Flexible metabolism in *Metarhizium anisopliae* and *Beauveria bassiana*: role of the glyoxylate cycle during insect pathogenesis

Israel Enrique Padilla-Guerrero,^{1,2} Larissa Barelli,²
Gloria Angélica González-Hernández,¹ Juan Carlos Torres-Guzmán¹
and Michael J. Bidochka²

Correspondence

Michael J. Bidochka
bidochka@brocku.ca

¹Laboratorio de Genética Molecular de Hongos, Departamento de Biología, Universidad de Guanajuato, Noria Alta s/n, Guanajuato, Gto., Mexico

²Department of Biological Sciences, Brock University, St Catharines, ON L2S 3A1, Canada

Insect pathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* have an increasing role in the control of agricultural insect pests and vectors of human diseases. Many of the virulence factors are well studied but less is known of the metabolism of these fungi during the course of insect infection or saprobic growth. Here, we assessed enzyme activity and gene expression in the central carbon metabolic pathway, including isocitrate dehydrogenase, aconitase, citrate synthase, malate synthase (MLS) and isocitrate lyase (ICL), with particular attention to the glyoxylate cycle when *M. anisopliae* and *B. bassiana* were grown under various conditions. We observed that ICL and MLS, glyoxylate cycle intermediates, were upregulated during growth on 2-carbon compounds (acetate and ethanol) as well as in insect haemolymph. We fused the promoter of the *M. anisopliae* ICL gene (*Ma-icl*) to a marker gene (mCherry) and showed that *Ma-icl* was upregulated when *M. anisopliae* was grown in the presence of acetate. Furthermore, *Ma-icl* was upregulated when fungi were engulfed by insect haemocytes as well as during appressorium formation. Addition of the ICL inhibitor 3-nitropropionate delayed conidial germination and inhibited appressorium formation. These results show that these insect pathogenic fungi have a flexible metabolism that includes the glyoxylate cycle as an integral part of germination, pathogenesis and saprobic growth.

Received 17 June 2010
Revised 20 September 2010
Accepted 5 October 2010

INTRODUCTION

The entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* encounter different niches within the host during the course of insect infection. These niches are highly variable with respect to the types and abundance of available nutrients. First, the fungal conidia adhere to the surface of the insect cuticle. This is followed by germination, production of an appressorium and hyphal penetration through the cuticle where the primary nutrients are bound as protein and chitin co-polymers in the cuticle matrix. Once in the nutrient-rich insect haemolymph, which contains accessible sugars, proteins and lipids, the fungi differentiate into blastospores and contend with phagocytic haemocytes. The fungus grows within the haemolymph and eventually hyphae re-emerge from the insect cadaver and conidiate on the surface of the cadaver. During each stage of pathogenesis, a specific

subset of virulence genes is upregulated (Clarkson & Charnley, 1996; Small & Bidochka, 2005; Thomas & Read, 2007; Wang & St Leger, 2006, 2007; Wang *et al.*, 2008).

During the course of insect infection, *M. anisopliae* and *B. bassiana* encounter different carbon sources; proteins, hydrocarbons, fatty acids and lipids may be found on the surfaces of insect cuticle, the fat body, haemolymph and within insect haemocytes (Arrese & Soulages, 2010; Jarrold *et al.*, 2007; Prasad *et al.*, 1986). In other fungi, utilization of non-fermentable carbon sources activates the upregulation of the glyoxylate cycle for cell metabolism and biosynthesis (Lorenz & Fink, 2001). The glyoxylate cycle is a modified shunt of the tricarboxylic acid cycle, the result of which is the production of malate and succinate from two molecules of acetyl-CoA derived from the metabolism of acetate or from the degradation of ethanol, fatty acids or poly- β -hydroxybutyrate (Dunn *et al.*, 2009). Several plant and human pathogenic fungi and bacteria utilise the glyoxylate cycle during host infection (Dunn *et al.*, 2009).

Here, we monitored the activity of the central carbon metabolic pathway of the glyoxylate cycle, in particular

Abbreviations: 3-NP, 3-nitropropionate; ICL, isocitrate lyase.

A supplementary figure showing the upregulation of the *Ma-icl* promoter is available with the online version of this paper.

isocitrate lyase (ICL), a key enzyme in the glyoxylate cycle in *M. anisopliae* and *B. bassiana*, under various growth conditions. We also monitored the upregulation of the glyoxylate cycle during appressorium formation and insect infection by *M. anisopliae*. ICL has been implicated in survival of the human pathogenic fungus *Candida albicans* within mammalian macrophages (Lorenz & Fink, 2001). We constructed a reporter gene (mCherry fluorescent protein or green fluorescent protein) under the control of the *icl* promoter in *M. anisopliae* in order to investigate the upregulation of the glyoxylate cycle in *M. anisopliae* during phagocytosis by insect haemocytes.

