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

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.

**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-b-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...
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 and stored at 20 °C for 10 days. *Escherichia coli* JM109 was used for DNA ligations and transformations.

**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*), aconitate (*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) using the SiteFinding-PCR method. PCR was performed using JumpStart Taq DNA polymerase (Sigma).

**Construction of the fluorescent protein expression vector using the Ma-icl promoter.** For construction of the pMa-Picl-gfp or pMa-Picl-mCherry expression vector, a 907 bp *M. anisopliae* promoter fragment was amplified from DNA with the primers ICL-Promo-F (5′-TGATCGGGTTTCGATGTTGGGCT, BanHI restriction site underlined) and ICL-ProFP (5′-GCCCTCGTTCTCCATTAGTGAC-GATGATGCACTA). The coding sequence of the fluorescent proteins eGFP (Fang et al., 2006) and mCherry (Araújo-Palomares et al., 2009) was amplified with the primer pair FP-U (5′-ATGATAGAACCGG-AGAGAAACCTT) and FP-L (5′-CTATTGTGATGTGATCAGT). 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 pMa-Picl-gfp and pMa-Picl-mCherry, respectively, were subcloned into pGEM-T Easy (Promega). After sequence verification, the resulting plasmids were digested with BanHI and EcoRI and ligated into plasmid pBARGEM7-2. The circular plasmids pMa-Picl-gfp and pMa-Picl-mCherry were used to transform *M. anisopliae* (Wang & St Leger, 2006). The transformants were selected in media containing 200 μg glufosinate-ammonium milligram−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-nitropropionic acid (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. bassiana*.


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

<table>
<thead>
<tr>
<th>Primer name (purpose)</th>
<th>Primer (5’–3’)</th>
<th>Gene</th>
<th>Amplification product (bp)</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Degenerate oligonucleotides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIT B-46</td>
<td>GTGACCCCTGGACCCGGGYNATAAGNGG</td>
<td>Citrate synthase</td>
<td>1327</td>
<td>FN796521</td>
</tr>
<tr>
<td>CIT D-42</td>
<td>TCGTGGGAAAGGCTCTGNNGKTYTC</td>
<td>ICL</td>
<td>841</td>
<td>FN796523</td>
</tr>
<tr>
<td>ICL B-10</td>
<td>CCAAGAAGTCTGGGCGAYTGACNGG</td>
<td>Malate synthase</td>
<td>706</td>
<td>FN796520</td>
</tr>
<tr>
<td>ICL E-28</td>
<td>GTATGTCGCGGCGGWCAYTYTYYT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLS D-34</td>
<td>GGGCACCGTGTCGTGTHAGARACNAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLS E-28</td>
<td>CGCGGTGGCCGGCRTCYTCCAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>M. anisopliae RT-PCR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME-CIT1</td>
<td>CAAGGTTGCCGCCCATCCACAGG</td>
<td>Citrate synthase 1</td>
<td>407</td>
<td>FN796521</td>
</tr>
<tr>
<td>ME-CIT2</td>
<td>GACGCAATGATCGGGGTACGTC</td>
<td>Citrate synthase 2</td>
<td>234</td>
<td>FN796522</td>
</tr>
<tr>
<td>ME-ICL</td>
<td>GCTTGCGCAAGCTGCTTGCTGCACTG</td>
<td>ICL</td>
<td>386</td>
<td>FN796523</td>
</tr>
<tr>
<td>ME-ICL</td>
<td>AATCACCGCAACTCGGACTATCTCGAT</td>
<td>Aconitase</td>
<td>369</td>
<td>FN796520</td>
</tr>
<tr>
<td>ME-ICL</td>
<td>GCCGACCCGAGCAGCCAGCCAGCAG</td>
<td>Isocitrate dehydrogenase</td>
<td>361</td>
<td>FN806962</td>
</tr>
<tr>
<td>ME-IDH</td>
<td>GCGGCCGCAACAGCAATCTCACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. bassiana RT-PCR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BE-CIT</td>
<td>CCCCGCCGCGCAAGACGACG</td>
<td>Citrate synthase 1</td>
<td>474</td>
<td>DT370663</td>
</tr>
<tr>
<td>BE-ACO</td>
<td>CGTGGGCGCTGCTGCTCAGTTGAAGTGATG</td>
<td>Aconitase</td>
<td>326</td>
<td>DT368904, DT370106, DT370219, DT372548, DT375952, DT375966</td>
</tr>
<tr>
<td>BE-IDH</td>
<td>GGGTGGGCTGCAGGTGTGTGTCTCT</td>
<td>Isocitrate dehydrogenase</td>
<td>379</td>
<td>DT376726</td>
</tr>
<tr>
<td><strong>Metarhizium and Beauveria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S U</td>
<td>AGGCCCGGTAATCTGT</td>
<td>18S rRNA</td>
<td>266</td>
<td>AY755540, AY755538</td>
</tr>
<tr>
<td>18S L</td>
<td>GACGTGTATGACCTTTACTCTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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-Piel-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-Piel-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-Piel-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 530-550/650 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 coincidently contained a shared bidirectional promoter region (Fig. 2). Putative transcriputional binding elements found in this region include a CREA region, responsive to glucose repression (SYGGRG), homologous to that in *Aspergillus* (Cubero & Scaccocchio, 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 saprobiic 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 10⁵ × μmol phenylhydrazine glyoxylate produced min⁻¹ (mg protein)⁻¹.
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...
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. 6. Upregulation of Ma-icl promoter. Time-course of pMa-Picl-mCherry construct expression in M. anisopliae grown in glucose (a) or acetate (b). Images were taken (×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 $10^{-5} \times \mu$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 ± SD of three independent experiments.
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 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).

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.

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.

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 A. fumigatus 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 A. 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 ICL within the haemocoel 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 Concejo Nacional de Ciencia y Tecnología (Mexico).

**REFERENCES**


Hallsworth, J. E. & Magan, N. (1994). Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of
conidia of three entomopathogenic fungi. Microbiology 140, 2705–2713.


Edited by: B. A. Horwitz