A regulator of a G protein signalling (RGS) gene, *cag8*, from the insect-pathogenic fungus *Metarhizium anisopliae* is involved in conidiation, virulence and hydrophobin synthesis

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Regulators of the G protein signalling (RGS) pathway have been implicated in the control of a diverse array of cellular functions, including conidiation in filamentous fungi. However, the regulatory processes involved in conidiation in insect-pathogenic fungi are poorly understood. Since conidia are the infective propagules in these fungi, an understanding of the regulatory processes involved in conidiation is essential to the development of an effective biocontrol fungus. Here, the cloning and characterization of an RGS protein gene, *cag8* (conidiation-associated gene), from the insect-pathogenic fungus *Metarhizium anisopliae* is reported. Phylogenetic analysis showed that CAG8 was orthologous to the RGS protein FlbA from *Aspergillus nidulans*. Complementation of *A. nidulans* $\Delta flbA$, which cannot conidiate, with *M. anisopliae* *cag8* restored conidiation. Gene disruption of *cag8* in *M. anisopliae* resulted in the lack of conidia on agar plates and on infected insects, reduced mycelial growth, decreased virulence, lysis during growth in liquid medium as well as lack of pigmentation and irregularly shaped blastospores. Transcript levels of *ssgA* (hydrophobin-encoding gene) were markedly reduced in a $\Delta cag8$ strain, while *pr1A* (subtilisin-like protease) transcription was unaffected. These results suggest that *cag8* is involved in the modulation of conidiation, virulence and hydrophobin synthesis in *M. anisopliae*.

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INTRODUCTION

Biological control agents, such as insect-pathogenic fungi, offer an environmentally compatible alternative to chemical pesticides; however, their use is limited mainly because of the relatively slow rate of kill (St Leger, 1993). To enhance their virulence, detailed knowledge of the mechanisms of fungal pathogenesis is needed. The conidium is the infective propagule, which comes into contact with, and adheres to, the insect cuticle via hydrophobic mechanisms (Boucias *et al.*, 1988) mediated by conidial surface proteins called hydrophobins (Bidochka *et al.*, 2001). Once attached, the conidium germinates and penetrates the insect cuticle via mechanical pressure and cuticle-degrading enzymes (St Leger *et al.*, 1996). Hyphae proliferate within, and emerge from, the dead insect and subsequently conidiate on the cadaver. Under favourable conditions, the newly produced conidia will disperse and infect other insects. Thus, the

conidium is the propagule that initiates pathogenesis and is involved in disease transmission. An understanding of the regulatory processes involved in conidiation is essential to the commercial development and improvement of this biocontrol fungus.

In several filamentous fungi, the heterotrimeric G protein signalling pathway is involved in conidiation, as well as morphogenesis and pathogenesis (Bölker, 1998; Borkovich *et al.*, 2004; Lengeler *et al.*, 2000). The genetic regulatory circuit for conidiation is initiated when the heterotrimeric G protein pathway is activated. This allows the $G\alpha$ subunit of the heterotrimeric G protein complex to substitute GDP for GTP, resulting in the dissociation of the $G\alpha$ and $G\beta\gamma$ subunits and the activation of downstream effectors by both $G\alpha$ and $G\beta\gamma$ (Hamm, 1998). $G\alpha$ and $G\beta\gamma$ subunits have been identified in several filamentous fungi and their implication in fungal conidiation is well established. The signal amplitude of G protein signalling is determined by the balance of the rates of GDP/GTP exchange (activation) and the rates of GTP hydrolysis (deactivation), which is achieved by the intrinsic GTPase of the $G\alpha$ subunit. However, the GTPase of the $G\alpha$ subunit requires a regulator of G protein signalling (RGS) to enhance GTPase activity (Ross & Wilkie, 2000).

Abbreviations: RACE, rapid amplification of cDNA ends; RGS, regulator of G protein signalling; STRE, stress-responsive element; YADE, Y-shaped adaptor-dependent extension.

The GenBank/EMBL/DBJ accession number for the sequence reported in this paper is DQ826044.