METHODS

Fungal and bacterial strains. *M. anisopliae* strain ARSEF 2575 and *B. bassiana* strain ARSEF 252 were obtained from the United States Department of Agriculture Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA. Cultures were grown on potato dextrose agar (PDA) at 27 °C for 10 days. *Escherichia coli* JM109 was used for DNA ligations and transformations.

ICL assay. *M. anisopliae* and *B. bassiana* conidia were inoculated into yeast extract–peptone–glucose (YPD) broth cultures, grown for 72 h, 27 °C at 180 r.p.m. The mycelia were harvested by vacuum filtration onto Whatman no. 2 filter paper, washed with sterile distilled water, and transferred into 0.025 % yeast extract (YE) broth for 24 h followed by another water wash. Previous experimentation determined that this ‘starvation phase’ in a low nutrient medium (0.025 % YE) was necessary to deplete fungal hyphae of carbohydrate reserves that obfuscated results if fungi were transferred directly into media containing various carbon sources from the nutrient-rich YPD broth. To determine the inductive effects under various growth conditions, approximately 0.2 g (wet weight) of washed mycelia were inoculated into 50 ml distilled, deionized water containing only the carbon source 2 % glucose, 2 % acetate, 2 % raffinose, 3 % ethanol, 0.05 % oleic acid or 2 % glucose–2 % acetate and grown for 6 h. The mycelia were again harvested by vacuum filtration onto Whatman no. 2 filter paper and stored at –80 °C.

We monitored ICL activity using an enzymic assay during germination of *M. anisopliae* and *B. bassiana*. Conidia (1×10^7 conidia ml⁻¹) were inoculated into 100 ml minimal medium (M-100) supplemented with 1 g glucose, 0.3 g KNO₃ and 6 ml M-100 salt solution and grown at 27 °C and 180 r.p.m. At 3, 6, 12, 18 and 24 h post-inoculation, the conidia were re-collected by centrifugation (6000 g for 5 min) and stored at –80 °C. The conidial sample was ground using a sterile mortar and pestle in liquid nitrogen. The finely ground sample was suspended and vortexed in 300 µl extraction buffer composed of 21.8 mM HEPES, 1.0 mM dithiothreitol, 1.0 mM KCl, 0.125 % Triton X-100 and mini EDTA-free protease inhibitor cocktail tablet (Roche). ICL activity was detected using the following final assay concentration: 25 mM imidazole buffer (pH 6.8), 5 mM MgCl₂, 4 mM phenylhydrazine and 1 mM isocitric acid. Enzymic assays were carried out in 96-well plates. Each well contained 200 µl reaction master mix and 50 µl protein extract. The absorbance was taken at 324 nm at 30 °C using a temperature-controlled KC4 96-well plate reader (Bio-Tek). ICL specific activity was reported as $10^{-5} \times \mu\text{mol phenylhydrazine glyoxylate generated min}^{-1} (\text{mg protein})^{-1}$.

Gene cloning. Fungal genomic DNA was isolated using the plant/fungus DNA isolation kit (Norgen). A fragment of the genes encoding citrate synthase-1 (*Ma-cit1*), malate synthase and ICL (*Ma-icl*) from *M. anisopliae* was cloned using degenerate hybrid primers constructed

with the program iCODEHOP v1 (Rose *et al.*, 2003) and identified using BLASTX (Table 1). We also found another citrate synthase gene (*Ma-cit2*) while gene-walking from the 5' end of *Ma-icl*. The full-length *Ma-icl* and *Ma-cit2* genes were subsequently cloned by PCR walking using the Y-shaped adaptor dependent extension (YADE) method and SiteFinding-PCR (Fang *et al.*, 2007; Tan *et al.*, 2005). We also cloned a 2206 bp DNA fragment, which contained the partial sequence for *Bb-icl*, using an EST identified in previous work (Cho *et al.*, 2006a, b) by using the SiteFinding-PCR method. PCR was performed using JumpStart *Taq* DNA polymerase (Sigma).

