoA was shown to inhibit the formation of polyketide-derived secondary metabolites such as sterigmatocystin, naphthopyrone, ascoquinone A and 1,8-dihydroxynaphthalene (DHN) melanin (Maerker *et al.*, 2005; Zhang *et al.*, 2004; Zhang & Keller, 2004), which seems to be mainly due to an imbalance between the intracellular ratio of propionyl-CoA and acetyl-CoA.

Plant-pathogenic fungi, such as various *Fusarium* species, contain genes coding for putative methylcitrate cycle enzymes in their genomes; however, experimental evidence of a functional methylcitrate cycle in these species has not been provided. Since it is possible that propionyl-CoA-generating carbon sources such as odd chain fatty acids or amino acids are consumed during plant infection, this pathway might play an important role during pathogenesis and therefore constitute a novel antifungal target. In addition, because several *Fusarium* species are known to produce highly toxic secondary metabolites during plant infection and colonization of stored foodstuffs (De Lucca, 2007; Jestoi, 2008; Schollenberger *et al.*, 2007; Völkl *et al.*, 2004), inhibition of the methylcitrate pathway could reduce the production of these toxins. For example, propionate, the direct precursor of propionyl-CoA, is frequently added to stored seeds, especially corn, to prevent mould growth and mycotoxin production (Selwet, 2008). Notably, low amounts of propionate are often well tolerated by fungi and used as a nutrient source, increasing rather than reducing growth rates. Therefore, detoxifying mechanisms do exist that allow growth in the presence of propionate.

Last but not least, several *Fusarium* species are able to cause severe invasive fusariosis in immunocompromised patients, with attributable mortality rates of up to 100%. The *Fusarium solani* species complex (teleomorph *Nectria haematococca*) is isolated from approximately two-thirds of patients with fusarial mycoses (Zhang *et al.*, 2006) and seems to be the most virulent *Fusarium* species complex (Mayayo *et al.*, 1999). This suggests that this species complex is able to readily adapt to growth within infected human tissues. In contrast, *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) is much less frequently isolated from patients, but is a major pathogen of maize and sorghum and can either grow as an endophyte or cause visible disease on the ears, stalks, roots and seedlings

(Brown *et al.*, 2008; Jurgenson *et al.*, 2002). Furthermore, *F. verticillioides* is a major producer of fumonisins, mycotoxins that have a strong carcinogenic potential (Gelderblom *et al.*, 1988). In contrast to *Fusarium solani*, which is frequently found as a soil saprophyte (Zhang *et al.*, 2006), a major natural reservoir of *F. verticillioides* is within maize seeds, which are colonized by vertical transmission during asymptomatic endophytic growth within plants (Bacon *et al.*, 2001). These lifestyle differences suggest that these two *Fusarium* species may possess different mechanisms for adapting to changing environmental conditions and growth under nutrient-limited conditions.

To investigate the ability of *Fusarium* species to cope with the formation of the toxic compound propionyl-CoA, we analysed the methylcitrate synthases from both *F. solani* and *F. verticillioides* and determined the methylcitrate cycle activity from cells grown *in vitro* under various conditions. Methylcitrate synthases from both species showed a high degree of sequence identity, but biochemical characteristics and activity induction differed between the species, which may influence their ability to utilize and detoxify propionyl-CoA.

## METHODS

**Chemicals and growth media.** Unless stated otherwise, all media and chemicals used in this study were obtained from Sigma-Aldrich Chemie.

**Media and culture conditions for *F. solani* and *F. verticillioides*.** In all experiments the isolates *F. solani* IP 2330.95 (Hue *et al.*, 1999) and *F. verticillioides* IP 2333.95, kindly provided by the Institut Pasteur culture collection (Paris, France), were used. Cultures were routinely incubated at 28–30 °C and propagated either on solid agar plates or in liquid cultures, which were shaken at 220 r.p.m. on a rotary shaker. For complex media, potato dextrose broth, potato broth, malt extract or Sabouraud medium were used. Defined minimal media, based on *Aspergillus* minimal medium pH 6.5, were supplemented with Casamino acids (MP Biomedicals), peptone (AppliChem), glucose, acetate, ethanol or propionate (all in a range between 10 and 100 mM or 1%). Media were inoculated with microconidia derived from cultivation of mycelia in peptone-containing liquid medium (24–48 h). Microconidia were harvested by filtration through Miracloth with subsequent centrifugation at 4000 g for 10 min. Conidia were washed twice with PBS and stored as spore suspensions for up to 7 days at 4 °C. To harvest mycelia, cultures were monitored microscopically for excessive microconidia formation. Mycelium was retained during filtration over Miracloth, pressed dry and shock frozen in liquid nitrogen until further use. Cell-free extracts were prepared by grinding the mycelia in the presence of liquid nitrogen, resuspension in 50 mM HEPES buffer pH 7.5 and removal of cell debris by 5 min centrifugation at 16 000 g.

**Identification of methylcitrate synthase coding sequences.** The protein sequence of the *A. nidulans* methylcitrate synthase (accession no. CAB53336) was used as a template for a TBLASTN search against the genome of *F. solani* (*Nectria haematococca*; <http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Necha2&advanced=1>). A predicted protein with 70% sequence identity was identified on chromosome 5 and the coding sequence was used for a BLAST search against the genome of *F. verticillioides* ([http://www.broadinstitute.org/annotation/genome/fusarium\\_verticillioides/Blast.html](http://www.broadinstitute.org/annotation/genome/fusarium_verticillioides/Blast.html)). A hit with high