Altogether, six RGS protein families (orthologues of RgsA, RgsB, RgsC, RgsD, FlbA and Gprk) have been identified from *Aspergillus* spp. and other fungal RGS proteins fall into one of these families (Lafon *et al.*, 2006). The first RGS protein-encoding gene identified in a filamentous fungus was *flbA* (fluffy low *brlA*) in *Aspergillus nidulans* (Lee & Adams, 1994). FlbA negatively regulates signalling for vegetative growth and activated conidiation, mediated through the G α subunit, FadA (Yu *et al.*, 1996), and the G β subunit, SfaD (Rosen *et al.*, 1999). Han *et al.* (2004) described a second RGS protein in *A. nidulans*, RgsA, which down-regulated stress responses and stimulated conidiation through attenuation of GanB (G α) signalling. Several RGS proteins with domain organizations similar to FlbA have been identified and implicated in conidiation in other filamentous fungi. The RGS protein encoded by the *thn-1* gene of *Schizophyllum commune* is necessary for fruit body formation in dikaryons and aerial hyphae formation in monokaryons (Fowler & Mitton, 2000). Most recently, an RGS protein, CPRGS-1, from the phytopathogenic fungus *Cryphonectria parasitica* has been implicated in fungal conidiation as well as virulence and hydrophobin synthesis (Segers *et al.*, 2004).

Metarhizium anisopliae is arguably one of the best studied insect-pathogenic fungi. Several genes implicated in cuticle penetration and pathogenesis have been characterized (Bogo *et al.*, 1998; Joshi & St Leger, 1999; Screen *et al.*, 1998, 2001; St Leger *et al.*, 1992); however, to our knowledge, genes regulating conidiation have not been identified. Here, we isolated an RGS protein gene, *cag8*, from *M. anisopliae* that showed significant homology to *flbA* (Lee & Adams, 1994). The characterization of *cag8* using gene disruption mutants established a role for its product in modulating conidiation, virulence and hydrophobin synthesis in *M. anisopliae*.

METHODS

Fungal and bacterial strains. *Metarhizium anisopliae* strain ARSEF 2575 was obtained from the United States Department of Agriculture Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA. Cultures were grown on potato dextrose agar (PDA; Difco) at 27 °C for 10 days. *Aspergillus nidulans* wild-type strain A26 was purchased from the Fungal Genetics Stock Center, School of Biological Sciences, University of Missouri, Kansas City, MO, USA, and *A. nidulans* $\Delta flbA$ (*yeA flbA::argB methG pyro veA*) was kindly provided by Dr Nancy Keller, Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI, USA) and Dr J. H. Yu (Department of Food Microbiology and Toxicology & Molecular and Environmental Toxicology Center, University of Wisconsin-Madison, Madison, WI, USA). On complete medium supplemented with methionine and pyridoxin, *A. nidulans* $\Delta flbA$ cannot conidiate and the colony undergoes autolysis as it matures. Yellow conidia of *A. nidulans* $\Delta flbA$ were obtained by culturing on complete medium agar containing 0.8 M NaCl (Yu *et al.*, 1996). *Escherichia coli* DH5 α was employed for DNA manipulations and transformations. *Agrobacterium tumefaciens* AGL-1 was used for fungal transformations.

Gene cloning. Fungal genomic DNA was isolated by using the DNeasy Isolation Kit (Qiagen). RNA was prepared by using the

RNeasy Mini Kit (Qiagen); genomic DNA was eliminated by loading RNase-free DNaseI onto the filter column (Qiagen). Nucleic acid extractions were performed according to manufacturer's instructions. Nucleic acid concentrations were measured with GeneQuantII (Pharmacia Biotech).