RT-PCR. RT-PCR was used to examine transcript levels of genes representative of the glyoxylate and TCA cycles. Primers for RT-PCR were designed for isocitrate dehydrogenase (*idh*), aconitase (*aco*), citrate synthase (*cit*), malate synthase (*mls*) and ICL (*icl*), and were identified in this study (*M. anisopliae*) or from an EST analysis of *B. bassiana* transcripts from previous work (Cho *et al.*, 2006a, b). As the reference, the 18S rRNA gene was utilized for the two fungi. The primers and GenBank accession numbers are listed in Table 1. *M. anisopliae* and *B. bassiana* conidia were inoculated into YPD broth cultures, grown for 72 h, 27 °C at 180 r.p.m. Growth conditions and mycelial recovery were identical to those of the enzymic assays except that fungi were also grown in *Manduca sexta* haemolymph in a 10 ml polycarbonate tube without air space for 1, 3, 6, 9 and 12 h. Fungi were also grown in 1 % w/v locust cuticle, giant cockroach cuticle, *Manduca sexta* cuticle, chitin, ground tomato stem, cellulose, starch and bean root exudate (Wang & St Leger, 2007). The RNA was extracted from ground mycelia using TRI-reagent (Sigma-Aldrich) and treated with RQ1 RNase-free DNase (Promega). Total RNA (1 µg) was used for cDNA synthesis according to manufacturer's instructions using the DyNAmo cDNA kit (Finnzymes). The PCR contained 1 µl cDNA and 1 µl each primer to 0.12 nmol µl⁻¹ in 25 µl final volume with 0.5 µl *Taq* DNA polymerase and ThermoPol buffer (New England Biolabs). The conditions for amplification were 95 °C for 1 min, followed by 30 cycles of 20 s denaturation at 95 °C, 45 s annealing at 60 °C and 1 min polymerization at 72 °C, followed by 72 °C for 3 min. The RT-PCR was performed independently twice.

Construction of the fluorescent protein expression vector using the *Ma-icl* promoter. For construction of the p*Ma-Picl-gfp* or p*Ma-Picl-mCherry* expression vector, a 907 bp *Ma-icl* promoter fragment was amplified from DNA with the primers ICL-Promo (5'-TGGATCCGGTTGTCGATGTTGGGCT, *Bam*HI restriction site underlined) and ICL-ProFP (5'-CGCCCTTGCTCACCATAGTGACGATGATGGCCAA). The coding sequence of the fluorescent proteins eGFP (Fang *et al.*, 2006) and mCherry (Araujo-Palomares *et al.*, 2009) was amplified with the primer pair FP-U (5'-ATGAGTAAAGGAGAAGAAGCTTT) and FP-L (5'-CTATTTGTATAGTTTCATCCATG). The two fragments (promoter and fluorescent protein) were fused by PCR using the primers ICL-Promo and FP-L. The 1635 bp and 1625 bp products for the fused p*Ma-Picl-gfp* and p*Ma-Picl-mCherry*, respectively, were subcloned into pGEM-T Easy (Promega). After sequence verification, the resulting plasmids were digested with *Bam*HI and *Eco*RI and ligated into plasmid pBARGEM7-2. The circular plasmids p*Ma-Picl-gfp* and p*Ma-Picl-mCherry* were used to transform *M. anisopliae* (Wang & St Leger, 2006). The transformants were selected in media containing 200 µg glufosinate-ammonium ml⁻¹ and screened for fluorescence. Integration of the construct into the genome was verified by PCR. Functionality of the promoter was evaluated by growing the transformants in 100 ml minimal medium (M-100) supplemented with 1 g glucose or acetate, 0.3 g KNO₃ and 6 ml M-100 salt solution.

Inhibition of ICL activity using 3-nitropropionate (3-NP). For inhibition of ICL activity we used 3-NP (Ebel *et al.*, 2006; Kim *et al.*, 2006) a carbanion form of 3-nitropropionic acid (Sigma) (Schloss & Cleland, 1982). Minimal medium, M-100, was supplemented with glucose or acetate and 8 mM or 6 mM of 3-NP for *M. anisopliae* or *B.*