identity was found at locus tag FVEG\_00189.3. Genome sequences for both *Fusarium* enzymes were used for ORF prediction by the program GENSCAN (<http://genes.mit.edu/GENSCAN.html>). To experimentally confirm the open reading frames for both methylcitrate synthases, RNA was isolated from peptone-grown mycelia using the NucleoSpin RNA L kit (Macherey-Nagel) with subsequent Baseline-ZERO DNase treatment (Biozym Scientific). RNA was reverse transcribed into cDNA using anchored Oligo(dT)<sub>20</sub> primers and reverse transcriptase SuperScript III (Invitrogen). RNA was removed by treatment with NaOH at 70 °C and cDNA was purified by ethanol precipitation in the presence of glycogen. The cDNA was used as template for gene amplification using the oligonucleotides FvMcsASequ\_upfo and FvMcsASequ\_dore for the *F. verticillioides* and FsMcsASequ\_upfo and FsMcsASequ\_dore for the *F. solani* methylcitrate synthases (Table 1). Gene-specific cDNAs were cloned into the pJET1.2 (Fermentas) cloning vector and sequenced from both strands.

**Cloning of methylcitrate synthase genes for recombinant overproduction in *E. coli*.** Analysis of the ORF for both methylcitrate synthases revealed a mitochondrial import sequence (<http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html>), which was removed before heterologous enzyme production in *E. coli*. The cDNAs from the respective species were used for amplification of the genes with a proofreading polymerase (Accuzyme; Bionline) using oligonucleotides FsMcsABam\_for and FsMcsAHind\_rev for the *F. solani* enzyme and FvMcsABam\_for and FvMcsANot\_rev for the *F. verticillioides* enzyme. The PCR products were cloned into the pJET1.2blunt vector (Fermentas) and sequenced from both strands. Internal *Bam*HI sites in both the *F. solani* and the *F. verticillioides* coding sequences prohibited direct subcloning into the expression vector. Therefore, the internal *Bam*HI sites were mutated using the FlipFlop Site-Directed Mutagenesis kit as recommended by the manufacturer (Bionline) and the oligonucleotides FsMutBamMcs\_for and FsMutBamMcs\_rev for *F. solani* *mcsA* and FvMcsMutBam\_for and FvMcsMutBam\_rev for *F. verticillioides* *mcsA*. Amplified vectors were transferred into XL1-Blue Supercompetent cells (Stratagene Europe) and the sequences of the mutated genes were confirmed by *Bam*HI restriction and sequencing. The genes were excised from the pJET1.2blunt vector with *Bam*HI and *Hind*III for the *F. solani* enzyme and with *Bam*HI and *Not*I for the *F. verticillioides* enzyme and subsequently ligated into a modified pET43.1a vector (Novagen/

Merck) containing an N-terminal His-tag and a TEV protease cleavage site (Hortschansky *et al.*, 2007). Plasmids were then transferred into *E. coli* BL21(DE3) Rosetta 2 cells (Novagen/Merck), which were used for overproduction of the recombinant enzymes.

**Heterologous methylcitrate synthase production and enzyme purification.** *F. solani* and *F. verticillioides* methylcitrate synthases were purified from *E. coli* BL21(DE3) Rosetta 2 cells harbouring the appropriate pET43.1 expression vectors. Cells were grown for 26 h at 28 °C in 15 ml Overnight express instant TB medium (Novagen/Merck) in 50 ml flasks on a rotary shaker at 220 r.p.m. Cells were harvested by centrifugation at 4000 g and cell pellets were resuspended in 3 ml of 50 mM HEPES buffer pH 7.5 (buffer A). Cells were sonicated on ice for 3 × 2 min at 70 % intensity with a pulse rate of 50 % and a sonotrode with a diameter of 3 mm (Sonoplus homogenizer; Bandelin Electronic). Debris was removed by centrifugation at 16800 g and the supernatant was loaded on a gravity-flow Ni-NTA agarose column (bed volume 1 ml; Qiagen) previously equilibrated with buffer A. The column was washed with 8 column volumes of buffer A containing 30 mM imidazole and methylcitrate synthase was eluted in buffer A with 200 mM imidazole. Purity of fractions was confirmed by SDS-PAGE analysis on NuPage Bistris 4–12 % gradient gels in a MES-buffered running system (Invitrogen). Pure fractions were combined and desalted against 40 mM potassium phosphate buffer pH 7.0 using centrifugal filter devices with a cut-off of 30 kDa (Millipore). Glycerol was added to a final concentration of 50 % (v/v) and samples were stored at –20 °C.

**Methylcitrate synthase assay and biochemical characterization of methylcitrate synthases.** The standard assay for methylcitrate synthase (citrate synthase) activity is based on the reduction of 5,5'-dithiobis-3-nitrobenzoate by CoASH released during the condensation of propionyl-CoA (acetyl-CoA) and oxaloacetate. Assays were performed as described previously (Brock *et al.*, 2000). Temperature stability was tested by incubation of the enzyme for different times at various temperatures followed by an activity determination under standard conditions. Temperature dependency was analysed by preheating the assay mixture to the desired temperature using a water bath connected to the UV/Vis double-beam spectrophotometer that was used in all enzymic analyses (Lambda 25 UV/VIS, Perkin Elmer). The effect of pH on activity was

**Table 1.** Oligonucleotides used in this study

Sequences of restriction sites are shown in bold letters. Bases altered for mutation of a restriction site are shown in bold italic.