To clone a fragment of the RGS protein gene from *M. anisopliae* by PCR, consensus degenerate primers RGSF1 and RGSRI (Table 1) were designed based on regions of homology to *A. nidulans* FlbA (Lee & Adams, 1994), *Schizophyllum commune* THN-1 (Fowler & Mitton, 2000) and *Saccharomyces cerevisiae* SST2 (Dohlman *et al.*, 1996). PCR was performed using hot start *Taq* DNA polymerase (Qiagen). The PCR cycles were 95 °C for 15 min, followed by 35 cycles of 1 min denaturation at 94 °C, 45 s annealing at 48 °C and 1 min polymerization at 72 °C, followed by 72 °C for 10 min. The resultant PCR product was shown to be a fragment of an RGS protein gene. The full length of this gene as well as the upstream and downstream regulatory sequence was subsequently cloned by PCR walking using the Y-shaped adaptor-dependent extension (YADE) method as described previously (Fang *et al.*, 2005). Restriction enzymes *DraI*, *EcoRV*, *ScaI*, *SmaI*, *XbaI*, *SpeI*, *NheI*, *BamHI* and *BglII* were used to digest the *M. anisopliae* genomic DNA. Three rounds of PCR walking were conducted for the isolation of the upstream sequences and one round for downstream sequences. Primers used for YADE are listed in Table 1. Hot start *Taq* DNA polymerase (Qiagen) was used and the reaction mixtures were prepared according to manufacturer's instructions. For YADE, all linear amplifications were performed at 95 °C for 15 min, followed by 40 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C and 3 min polymerization at 72 °C, followed by 72 °C for 3 min. All exponential amplifications were conducted at 95 °C for 15 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C and 2 min polymerization at 72 °C, followed by 72 °C for 10 min. All PCR products were cloned into the A-T cloning vector pGEM-T (Promega), according to manufacturer's instructions, and subsequently sequenced. The RGS protein-encoding gene from *M. anisopliae* was named *cag8*.

The full-length *cag8* gene as well as its upstream and downstream sequences were resolved by using the PCR and YADE products based on overlapping regions. Primers (Table 1) were designed to clone the cDNA sequence of this gene using Second Generation 5' and 3' RACE (rapid amplification of cDNA ends) kits (Roche).

Reverse-transcribed PCR (RT-PCR). RT-PCR was conducted with the One-Step RT-PCR kit (Qiagen). Total RNA (250 ng) was subjected to RT-PCR and the reaction components were prepared according to manufacturer's instructions. RT-PCR cycles were 50 °C for 30 min, 95 °C for 15 min, followed by 30 cycles of 45 s denaturation at 94 °C, 30 s annealing at 60 °C and 45 s polymerization at 72 °C, followed by 72 °C for 10 min. RT-PCR primers for *cag8* and *gpd* are listed in Table 1. Expression of *cag8* was investigated during growth in broth culture (three developmental stages), agar medium (four developmental stages), as well as during insect pathogenesis (three developmental stages). In YPD broth (0.2 % yeast extract, 1 % peptone, 2 % glucose), the developmental stages were (i) conidium, (ii) swollen conidia (7 h) after inoculation and (iii) conidial germination (16 h) with germ tube lengths of 40–100 μ m. In YPD agar (0.2 % yeast extract, 1 % peptone, 2 % glucose, 1.5 % agar), the developmental stages were (i) mycelial growth (2 days), (ii) early conidiophore development with no conidia (3 days), (iii) late conidiophore development with few conidia (4 days) and (iv) fully mature conidiophore with conidia (6 days). During insect pathogenesis, the developmental stages were (i) emergent mycelia from insect (*Galleria mellonella*) cadavers (3 days after insect death), (ii) emergent mycelia with few conidia (5 days after insect death) and (iii) insect cadavers mummified with mycelia and conidia (10 days after insect death). Insects (*G. mellonella*) were infected by immersion in