Table 1. Oligonucleotides used in this study

Primer name (purpose)	Primer (5'–3')	Gene	Amplification product (bp)	GenBank accession no.
Degenerate oligonucleotides				
CIT B-46	GTGACCCTGGACCAGGYNTAYGGNNG	Citrate synthase	1327	FN796521
CIT D-42	TCGGTGGAGAAGGACTTNGGNCKYTC			
ICL B-10	CCAAGAAGTGC GGCCAYATGGCNGG	ICL	841	FN796523
ICL E-28	GTAGTTGGCGCCGSWCCAYTTYTG			
MLS D-34	GGGCACCGTGCTGATHGARACNAT	Malate synthase	706	FN796520
MLS E-28	CGGCGGTGGCGGCRTCTCTCCAT			
<i>M. anisopliae</i> RT-PCR				
ME-CIT1 U	CAAGGTTGCCCCATCCAGAAGG	Citrate synthase 1	407	FN796521
ME-CIT1 L	GAGCCATGTATCGGGGGTCAGTC			
ME-CIT2 U	GCCTTGGCAAACCTGGATTCTGTAG	Citrate synthase 2	234	FN796522
ME-CIT2 L	CAAGGGCAAGACTGGCACTGAGATG			
ME-ACO U	ACCTCCCCTGATCTCGTAGTCGCTCTC	Aconitase	364	FN773487
ME-ACO L	TGTCCAGGTGGCCACGGTATTTCAAC			
ME-IDH U	GAGCAGACCGGAACAGCCCTACCAG	Isocitrate dehydrogenase	361	FN806962
ME-IDH L	CGCGCCGAACAGCAATCTCACC			
ME-ICL U	GCCGGCCCCGGAAGTGGTAG	ICL	386	FN796523
ME-ICL L	AGTCCGGCAAGCTGCTTTCTTCC			
ME-MLS U	GTCCTCGCGGCGCACAAACAG	Malate synthase	369	FN796520
ME-MLS L	TGCGGCCGCTGGGACTACATCT			
<i>B. bassiana</i> RT-PCR				
BE-CIT U	CCCCCGGCGGCAAAGAGC	Citrate synthase	474	DT370663
BE-CIT L	CGTGGTGGGTGTGGATGGTCAGGTAG			
BE-ACO U	CATTTGAGGAGTTCGGTGGCATTGTTCTT	Aconitase	326	DT368904, DT370106, DT370219, DT372548, DT375952, DT375966
BE-ACO L	AGTGTTGTTGCCAGGGTCATAGCCATCAGT			
BE-IDH U	GCCCGTTTCGCCTTTTCCTTTGCTCT	Isocitrate dehydrogenase	379	DT376726
BE-IDH L	CGGTGGGGTTGGCCTGGTCCTT			
BE-MLS U	CGCGGCGTTGGCTGTGTT	Malate synthase	370	DT371106
BE-MLS L	CGGCGTGGCGTTGATGC			
BE-ICL U	GGCCCAGCTCTTCCACGAC	ICL	337	FN796519
BE-ICL L	GTACGGGCGACGGCAATGAG			
<i>Metarhizium</i> and <i>Beauveria</i>				
18S U	AGGCCCGGGTAATCTTGT	18S rRNA	266	AY755540, AY755538
18S L	GACCTTGTTACGACTTTTACTTCTCT			

bassiana, respectively. We also observed the effect of 8 mM 3-NP on *M. anisopliae* appressorium formation in 0.1 % alanine in a Petri dish.

Glyoxylate cycle during the infection process. Conidia (20 µl of an aqueous suspension containing 5×10^7 conidia ml⁻¹) derived from fungi expressing *Ma-PicI-mCherry* were injected into the first proleg of fifth-instar larvae of *Manduca sexta*. Haemolymph was then collected at 0, 3, 6 and 9 h post-inoculation and haemocytes were concentrated by centrifugation at 150 g for 5 min; the samples were observed microscopically. We also determined if *Ma-PicI-mCherry* was expressed by *M. anisopliae* 48 h post-inoculation in *Manduca sexta* and during fungal re-emergence from the insect cadaver (5 days). We also determined if *Ma-PicI-mCherry* was expressed during appressorium formation when fungi were grown in 0.1 % alanine in a Petri dish.

Microscopy. Imaging was performed using a Nikon OPTIPHOT-2 microscope equipped with epifluorescent illumination. Cells expressing

mCherry or eGFP were imaged using TRITC HYQ 530-550/565/690-650 or GFP HYQ (LP) 425-475/480/485LP filter cubes (Chroma). The pictures for bright-field and fluorescence were obtained using a SPOT-RT colour digital camera and the SPOT advanced software version 4.6. In all the microscopic studies, at least 200 cells were observed.