Name	Sequence 5'→3'	Function
Anchored Oligo(dT) <sub>20</sub>	NVT TTT TTT TTT TTT TTT T	cDNA synthesis
<i>Fusarium solani</i>		
FsMcsASequ_upfo	CCA ATT CAT CTC CTC TCA CAC	Amplification <i>mcsA</i>
FsMcsASequ_dore	GAC ATG TCC CAA ATT CCA ATC G	Amplification <i>mcsA</i>
FsMcsABam_for	<b>GGA TCC</b> ACG GCT GAG CCC GAC	Overexpression <i>mcsA</i>
FsMcsAHind_rev	<b>AAG CTT</b> ACT GGC CCT CAA CCT GC	Overexpression <i>mcsA</i>
FsMutBamMcs_for	GAT GGA <b>TTC</b> TCC AGA TGA AG	Mutation <i>Bam</i> HI site
FsMutBamMcs_rev	CTT CAT CTG GAG <b>AAT</b> CCA TC	Mutation <i>Bam</i> HI site
<i>Fusarium verticillioides</i>		
FvMcsASequ_upfo	CTC CTC ATA CTT GAT CTC TTC	Amplification <i>mcsA</i>
FvMcsASequ_dore	CTC TGT CAC GAC GCT ATA TAC	Amplification <i>mcsA</i>
FvMcsABam_for	<b>GGA TCC</b> GCA GAG CCT GAT CTC AAG	Overexpression <i>mcsA</i>
FvMcsANot_rev	<b>GCG GCC</b> GCT TAT TGA CCC TCA ACC TGC	Overexpression <i>mcsA</i>
FvMcsMutBam_for	GTG CTC TGT <b>CTG</b> ATC CTT TCC	Mutation <i>Bam</i> HI site
FvMcsMutBam_rev	GGA AAG GAT CAG ACA GAG CAC	Mutation <i>Bam</i> HI site

determined by replacing the standard Tris/HCl assay buffer pH 8.0 with a buffer system containing a mixture of boric acid, acetic acid and phosphoric acid (all 0.1 M) adjusted with NaOH to the desired pH values (500  $\mu$ l buffer per 1 ml assay).  $K_m$  values for propionyl-CoA, acetyl-CoA and oxaloacetate were determined using the standard assay in which the concentration of one substrate was held constant whereas that of the other was varied.  $K_m$  values were calculated from double reciprocal Lineweaver–Burk plots.

**Purification of methylcitrate from *A. nidulans*  $\Delta mcsA$  culture broth and determination of methylcitrate cycle and isocitrate lyase activity.** Methylcitrate cycle activity was determined by following the conversion of methylcitrate to pyruvate and subsequent reduction to lactate. To obtain pure 2S,3S-2-methylcitrate, an *A. nidulans* methylcitrate dehydratase deletion strain  $\Delta mcdA$  (unpublished) was grown for 72 h on a medium containing 50 mM acetate and 20 mM propionate. The  $\Delta mcdA$  strain is unable to convert methylcitrate to methylnaconitate, thus excreting methylcitrate into the growth medium. After removing the mycelium by filtration, a 2 M BaCl<sub>2</sub> solution was added to the medium until no further precipitation occurred. The precipitate was collected, dried and mixed with Dowex W 50  $\times$  8 (H<sup>+</sup>-form; Fluka). The supernatant contained free methylcitric acid, which was subsequently concentrated on a rotary evaporator, from which a yellowish oil was recovered. GC-MS analysis revealed no major contaminants in the methylcitric acid preparation. A 20 mM methylcitrate stock solution was prepared and used as substrate for methylcitrate cycle activity determination. The assay, performed in a final volume of 1 ml, contained the following: 0.2 mM methylcitric acid, 2 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 2 U lactate dehydrogenase from rabbit muscle (Fluka), 0.2 mM NADH, different amounts of cell-free extract and 50 mM HEPES buffer pH 7.5. The reaction was started by the addition of either methylcitric acid or cell-free extract and the decline in absorbance at 340 nm was monitored over a period of 15 min. Specific activity was defined as the oxidation of 1  $\mu$ mol NADH min<sup>-1</sup> (mg protein)<sup>-1</sup> using a millimolar absorption coefficient of 6.2 mM<sup>-1</sup> cm<sup>-1</sup>.

Isocitrate lyase activity was determined based on the formation of glyoxylate-phenylhydrazine from glyoxylate and phenylhydrazine. The assay was performed as previously described (Ebel *et al.*, 2006) and a millimolar absorption coefficient of 16.8 mM<sup>-1</sup> cm<sup>-1</sup> at 324 nm was used for calculation of enzyme activity.

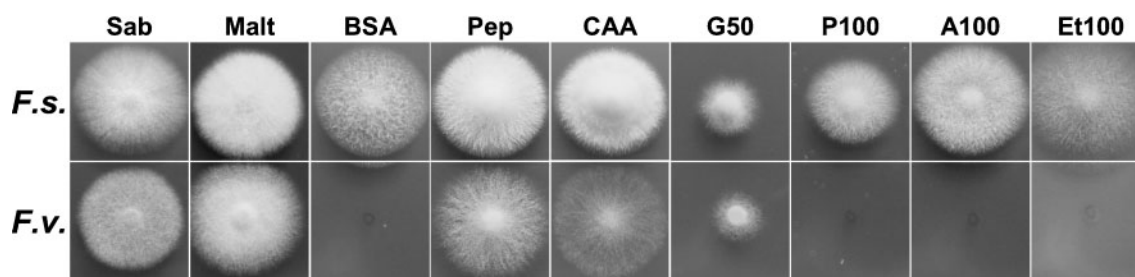
**Data reproducibility.** All growth experiments on solid media were performed in biological triplicates and representative photographs are

shown. For determination of enzymic activities after growth on different carbon sources, two independent cell-free extracts were prepared from each condition. From each extract three activity determinations were performed. Tables represent mean values and their standard deviation.