Table 1. Oligonucleotides used in this study

Purpose and oligonucleotide	Sequence (5′–3′)*	Remarks
PCR-based cloning of a fragment of <i>cag8</i>		
RGSF1	WSNAAVMGNATGCCNGAYCC	
RGSR1	NGTNSWRCARTCCATNARCC	
YADE-based PCR walking		
cag8-3-1	CGCCGACTCGGACTCCCTACAC	Used for cloning 3′ end of <i>cag8</i>
cag8-3-2	GAGCGGAAGGTGAACGGCAAG	
cag8-5-1	GAGTCAATTGCCAAACAGATCCC	Used for cloning 5′ end of <i>cag8</i>
cag8-5-2	CACGAAGCGTTGGCAGATGG	
cag8-5c-1	AGAGGTGTAGTGCTTCAATGG	Used for cloning 5′ end of fragment obtained with cag8-5-1 and cag8-5-2
cag8-5c-2	TTGCGAGGGAGTGTCTCTTC	
cag8-5cc-1	ACATGAGGCGTGAGGGTGGG	Used for cloning 5′ end of fragment obtained with cag8-5c-1 and cag8-5c-2
cag8-5cc-2	GAGTTGTCTGGACGAGGAAGACC	
Cloning cDNA of <i>cag8</i> using RACE		
cag8-3	CAAATCGAATGCCGATCCCAAG	Used for 3′ RACE
cag8-5	CTGCTTCTTTGGATGCCATTCC	Used for 5′ RACE with cag8-5-1 and cag8-5-2
RT-PCR		
cag8RT-1	AAGCTGATGGCTAGCGATTCT	Used for RT-PCR and real-time RT-PCR analysis of <i>cag8</i>
cag8RT-2	TTGCGGTTGGAACGACTTTG	
gpdRT-1	GACTGCCCGCATTGAGAAG	Used for RT-PCR analysis of <i>gpd</i>
gpdRT-2	AGATGGAGGAGTTGGTGTG	
Cloning <i>cag8</i> for complementation of <i>A. nidulans</i> $\Delta flbA$		
cag8F	CCGGATCCCCAAGAGCCCCAAATCAAGG	Used for amplification of <i>cag8</i> and its upstream and downstream regulatory sequence
cag8R	GGAAGCTTATCAACGCATGCCATCCATGTTT	
For <i>cag8</i> disruption vector construction		
Hcag8-1	CCCACGTGGTACTTAAGTAAGTATGCGTG	Used for amplification of 5′ end of <i>cag8</i>
Hcag8-2	GGACTAGTGATGGAAGGGCCGGGGCTTC	
Hcag8-3	GGGCGGCCGCGTCTGCCCGCACCTATCAGAG	Used for amplification of 3′ end of <i>cag8</i>
Hcag8-4	GGGAATTCGCCAACGTCCTTGTAATAATG	
For verification of <i>cag8</i> disruption		
Dcag8	GGGGGCACAAGGGTAAGAGG	Used for confirmation of the disruption of <i>cag8</i>
Dbar	GACGTGGGTTTCTGGCAGCTGG	

*W=A or T; S=G or C; Y=C or T; N=A, G, C or T; M=A or C; R=A or G.

a conidial suspension (2×10^7 conidia ml^{-1}). At death, the insect was surface-sterilized in 1% sodium hypochlorite (1 min) and rinsed twice in sterile distilled water. The sterilized cadavers were then placed in a Petri dish with moist paper to promote fungal emergence from the cadaver. RNA was extracted from emergent mycelia and conidia from infected insects.

Real-time RT-PCR. First-strand cDNA was synthesized using hexamers and oligo-dT primers with iScript reverse transcriptase, according to manufacturer's instructions (Bio-Rad). One microgram of total RNA was used for cDNA synthesis and the cDNA was subsequently diluted with nuclease-free water (Qiagen) to $10 \text{ ng } \mu\text{l}^{-1}$. Real-time RT-PCR amplification mixtures (25 μl) contained 10 ng template cDNA, $2 \times$ SYBR GreenI master mix buffer with fluorescein for dynamic well factor collection (12.5 μl) (Bio-Rad) and 200 nM each of the forward and reverse primers (Sigma). The reaction was performed on the iCycler system (Bio-Rad). PCR was accomplished

after a 1.5 min activation/denaturation step at 95°C , followed by 40 cycles of 15 s at 95°C , 30 s at 60°C and 30 s at 72°C . Fluorescence was detected and recorded at each polymerization step. The expression of *cag8*, *ssgA* (Bidochka *et al.*, 2001) and *pr1A* (St Leger *et al.*, 1992) were analysed using *gpd*, *tef* and *try* as reference genes, and expression levels were calculated using geNORM (Fang & Bidochka, 2006). Primers are listed in Table 1.