RESULTS

Detection of ICL activity

ICL and malate synthase are intermediate enzymes in the glyoxylate cycle involved in the metabolism of 2-carbon (C₂) compounds, such as acetate, and hydrolytic products of fatty acids. Although the glyoxylate cycle has been found in many fungi, we first determined whether at least one

intermediate enzyme of the glyoxylate cycle, ICL, was present in *M. anisopliae* and *B. bassiana*. Enzymic assays determined that ICL activity was present in these fungi and that the expression of ICL differed in various media (Fig. 1). Generally, ICL enzymic activities in *M. anisopliae* and *B. bassiana* were greatest when grown in the presence of a C₂ compound (acetate and ethanol) and were reduced when glucose and raffinose were used as sole carbon sources. The addition of glucose and acetate reduced ICL activity in *M. anisopliae*, suggesting catabolic repression, which was not observed in *B. bassiana*. Both fungi also showed ICL activity when grown in the presence of oleic acid.

Isolation of genes in the central carbon metabolic pathway

Once we showed the existence of ICL activity, we cloned fragments of the genes from *M. anisopliae* and *B. bassiana* involved in the glyoxylate cycle (ICL and malate synthase) as well as genes that code for enzymes in the TCA cycle (citrate synthase, aconitase and isocitrate dehydrogenase; Table 1), which occurred before or after the bifurcation of the central carbon pathway to the glyoxylate cycle. For *M. anisopliae*, we isolated a 4509 bp DNA fragment that contained two genes, the ORF (1854 bp) for ICL (*Ma-icl*) and, fortuitously, a second gene encoding citrate synthase (*Ma-cit2*), which has two introns and an ORF of 1416 bp. The fragment also contained an intervening region between the ORFs of *Ma-icl* and *Ma-cit2* of just over 907 bp, which coincidentally contained a shared bidirectional promoter region (Fig. 2). Putative transcriptional binding elements found in this region include a CREA region, responsive to glucose repression (SYGGRG), homologous to that in *Aspergillus* (Cubero & Scazzocchio, 1994), a nitrogen

regulatory element, AREA (HGATAR) (Ravagnani *et al.*, 1997), and an acetate regulatory element, FacB (TCC/G N8–10 C/GGA or GCA/C N8–10 T/GGC) homologous to that found in *Aspergillus* (Todd *et al.*, 1998). The bidirectional *icl-cit2* promoter of *Metarhizium* is rich in these elements (Fig. 2), particularly the acetate-responsive element, FacB.

Phylogenetic analysis of *Ma-icl* and *Bb-icl*

Fig. 3 shows the phylogenetic relationship of the *icl* genes from *B. bassiana* (*Bb-icl*) and *M. anisopliae* (*Ma-icl*) as well as *icl*-like ORF genes from other ascomycete and basidiomycete fungi obtained using CLUSTAL_X. The *Bb-icl* gene was most closely related to *Chaetomium globosum* in the ascomycete clade. Meanwhile *Ma-icl* was most closely related to the *icl* gene from *Trichoderma reesei* in the basidiomycete clade.

Gene expression

M. anisopliae and *B. bassiana* were grown in various media to study the induction of the glyoxylate cycle (Fig. 4). RT-PCR analyses show high expression of *icl* and *mls* in acetate or ethanol (C₂ compounds), and low expression in glucose or raffinose. These results support those observed in the enzymic assays. However, differences in the regulation of the other representative genes in the central carbon metabolic pathways between *M. anisopliae* and *B. bassiana* were observed. In *B. bassiana*, the presence of glucose with acetate did not repress gene expression of the glyoxylate cycle genes. In contrast with this, glyoxylate cycle genes in *M. anisopliae* were repressed in the presence of glucose with acetate (Fig. 4). These differences were also observed in the regulation of the glyoxylate cycle between *M. anisopliae* and *B. bassiana*, when grown in insect haemolymph. The expression and regulation of *Ma-icl* and *Ma-cit2* were identical in the presence of various carbon sources, possibly since they shared a bidirectional promoter (Fig. 4).

A time-course experiment to determine the expression of *M. anisopliae* and *B. bassiana* genes in the central metabolic pathway was performed when fungi were grown in insect (*Manduca sexta*) haemolymph in limiting oxygen for up to 12 h (Fig. 4). This shows that the *Ma-icl* and *Ma-cit2* genes of *M. anisopliae* in haemolymph are upregulated and that *Ma-cit1* is downregulated. In *B. bassiana*, the level of transcripts indicated that genes involved in the glyoxylate cycle were not induced when grown in the haemolymph.