## RESULTS

### Growth of *F. solani* and *F. verticillioides* on different carbon sources

To test the general ability of *F. solani* (strain IP 2330.95) and *F. verticillioides* (strain IP 2333.95) to grow on defined media, we point-inoculated agar plates, containing different carbon sources, with  $1 \times 10^4$  conidia from fresh microconidia suspensions and incubated plates at 30 °C for 3 days (Fig. 1). Both strains grew well on the complete media Sabouraud dextrose and malt extract agar. Furthermore, both strains were able to grow on partially or completely hydrolysed proteins (peptone and Casamino acids, respectively). Interestingly, on serum albumin, a potential nutrient source during infection of a human host, rapid germination of *F. solani*, but not *F. verticillioides*, was observed. On all other tested media, growth of *F. solani* was much more pronounced than that of *F. verticillioides*. On glucose, both strains showed some biomass formation, whereas on propionate (propionyl-CoA forming) and also on acetate and ethanol, biomass formation was only observed for *F. solani*. Furthermore, growth of *F. solani* was more pronounced on these gluconeogenic carbon sources than on glucose. This observation fits with the saprophytic lifestyle of *F. solani* and suggests that the degradation of carboxylic acids such as acetate and propionate provides nutrient sources during growth in the soil. In contrast, *F. verticillioides*, which is mainly associated with plants, seems to prefer rich media containing glucose and peptides/amino acids, whereby the latter would also lead to the formation of toxic propionyl-CoA. In order to test whether the rapid adaptation of *F. solani* to



**Fig. 1.** Growth analysis of *F. solani* (*F.s.*) and *F. verticillioides* (*F.v.*) on different carbon sources. Plates were inoculated with 10 000 microconidia per spot, incubated for 3 days at 30 °C and photographed. Sab, Sabouraud dextrose medium; Malt, malt extract agar; BSA, 1% (w/v) BSA; Pep, 1% (w/v) peptone; CAA, 1% (w/v) Casamino acids; G50, 50 mM glucose; P100, 100 mM propionate; A100, 100 mM acetate; Et100, 100 mM ethanol. *F. solani* forms colonies under all conditions, whereas *F. verticillioides* only grows in the presence of glucose and/or hydrolysed proteins. No colony formation by *F. verticillioides* is observed on BSA after the 3 days incubation, indicating a low proteolytic activity.

propionate is due to a more efficient methylcitrate synthase, which would remove even low amounts of toxic propionyl-CoA, we sought to identify and characterize the respective enzymes from both species.

### Identification of methylcitrate synthase coding regions

The genomes of *F. solani* (teleomorph *Nectria haematococca*) and *F. verticillioides* (teleomorph *Gibberella moniliformis*) have recently been sequenced and their genome annotation is in progress. To date, only the coding sequences of the methylcitrate synthases (*mcsA*; EC 2.3.3.5) from the filamentous fungi *A. nidulans* (Brock *et al.*, 2000) and *A. fumigatus* (Maerker *et al.*, 2005) have been experimentally verified and, due to their high sequence identity to the respective citrate synthases accompanied by a mitochondrial localization signal for both methylcitrate and citrate synthases, an unambiguous annotation for other fungal species is rather difficult. Nevertheless, we expected that among filamentous fungi the *mcsA* sequences should display higher conservation than those coding for citrate synthases. Therefore, we performed a translated BLAST search against the genome of *F. solani* using the protein sequence of the *A. nidulans* McsA as a query. The search identified a sequence (ID 69757) of 472 amino acids that displayed 70 % identity to the *A. nidulans* McsA query. In addition, the protein showed a sequence identity of 96 % to a conserved hypothetical protein from the close relative *Fusarium graminearum* (*Gibberella zeae*; accession no. XP\_380351).

The same search against the *F. verticillioides* genome revealed a predicted protein (locus tag FVEG\_00189.3) with 71 % identity to the *A. nidulans* McsA. However, the *F. verticillioides* protein consisted of only 393 amino acids. A BLAST search of the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) implied that the predicted protein sequence contained N-terminal and C-terminal truncations. Therefore, the genomic sequences surrounding locus tag FVEG\_00189.3 were downloaded and manually checked for the missing coding sequences using the gene prediction program GENSCAN.

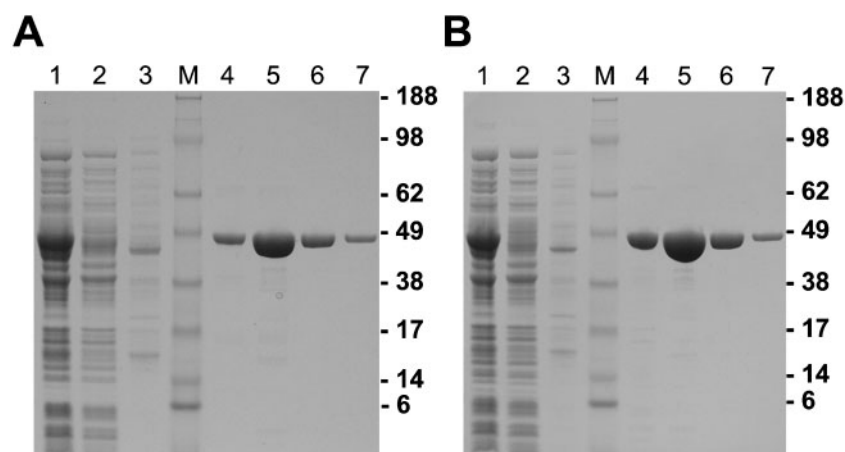
To experimentally confirm the open reading frames from both species, oligonucleotides located approximately 50 nucleotides upstream and downstream of the 'self-predicted' start and stop codons were designed. After PCR amplification from total cDNA and subcloning of the PCR products, two independent clones from each species were sequenced. Interestingly, the cDNA sequence of *F. solani* IP 2330.95 (accession no. FN400886) showed a total of 38 nucleotide exchanges (mainly in the wobble position three of the codons) when compared to the published sequence of strain *F. solani* MPVI 77-13-4. Only two nucleotide exchanges (codon position 2 affected) led to a modification of the deduced amino acid sequence (at amino acid 138, glycine to valine, and position 390, arginine to glutamine). However, due to the phylogenetic diversity of the *F. solani*

species complex, which includes more than 45 phylogenetic and/or biological species (O'Donnell *et al.*, 2008; Zhang *et al.*, 2006), we expected such minor differences in the coding region of the methylcitrate synthase gene when compared to the sequenced strain.