Disruption of *cag8*. To disrupt *cag8* in *M. anisopliae* based on homologous recombination, the herbicide resistance gene *bar* was inserted into *cag8*. The 5' end of *cag8* was cloned by PCR with primers Hcag8-1 and Hcag8-2 (Table 1) using Jumpstart AccTaq LA DNA polymerase (Sigma). The resultant PCR product was digested with *SpeI* and *PmlI*, and inserted into *XbaI* and *PmlI* sites of pBARGPE1 (Ponting & Bork, 1996) to form pGPE-cag8-5:bar. The 3' end of *cag8* was amplified with primers Hcag8-3 and Hcag8-4 with Jumpstart AccTaq LA DNA polymerase (Sigma). The PCR

product was then digested with *EcoRI* and *NotI* and cloned into the corresponding sites of pGPE-cag8-5:bar to form pGPE-cag8-5:bar:cag8-3. From this, the cag8-5:bar:cag8-3 portion was excised using *EcoRI* and *PmlI* and inserted into *EcoRI/EcoRV*-digested binary vector pPK2 (McCluskey, 2003) in which an *hph* cassette was deleted using *KpnI* and *XbaI* to form pPK-cag8-5:bar:cag8-3. The *gfp* cassette was excised from TEFGFP (Spear *et al.*, 1999) using *EcoRI* and subsequently inserted into the same site of pPK-cag8-5:bar:cag8-3 to form pPKGFP-cag8-5:bar:cag8-3, which was then mobilized into *Ag. tumefaciens* AGL-1 for fungal transformation (Fang *et al.*, 2006).

cag8-deficient mutants were selected as herbicide-resistant and observed for GFP expression. PCR with primer combinations Dcag8-1/Dcag8-2 and Dbar/Dcag8-2 as well as Southern blotting was used to confirm disruption of *cag8* in *M. anisopliae* transformants.

Complementation experiments. To complement $\Delta flbA$ of *A. nidulans* with *cag8*, a plasmid vector containing *cag8* was constructed. The ORF of *cag8*, as well as the upstream and downstream sequences, were amplified with primers cag8F and cag8R (Table 1) using High-Fidelity *Taq* DNA polymerase (Qiagen) and sequenced for verification. The PCR product was digested with *EcoRV* and inserted into the *SmaI* site of vector pUG11-41 which contains the methionine gene (kindly provided by Dr Nancy Keller) to form the vector pUG11-41-cag8.

Conidia of *A. nidulans* $\Delta flbA$ were collected from complete medium agar supplemented with 0.8 M NaCl and cultured overnight in complete medium broth at 37 °C. Protoplast preparation and transformations were performed as described previously (Ballance *et al.*, 1983; Ballance & Turner, 1985). Plasmids pUG11-41 and pUG11-41-cag8 were used for transformations. Transformants were selected based on methionine utilization and further confirmed by PCR with primers Hmacag8F and Hmacag8R (Table 1) as well as Southern hybridization. Expression of *cag8* in transformants was verified by RT-PCR with primers cag8RTF and cag8RTR (Table 1).

Bioassays. Virulence of fungal blastospores was tested against *G. mellonella* by injecting 3 µl blastospore suspension (10^4 ml⁻¹) or by immersion of insects into a blastospore suspension (3×10^6 ml⁻¹). Blastospores were obtained from wild-type transformants with cag8-5:bar:cag8-3 integrated ectopically, and *cag8*-deficient mutants which were cultured in 100 ml YPD broth. After a 4 day incubation at 27 °C with 200 r.p.m. shaking, cultures were filtered through glass wool to obtain blastospores. Mortality was recorded daily and LT₅₀ values were estimated by probit analysis.

Each treatment was replicated three times with 10 insects per replicate. All bioassays were repeated three times.

RESULTS

Isolation of *cag8* from *M. anisopliae*

A 5400 bp DNA fragment, which contained the full-length RGS protein gene, named *cag8*, as well as upstream and downstream regulatory sequences was cloned and sequenced.

The putative coding sequence was identified using BLASTX and based on this, primers (described in Methods) were designed to clone the concordant cDNA using 5' and 3' RACE. A 1933 bp cDNA fragment was resolved based on RACE products, which contained a 1326 bp ORF and a 123 bp 5' UTS and 484 bp 3' UTS. A protein of 441 aa with a predicted molecular mass of 49.9 kDa and a pI of 8.7 was

deduced. Two introns of 451 and 122 bp, respectively, were identified. Southern blotting showed that *cag8* existed as a single copy in the *M. anisopliae* genome (data not shown). Three stress-responsive element (STRE) domains were identified in the 2838 bp upstream regulatory sequence. STREs have previously been shown to mediate transcriptional activation in response to various stresses, particularly heat stress, osmotic stress, low pH and nutrient starvation in *Saccharomyces cerevisiae* (Siderius & Mager, 1997).