M. anisopliae is a facultative insect pathogen that can also occupy non-insect host niches such as saprobic growth and the plant rhizosphere (Hu & St Leger, 2002; St Leger, 2008). Patterns of regulation of *Ma-icl* and *Ma-cit2* during growth on insect (locust, giant cockroach or *Manduca sexta*) cuticles and plant-derived compounds (bean root exudate, tomato stems, cellulose or starch) show that *Ma-icl* and *Ma-cit2* have similar patterns of regulation (Fig. 5).

Fig. 1. ICL activities in *M. anisopliae* (grey) and *B. bassiana* (black) when grown on different carbon sources. 1, 2% Glucose, 2% sodium acetate; 2, 2% glucose; 3, 2% raffinose; 4, 3% ethanol; 5, 2% sodium acetate; 6, 0.5% oleic acid. Specific activity is measured as $10^{-5} \times \mu\text{mol}$ phenylhydrazine glyoxylate produced min^{-1} (mg protein^{-1}).

Fig. 2. Organization and potential core promoter elements for *Ma-icl* and *Ma-cit2*. The diagram shows the intergenic region between the *Ma-cit2* and *Ma-icl* genes. The ATG codons of both genes and their orientation are shown. Squares (■) show the position and putative transcriptional binding elements involved in the regulation for glucose (CREA), acetate (FacB) and nitrogen (AREA).

Determination of *Ma-icl* promoter activity

The *Ma-icl* promoter and fluorescent protein fusions were constructed to monitor the *in vivo* regulation of the glyoxylate cycle of *M. anisopliae* in pathogenic and saprophytic conditions. First, the expression patterns of these promoter fusions were examined when transformants were grown in glucose or acetate. A greater level of fluorescence was observed when the fungi were grown in acetate, particularly at 48 h, but not in glucose. Some fluorescence was observed in conidia, which suggests constitutive levels of *Ma-icl* (Fig. 6). Enzymic

ICL assays in conidia and during conidial germination concurred with these results (Fig. 7a).

Role of the glyoxylate cycle in the conidial germination and appressorium formation

We monitored the enzymic activity of the glyoxylate cycle intermediate ICL during conidial germination in *M. anisopliae* and *B. bassiana* throughout the 24 h after germination in medium containing glucose. Glucose was

Fig. 3. Phylogenetic analysis of fungal ICL including *B. bassiana* and *M. anisopliae* (asterisks). Relationships were inferred by using the CLUSTAL_X method. The GenBank accession numbers of the sequences are shown alongside the names of the fungal species.

Fig. 4. RT-PCR analysis of the expression of genes representative of the central carbon metabolic pathway of *M. anisopliae* and *B. bassiana* grown with different carbon sources and in haemolymph. (a) *M. anisopliae* and (b) *B. bassiana* with various carbon sources after 6 h: G, glucose; R, raffinose; A, acetate; E, ethanol; O, oleic acid; G-A, glucose-acetate. (c) *M. anisopliae* and (d) *B. bassiana* in insect (*Manduca sexta*) haemolymph under low oxygen conditions up to 12 h. *cit*, Citrate synthase; *aco*, aconitase; *idh*, isocitrate dehydrogenase; *icl*, ICL; *mls*, malate synthase; *18S*, 18sRNA control). Experiments were performed independently twice.

utilized since it did not exogenously induce the glyoxylate cycle (see previous results) thus allowing the determination of constitutive levels of ICL. The experiment revealed the presence of ICL in germinating conidia of *M. anisopliae* and *B. bassiana* (Fig. 7a). A peak in ICL activity was observed at 9 and 18 h post-germination for *M. anisopliae* and *B. bassiana*, respectively.

First, we tested the concentrations of 3-NP, which resulted in 100% loss of germination (after a 24 h incubation) in acetate medium and determined that 8 and 6 mM 3-NP for *M. anisopliae* and *B. bassiana*, respectively, could stimulate this loss. We then used these concentrations to monitor fungal germination in medium supplemented with glucose. We observed a delay in the rate of germination for *M. anisopliae* and *B. bassiana* conidia in glucose medium with 3-NP compared with the control (without 3-NP) after a 24 h incubation (Fig. 7b).

We also evaluated the effect of 3-NP on appressorium formation in *M. anisopliae* in a Petri dish containing 0.1% alanine (St Leger *et al.*, 1989). Appressorium formation was inhibited in the presence of 3-NP (Fig. 7c). However, no differences were observed in conidial germination with or without 3-NP under these conditions (Fig. 7d).