Sequencing of the *F. verticillioides* cDNA (accession no. FN400887) confirmed the manually deduced amino acid sequence and revealed a protein of 472 amino acids, the same size as determined for the *F. solani* enzyme. Only five nucleotides of the cDNA sequence did not match with those of the sequenced strain *F. verticillioides* 7600. These differences only occurred in wobble positions and did not alter the final amino acid sequence. Like the previously studied *Aspergillus* enzymes, the methylcitrate synthases from *F. solani* and *F. verticillioides* contained a mitochondrial import sequence with a cleavage site at amino acid position 30, as predicted by the programs MITOPROT and PSORT ([www.expasy.ch](http://www.expasy.ch)). Removal of the 29 amino acid leader peptide leads to mature enzymes containing 443 amino acids and with molecular masses of 49 kDa. Comparison of the genomic sequences of the methylcitrate synthases revealed two introns at similar positions. The first intron is located within the mitochondrial import sequence at the same position as the first introns of the methylcitrate synthases of *A. nidulans* and *A. fumigatus* (accession no. CAI61947). The second intron is located within the coding sequence. Both introns from both *Fusarium* species follow the GT-AG rule for intron splicing (Breathnach *et al.*, 1978).

### Heterologous production of recombinant methylcitrate synthases

For heterologous overproduction of the *Fusarium* methylcitrate synthases in *E. coli* we subcloned the respective cDNAs without the mitochondrial import sequence to yield correctly processed mature enzymes. To facilitate cloning into the modified pET43.1 expression vector, which contains an N-terminal His-tag and a TEV protease cleavage site (Hortschansky *et al.*, 2007), we removed the internal *Bam*HI restriction site from both genes by mutation without altering the encoded amino acids. This strategy enabled the cloning of the *F. solani* enzyme using *Bam*HI and *Hind*III restriction sites, whereas the *F. verticillioides* enzyme was subcloned using *Bam*HI and *Not*I restriction sites. Overproduction was performed at 26 °C in Overnight express instant TB medium and crude extract measurements showed high methylcitrate synthase activity of 3.0 U mg<sup>-1</sup> for the *F. solani* and 2.0 U mg<sup>-1</sup> for the *F. verticillioides* overproduction approach. The background activity in *E. coli* cells harbouring an empty pET43.1a vector was <0.1 U mg<sup>-1</sup>. When cells were grown at temperatures above 28 °C, significant proportions of the enzymes accumulated within insoluble inclusion bodies. The enzymes were purified from cell-free extracts by Ni-chelate chromatography and the eluted fractions analysed for enzyme purity by SDS-PAGE analysis (Fig. 2). Both



**Fig. 2.** SDS gel analysis of purified recombinant methylcitrate synthases from *F. solani* (A) and *F. verticillioides* (B) heterologously produced in *E. coli*. For both purifications the gels were loaded using the same scheme. Lanes: 1, cell-free extract; 2, flowthrough; 3, wash fraction; 4–7, eluted fractions 1–4; M, molecular mass marker (SeeBlue Plus2 Pre-stained mass marker; Invitrogen). The purified proteins show a molecular mass of approximately 49 kDa, which fits well with the predicted mass of a single subunit of the methylcitrate synthases.

enzymes were purified to approximately 99 % homogeneity and showed the expected subunit molecular mass of approximately 49 kDa. The specific activity of the purified methylcitrate synthase from *F. solani* (FsMcsA) was  $19.55 \text{ U mg}^{-1}$ , whereas that of the *F. verticillioides* enzyme (FvMcsA) was  $15.31 \text{ U mg}^{-1}$ . Treatment with TEV protease (kindly provided by P. Hortschansky, HKI Jena, Germany) for the removal of the N-terminal His-tag did not alter the specific activity of either enzyme (data not shown) and was therefore omitted for further biochemical characterizations. To verify that the purified enzymes originated from the respective cDNAs, protein spots were excised from the gels and subjected to tryptic digestion followed by peptide mass analysis and peptide sequencing by MS/MS analysis. For FsMcsA, 13 peptides exactly matched the predicted sequence, with a total sequence coverage of 33.4 %. The sequencing of two peptides by MS/MS analysis additionally confirmed the origin of the purified enzyme. For FvMcsA, peptide analyses showed a sequence coverage of 31.7 % with 11 peptides matching to

the predicted sequence, and sequencing of two peptides also confirmed the origin of the protein.

### Biochemical characterization of methylcitrate synthases

Methylcitrate synthases from aspergilli have been shown to be active not only with propionyl-CoA but also with acetyl-CoA, thereby acting as both methylcitrate and citrate synthases. Determination of maximum activities showed that citrate synthase activity exceeded that of methylcitrate synthase activity in *Aspergillus*, but the  $K_m$  value for propionyl-CoA was always lower than that for acetyl-CoA, leading to similar catalytic efficiencies for both enzymic activities (Brock *et al.*, 2000; Maerker *et al.*, 2005). We therefore determined both the methylcitrate and citrate synthase biochemical parameters of the *Fusarium* enzymes (Table 2). Interestingly, the *Fusarium* methylcitrate synthases differed in their biochemical parameters, despite sharing 96 % amino acid identity. For FsMcsA, citrate

**Table 2.** Comparison of the biochemical parameters of the methylcitrate synthases from *F. solani* and *F. verticillioides*