Ma-icl in the infection process

M. anisopliae can avoid insect haemocyte phagocytosis, perhaps originating as the ability to survive amoebic phagocytosis (Bidochka *et al.*, 2010). We followed the expression of ICL in *M. anisopliae* during insect haemocytic phagocytosis using mCherry linked to the *Ma-icl* promoter. We also used *gfp* linked to the *Ma-icl* promoter but observed less intense fluorescence than in *Ma-Picl-mCherry* (Supplementary Fig. S1). We observed upregulation of *icl* in *M. anisopliae* when it was phagocytosed by insect haemocytes (Fig. 8b–d), when the mycelium re-emerged in the insect cadaver (Fig. 8g) and in the formation of appressorium (Fig. 8f), but not when the fungus was proliferating within the insect haemocoel (Fig. 8e).

DISCUSSION

We investigated the role of metabolism, particularly the glyoxylate cycle, in *M. anisopliae* and *B. bassiana* during the occupation of various niches, as insect pathogens, under saprobic conditions or during growth in plant-derived compounds. The glyoxylate cycle has previously been reported to play an important role in the virulence of fungi

Fig. 5. Expression of *Ma-cit2* and *Ma-icl*. RT-PCR analysis from *M. anisopliae* grown in 1% locust cuticle (L), 1% giant cockroach cuticle (GC), 1% *Manduca sexta* cuticle (MS), 1% chitin (CH), 0.1%, 1% and 10% bean root exudate (BRE), 1% tomato stem (TS), 1% cellulose (CE) and 1% starch (S) grown for 12 h.

Fig. 6. Upregulation of *Ma-icl* promoter. Time-course of *pMa-PicI-mCherry* construct expression in *M. anisopliae* grown in glucose (a) or acetate (b). Images were taken ($\times 40$) by using light and fluorescence microscopy.

Fig. 7. ICL activity in germinating conidia and effects of the ICL inhibitor 3-NP. (a) ICL specific activity during germination in glucose of *M. anisopliae* and *B. bassiana*. Specific activity is measured as $10^{-5} \times \mu\text{mol}$ phenylhydrazine glyoxylate produced min^{-1} (mg protein) $^{-1}$. (b) Conidial germination in *M. anisopliae* and *B. bassiana* on acetate and glucose medium with 3-NP added 24 h after inoculation. (c) Effect of 3-NP on appressorium (Ap) formation from germinated conidia (Co) of *M. anisopliae* in 0.1% alanine. (d) Relative effect of 3-NP on germination (G) and appressorium (Ap) in *M. anisopliae*. (a) and (d) are means $\pm$ SD of three independent experiments.

Fig. 8. Expression of *M. anisopliae* ICL (*Ma-icl*) in vivo. *M. anisopliae* was transformed with *Ma-Picl-mCherry* and expression was monitored 0 h (a), 3 h (b), 6 h (c), 9 h (d) and 48 h (e) post-infection in *Manduca sexta*. In (b)–(d), note that *M. anisopliae* has been phagocytosed by insect haemocytes and there is upregulation of the *Ma-icl* promoter (arrows). In (e), *M. anisopliae* mycelia have ramified in the haemocoel and *Ma-icl* is not expressed. (f) Expression of *Ma-icl* 20 h after growth on 0.1 % alanine on a Petri dish. Note the presence of appressorium (arrow). (g) Expression of *Ma-icl* 5 days post-infection as mycelia emerged from the insect cadaver.

pathogenic to humans or plants (Dunn *et al.*, 2009). We demonstrated the enzymic presence of glyoxylate cycle intermediates in *M. anisopliae* and *B. bassiana* and confirmed this with RT-PCR. The presence of C₂ compounds resulted in the upregulation of the glyoxylate cycle in these entomopathogenic fungi. The regulation of the glyoxylate cycle in *M. anisopliae* and *B. bassiana* was found to be comparable to those reported in other fungi, with respect to glucose and acetate catabolism (Bibbins *et al.*, 1998; Chaure & Connerton, 1995; De Lucas *et al.*, 1994). In *B. bassiana*, the presence of glucose with acetate did not repress gene expression of the glyoxylate cycle genes, which has been observed in the fungus *Leptosphaeria maculans* (Idnurm & Howlett, 2002).

One of the more interesting findings of this study was that the *Ma-icl* and *Ma-cit2* genes share a bidirectional promoter. To our knowledge, this is first bidirectional promoter observed in *M. anisopliae* and the first bidirectional promoter involved in the regulation of glyoxylate cycle genes in any fungus. Bidirectional promoters have been described in other fungi for other genes, such as the *DAC1–NAG1* promoter of *C. albicans* (Kumar *et al.*, 2000), the *GAL1–GAL10* promoter of *Saccharomyces cerevisiae* (Bash & Lohr, 2001), and the *niiA–niaD* and *prnD–prnB* promoters of *Aspergillus nidulans* (Amaar & Moore, 1998;

García *et al.*, 2004). Studies of the human genome and the *Arabidopsis thaliana* genome show that genes with shared bidirectional promoters are often co-expressed and tend to be involved in similar biological functions (Trinklein *et al.*, 2004; Wang *et al.*, 2009). We also report two genes that encode citrate synthase, *Ma-cit1* and *Ma-cit2*, which showed differential expression. In *S. cerevisiae*, three genes have been reported that code for this enzyme, induction of which was differentially induced by carbon source and different subcellular localization (Kim *et al.*, 1986; Jia *et al.*, 1997). In *M. anisopliae*, the bidirectional promoter *Ma-icl–Ma-cit2* and the citrate synthase genes may facilitate carbon flow between the TCA and the glyoxylate cycle.

We also investigated the contribution of the glyoxylate cycle in conidial germination and appressorium formation. We used acetate or glucose as a carbon source and the ICL inhibitor, 3-NP, to show the utilization of the glyoxylate cycle in conidial germination. Germinating conidia of *M. anisopliae* and *B. bassiana* contained constitutive levels of ICL and germination was delayed in the presence of an ICL inhibitor, suggesting a role in conidial germination also described for *Aspergillus fumigatus* and *Magnaporthe grisea* (Ebel *et al.*, 2006; Wang *et al.*, 2003). The glyoxylate cycle plays a fundamental role in the utilization of C₂ compounds, possibly in the form of

storage lipids or fatty acids present in the conidia, which can be up to 48 % of the conidial dry weight in *Metarhizium* (Pupin *et al.*, 2000). The reserves of polyols and trehalose present in conidia of *M. anisopliae* and *B. bassiana* are not sufficient to generate the energy needed for germination (Hallsworth & Magan, 1994). The glyoxylate cycle was upregulated during appressorium formation and during insect pathogenesis, but not during hyphal proliferation within the haemocoel. We detected the upregulation of *Ma-icl* in the appressorium and confirmed the role of ICL by inhibiting appressorium formation using 3-NP. The role of the glyoxylate cycle in appressorium formation has been described in *Magnaporthe grisea* (Wang *et al.*, 2003; Kim *et al.*, 2006).

The human pathogen *C. albicans* can survive within mammalian macrophages, but *icl* mutants are unable to do so and are markedly less virulent in mice compared with wild-type strains (Lorenz & Fink, 2001). Previously, we have shown that *M. anisopliae* can evade phagocytosis (Bidochka *et al.*, 2010) and here we show that the glyoxylate cycle is upregulated when the fungus is within the insect haemocyte. Presumably, *M. anisopliae* is able to use C₂ compounds from the hydrolysis of intracellular lipids within insect haemocytes. However, once the fungus evades phagocytosis and proliferates within the haemocoel, the glyoxylate cycle was repressed, presumably since readily metabolizable sugars, such as trehalose, are present. Once these sugars are depleted, the glyoxylate cycle is again upregulated in order to metabolize more complex carbon sources, such as lipids and fatty acids, during hyphal re-emergence from the insect cadaver, which is followed by conidiation.

The glyoxylate cycle allows for metabolic flexibility in *M. anisopliae* and *B. bassiana* and allows for the utilization of various carbon sources during insect pathogenesis. These findings suggest that the glyoxylate cycle could also be used during saprobic growth and/or plant rhizosphere colonization. An understanding of the contribution of the glyoxylate cycle in these fungi could allow for the development of strategies to generate hypervirulent strains. Particularly, more information on how the glyoxylate cycle interacts with fatty acid degradation pathways is needed. This could be of significant importance in elucidating the mechanisms and energetics of insect cuticular lipid degradation as well as haemocyte lipids, haemolymph and fat body utilization.

ACKNOWLEDGEMENTS

This research was conducted with the assistance of a Natural Sciences and Engineering Research Council of Canada Discovery Grant to M. J. B. We thank D. Gonzalez for enzyme assay experiments. I. E. P.-G. received a doctoral fellowship from Consejo Nacional de Ciencia y Tecnología (Mexico).

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Edited by: B. A. Horwitz