Parameter	McsA <i>F. solani</i>	McsA <i>F. verticillioides</i>
Specific activity (propionyl-CoA)	$19.55 \text{ U mg}^{-1}$	$15.31 \text{ U mg}^{-1}$
Specific activity (acetyl-CoA)	$19.05 \text{ U mg}^{-1}$	$36.6 \text{ U mg}^{-1}$
Turnover number (propionyl-CoA)	$15.9 \text{ s}^{-1}$	$12.5 \text{ s}^{-1}$
Turnover number (acetyl-CoA)	$15.5 \text{ s}^{-1}$	$29.9 \text{ s}^{-1}$
$K_m$ value propionyl-CoA	$1.94 \text{ }\mu\text{M}$	$1.88 \text{ }\mu\text{M}$
$K_m$ value acetyl-CoA	$1.26 \text{ }\mu\text{M}$	$2.71 \text{ }\mu\text{M}$
$K_m$ value oxaloacetate (propionyl-CoA)	$2.58 \text{ }\mu\text{M}$	$2.81 \text{ }\mu\text{M}$
$K_m$ value oxaloacetate (acetyl-CoA)	$3.85 \text{ }\mu\text{M}$	$9.64 \text{ }\mu\text{M}$
Catalytic efficiency (propionyl-CoA)	$8.2 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$	$6.6 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$
Catalytic efficiency (acetyl-CoA)	$1.2 \times 10^7 \text{ s}^{-1} \text{ M}^{-1}$	$1.1 \times 10^7 \text{ s}^{-1} \text{ M}^{-1}$
Catalytic efficiency (oxaloacetate with propionyl-CoA)	$6.2 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$	$4.4 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$
Catalytic efficiency (oxaloacetate with acetyl-CoA)	$4.0 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$	$3.1 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$
$V_{\max}$ temperature	49–54 °C	49–54 °C
$V_{\max}$ pH	8.0–9.0	8.0–9.0
Temperature stability 50 °C (residual activity)	50 % after 15 min	88 % after 60 min

synthase activity was slightly lower than methylcitrate synthase activity, while the  $K_m$  value for acetyl-CoA was lower than that for propionyl-CoA, leading to similar catalytic efficiencies for both activities. Due to the low  $K_m$  for propionyl-CoA ( $<2 \mu\text{M}$ ) together with the low  $K_m$  for oxaloacetate ( $2.6 \mu\text{M}$ ) in the presence of propionyl-CoA, it can be assumed that the enzyme is capable of efficiently removing intracellular propionyl-CoA and thus avoiding toxic accumulation of this metabolite.

In contrast, the FvMcsA displayed a two times higher specific activity for acetyl-CoA than for propionyl-CoA and a lower  $K_m$  value for propionyl-CoA than for acetyl-CoA. In addition, the  $K_m$  value for oxaloacetate in the presence of propionyl-CoA was approximately 4.3 times lower than that in the presence of acetyl-CoA. This indicates that, despite the higher citrate synthase maximum activity, the enzyme can also function as an efficient methylcitrate synthase under *in vivo* conditions. Therefore, methylcitrate synthase removes toxic propionyl-CoA and produces methylcitrate for the methylcitrate cycle. In contrast, the citric acid cycle specific citrate synthase catalyses the condensation of acetyl-CoA and oxaloacetate for citrate production. Further differences in the biochemical properties of the two enzymes were noted: although both enzymes displayed maximum activity between 49 and 54 °C and between pH 8.0 and 9.0, the *F. verticillioides* enzyme turned out to be much more thermostable than the *F. solani* enzyme (Table 2). However, both enzymes were stable for at least 2 h at 30 °C, which is the upper optimum growth temperature for both species.

#### Determination of methylcitrate synthase, methylcitrate cycle and citrate synthase activity under different growth conditions

To confirm the *in vivo* presence of a functional methylcitrate synthase (and of the enzymes of the entire methylcitrate cycle), both *Fusarium* species were grown on different carbon sources and tested for enzymic activities. Preparation of mycelial crude extracts from cells grown directly on different media was not possible because: (i) *F. verticillioides* did not grow on ethanol or propionate to biomasses sufficient for crude protein extract; (ii) *F. verticillioides* tended to produce large amounts of microconidia on several media rather than producing mycelium.

To circumvent these problems, we decided to pre-grow both species on media that allowed optimal mycelia production. Growth tests revealed that *F. solani* produced high amounts of mycelia within 24 h when inoculated into minimal medium containing 100 mM glucose as the sole carbon and energy source. In contrast, the most favourable condition for *F. verticillioides* mycelia production was incubation for 24 h in Sabouraud dextrose broth. Mycelia were harvested, washed with 0.6 M KCl solution and aliquots were transferred to media containing different carbon sources (glucose, Casamino acids, ethanol or propionate). The mycelia were incubated in these media

for 7 h at 30 °C on a rotary shaker. The presence of intact mycelia and the absence of significant microconidia formation was confirmed regularly by microscopy.

To confirm that 7 h incubation was sufficient for adaptation to the new carbon sources, we tested cell-free extracts of mycelia shifted to ethanol for activity of the glyoxylate cycle marker enzyme isocitrate lyase (Icl). This cycle is essential for the provision of oxaloacetate during growth on ethanol, and high Icl activity denotes an adaptation to the growth medium. The specific Icl activity for *F. solani* increased from  $1.0 \pm 0.1 \text{ mU mg}^{-1}$  to  $295 \pm 7 \text{ mU mg}^{-1}$  after the 7 h shift to ethanol. For *F. verticillioides* the Icl activity increased from  $9.7 \pm 1.0 \text{ mU mg}^{-1}$  to  $342 \pm 4 \text{ mU mg}^{-1}$ . This indicates that both strains produced glyoxylate cycle enzymes for the utilization of ethanol. Therefore, methylcitrate synthase and methylcitrate cycle, as well as citrate synthase activities, were determined for both species under all growth conditions (Table 3).

The overall activity determination of the methylcitrate cycle consisted of the actions of methylcitrate dehydratase, methylisocitrate dehydratase and methylisocitrate lyase and hence represented the activity of the rate-limiting enzyme. Moreover, because only low levels of methylcitrate were present during determination of cycle activity, whilst methylcitrate synthase activity was measured under  $V_{\text{max}}$  conditions (substrate saturation), measured cycle activity never exceeded that of synthase activity. Nevertheless, increase of methylcitrate synthase activity was always accompanied by increased methylcitrate cycle activity, implying that the enzymes of the entire pathway follow the same scheme of regulation.

We also determined citrate synthase activity, because methylcitrate synthases have both methylcitrate and citrate synthase activity, whereas the citric acid cycle citrate synthases (characterized so far) are substrate-specific. Purified FsMcsA displayed similar methylcitrate and citrate synthase activity, and citrate synthase activity exceeding a ratio of 1:1 (Cs:Mcs) signified production of the citric acid cycle specific citrate synthase. In contrast, the purified FvMcsA displayed a higher citrate synthase maximum activity, and a ratio exceeding 2.4:1 (Cs:Mcs) was indicative of the formation of the specific citrate synthase. In both cases, the smaller the Cs:Mcs ratios, the higher the specific production of methylcitrate synthase activity. As shown in Table 3, the strongest methylcitrate synthase activity was observed on Casamino acids and propionate – both propionyl-CoA-generating nutrient sources. Surprisingly, *F. verticillioides* displayed an extremely high citrate synthase activity on Casamino acids. This was unexpected because Casamino acids are also rich in amino acids such as glutamate, which lead to citric acid cycle intermediates and generally suppress citrate synthase activity (as observed for *F. solani*). In addition, *F. verticillioides* displayed the highest specific methylcitrate synthase activity on Casamino acids, whereas *F. solani* showed strongest activity on propionate.

**Table 3.** Methylcitrate synthase, citrate synthase and methylcitrate cycle activity of *F. solani* and *F. verticillioides* grown for 7 h on different carbon sources

Growth condition	Methylcitrate cycle (MC) (mU mg <sup>-1</sup> )	Methylcitrate synthase (Mcs) (mU mg <sup>-1</sup> )	Citrate synthase (Cs) (mU mg <sup>-1</sup> )‡	Ratio Cs:Mcs‡	Ratio MC:Mcs
<i>F. solani</i> *					
Glucose (50 mM)	8 ± 1.0	21.7 ± 0.2	274 ± 5 (260)	12.6:1 (12.0:1)	1:2.7
Ethanol (50 mM)	14.6 ± 0.2	32.4 ± 0.2	400 ± 10 (368)	12.3:1 (11.6:1)	1:2.2
Casamino acids (1%)	10.0 ± 0.2	35.6 ± 2.0	210 ± 13 (175)	5.9:1 (4.9:1)	1:3.6
Propionate (20 mM)	37.4 ± 2.0	145 ± 3	615 ± 27 (474)	4.2:1 (3.3:1)	1:3.9
<i>F. verticillioides</i> †					
Glucose (50 mM)	3.8 ± 0.5	6.2 ± 0.6	369 ± 9 (354)	59.5:1 (57.1:1)	1:1.6
Ethanol (50 mM)	17.1 ± 1.0	29.5 ± 1.0	681 ± 13 (610)	23.1:1 (20.7:1)	1:1.7
Casamino acids (1%)	27.7 ± 2.5	81.7 ± 1.5	867 ± 28 (672)	10.6:1 (8.2:1)	1:2.9
Propionate (20 mM)	38.2 ± 3.0	69.8 ± 1.0	683 ± 24 (516)	9.8:1 (7.4:1)	1:1.8

\*Mycelia were harvested from 100 mM glucose minimal medium, washed and transferred to fresh medium as indicated.

†Mycelia were harvested from Sabouraud glucose medium, washed and transferred to fresh medium as indicated.

‡Numbers in parentheses denote citrate synthase activity after subtraction of activity deriving from methylcitrate synthases (factor 0.97 *F. solani*; 2.39 *F. verticillioides*).

Calculation of the ratios of methylcitrate cycle to methylcitrate synthase activity revealed that *F. solani* displayed a 3.6- and 3.9-fold higher methylcitrate synthase activity on Casamino acids and propionate, respectively, whereas a similar increase (2.9-fold) for *F. verticillioides* was only observed on Casamino acids and not on propionate. This suggests that, during growth of *F. verticillioides* on propionate, methylcitrate synthase may be the rate-limiting enzyme in the degradation of propionyl-CoA.

## DISCUSSION

In this study we showed that two *Fusarium* species contain functional methylcitrate synthases and methylcitrate cycle enzymes, which are induced in the presence of propionyl-CoA-generating carbon sources. Heterologous production of recombinant methylcitrate synthases in *E. coli* allowed the biochemical characterization of the purified enzymes, revealing that both enzymes, although sharing 96% identity at the amino acid level, displayed some differences in their biochemical properties. The most striking difference was the balanced methylcitrate and citrate synthase activities of FsMcsA under  $V_{\max}$  conditions compared to FvMcsA, which displayed significantly higher citrate synthase activity, as has been described for *A. nidulans* and *A. fumigatus* (Maerker *et al.*, 2005). Furthermore, at the substrate-specificity level, the *F. solani* enzyme behaved differently from methylcitrate synthases of *Aspergillus* species, displaying a lower  $K_m$  value for acetyl-CoA than for propionyl-CoA, whereas the *F. verticillioides* enzyme had a higher affinity for propionyl-CoA (Maerker *et al.*, 2005). Despite these differences, both *Fusarium* enzymes bound oxaloacetate more efficiently in the presence of

propionyl-CoA than in the presence of acetyl-CoA. Such high-efficiency binding of oxaloacetate is a prerequisite for an efficient methylcitrate synthase, because in the cell oxaloacetate may be the rate-limiting factor in the removal of propionyl-CoA. It has been shown for *A. niger* that the available oxaloacetate concentration for enzymic reactions is below 2–3  $\mu\text{M}$  (Ruijter *et al.*, 2000), indicating that methylcitrate synthases might act near or below their  $K_m$  values for this substrate. Taken together, the results indicate that although both enzymes display differences in their substrate affinities for the three substrates (acetyl-CoA, propionyl-CoA and oxaloacetate), they display very similar methylcitrate synthase catalytic efficiencies. Nevertheless, the different temperature stabilities of the *Fusarium* enzymes indicate a different stabilization of the protein folding, which seems to be mediated by only a few amino acid exchanges. In this context, it is difficult to predict which of the amino acids are responsible for the observed differences, but further analysis of methylcitrate synthases from other *Fusarium* species could help answer this question.

A possible candidate gene for comparison derives from *F. graminearum* (*Gibberella zeae*). The genome annotation of this fungus reveals a putative 'citrate synthase' (protein accession no. XP\_380351). This 'citrate synthase' also consists of 472 amino acids with a predicted mitochondrial import sequence encoded by the first 29 amino acids, which is exactly the same as predicted for the methylcitrate synthases from *F. solani* and *F. verticillioides*. A more detailed comparison of the *F. graminearum* enzyme with those of the other two species shows that it displays only eight amino acid differences from the *F. verticillioides* enzyme (four of these located within the mitochondrial import sequence), but 18 differences from the *F. solani*

enzyme (one difference located within the mitochondrial import sequence). Therefore, we predict that the *F. graminearum* enzyme also acts as a methylcitrate synthase and its biochemical parameters likely reflect those of the *F. verticillioides* enzyme.

In addition to the biochemical characterization of the recombinant methylcitrate synthases, which showed that functional methylcitrate synthases are indeed present within the genomes of both *Fusarium* species, we also determined whether functional enzymes of the entire methylcitrate cycle are in fact produced in the presence of propionyl-CoA-generating carbon sources. Our results showed that *F. solani* displayed the strongest formation of methylcitrate synthase and also the highest cycle activity when cells were grown in the presence of propionate. This result is in agreement with the rapid germination and growth of *F. solani* on propionate-containing medium (Fig. 1). However, although methylcitrate synthase and cycle activity were also present when *F. solani* was grown on Casamino acids, the increase in activity was rather weak, similar to that on ethanol, and only slightly higher than on glucose. This suggests that the *F. solani* methylcitrate cycle has quite a high basal activity and that this level is sufficient to remove propionyl-CoA derived from amino acid degradation. Another possible explanation is the preferred metabolism of amino acids other than isoleucine, valine and methionine. This would diminish the formation of propionyl-CoA, thus reducing the need for high methylcitrate cycle activity.

In *F. verticillioides*, activity of the methylcitrate synthase and of the entire cycle behaved somewhat differently. On glucose, it appears that carbon catabolite repression occurs, as hardly any methylcitrate synthase and cycle activity was detectable on this carbon source. Slightly higher activity was observed on ethanol, accounting for a release of repression without induction. Interestingly, the highest methylcitrate synthase activity for *F. verticillioides* was observed not on propionate, but on Casamino acids, accompanied by an extremely high citrate synthase activity. Such high citrate synthase activity was unexpected because Casamino acids contain a significant amount of glutamate (14.2% of all amino acids), which generally represses citrate synthase induction (Burand *et al.*, 1975). Furthermore, citrate synthase activity from *F. verticillioides* was always higher than that from *F. solani*, which accounted for the increased ratio of citrate synthase to methylcitrate synthase activity under all tested growth conditions (Table 3). However, methylcitrate synthase activity was strongly present on Casamino acids and was even higher than that observed on propionate. The differential enhancement of methylcitrate synthase activity by *F. verticillioides* was reflected in the growth of this strain, as *F. verticillioides* IP 2333.95 hardly grew on propionate but grew well on Casamino acids. Therefore, *F. verticillioides* would appear to use its methylcitrate cycle to remove propionyl-CoA derived from amino acids such as methionine, isoleucine and valine, which constitute 1.7%,

2.7% and 4.1%, respectively, of the total amino acids present in the Casamino acid preparation ([http://www.mpbio.com/product\\_info.php?products\\_id=3060-012](http://www.mpbio.com/product_info.php?products_id=3060-012)) used in our experiments. From these analyses we conclude that specific levels of methylcitrate synthase activity reflect the growth behaviour of both *Fusarium* species.

In summary, we have shown, we believe for the first time, that *Fusarium* species contain a functional methylcitrate cycle, important for the removal of propionyl-CoA. Interestingly, despite their strong homology, the biochemical properties of these two methylcitrate synthases resulted in quite different phenotypes of the two species during growth on different carbon sources. *F. solani*, but not *F. verticillioides*, was able to rapidly adapt to and assimilate multiple nutrient sources, perhaps reflecting its saprophytic lifestyle, where, in the soil, it must possess the metabolic flexibility to meet the demands of this environmental niche. *F. verticillioides*, on the other hand, is in almost constant association with plants and has therefore specialized its metabolic machinery. The high metabolic flexibility of *F. solani* may additionally explain why this species is most frequently isolated from patients with invasive fusariosis. To further analyse the relevance of propionyl-CoA detoxification in plant infection, saprophytic growth and the production of toxic secondary metabolites, deletion of the coding methylcitrate synthase sequences from the genomes of both species will be required. Such future analyses will show whether the methylcitrate cycle and the removal of toxic propionyl-CoA are essential during these processes.

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