RT-PCR analysis demonstrated that *cag8* was constitutively expressed throughout various growth stages in broth culture and agar medium as well during emergence of fungal mycelia and conidiation on the infected insect cadaver (data not shown).

Homology and phylogenetic analysis of CAG8

Six RGS protein families have been identified from *Aspergillus* spp. (Lafon *et al.*, 2006). They are orthologues of RgsA, RgsB, RgsC, RgsD, FlbA and GprK. Other fungal RGS proteins show homology to one of these six *Aspergillus* RGS proteins (Lafon *et al.*, 2006). Fig. 1(a) shows that CAG8 is most closely related to FlbA with a well supported bootstrap value of 100 %. Also, FlbA and CAG8 feature two DEP domains and one RGS domain. Fig. 1(b) shows the relationship of FlbA-like proteins from various ascomycetous and basidiomycetous fungi. The ascomycetous clade contains *A. nidulans* and *M. anisopliae*, *Gibberella zeae*, *Neurospora crassa* and *Cryphonectria parasitica* and is well supported (bootstrap 100 %). The RGS protein, CAG8, from *M. anisopliae* is most closely related to the RGS protein from the plant pathogen *Gibberella zeae*. The other major clade consists of the basidiomycetes *Schizophyllum commune*, *Ustilago maydis*, *Cryptococcus neoformans* and *Cryptococcus bacillisporus*.

Conidiation, morphology and virulence of *cag8* disrupted mutants

The *cag8* gene in *M. anisopliae* was disrupted with the *bar* gene followed by homologous recombination using *Ag. tumefaciens*-mediated transformation. Six *cag8* disruptants ($\Delta cag8$), D1–D5 and D7, were utilized for further study. These six $\Delta cag8$ strains, which were randomly selected from the transformants, were confirmed by PCR and Southern blotting and showed identical patterns of genomic integration (Fig. 2).

When grown on agar medium, the $\Delta cag8$ strain did not produce conidia (Fig. 3a) and mycelial growth was slower than the wild-type strain or transformants with the cag8-5:bar:cag8-3 integrated ectopically (Bar). In YPD broth, $\Delta cag8$ produced fewer blastospores than the wild-type strain (Table 2). Blastospores of $\Delta cag8$ (mean length 19.1 ± 1.3 µm, $n=100$) were about twice as long as the wild-type (mean length 8.9 ± 0.9 µm, $n=100$) (Fig. 3b). After 8 days of growth in YPD broth, the cultures of the wild-type strain or transformants with cag8-5:bar:cag8-3

Fig. 1. (a) Phylogenetic relationship of *M. anisopliae* CAG8 to RGS protein sequences identified in *A. nidulans* and *A. oryzae*. The unrooted tree was obtained using CLUSTALX with 1000 repetitions. The numbers shown are bootstrap values. The accession numbers of the RGS proteins are *A. nidulans* RgsA, AN5755.2; *A. nidulans* RgsB, AN3622.2; *A. nidulans* RgsC, AN1377.2; *A. nidulans* FlbA, EAA58402; *A. nidulans* GprK, AN7795.2; *A. oryzae* RgsD, AO0702980 00074. (b) Phylogeny of FlbA orthologues from 11 fungal species, including *M. anisopliae* CAG8, created using CLUSTALX with 1000 repetitions. The numbers shown are bootstrap values. The accession numbers of the RGS proteins are *Neurospora crassa*, XP_329025; *Gibberella zeae*, EAA73850; *A. nidulans*, EAA58402; *Cryphonectria parasitica*, AAT92283; *M. anisopliae*, DQ826044; *Saccharomyces cerevisiae*, AAA35104; *Schizosaccharomyces pombe*, CAA91077; *Schizophyllum commune*, AAF78951; *Cryptococcus bacillispora*, AAQ97625; *Cryptococcus neoformans*, AAR06255; *Ustilago maydis*, EAK83159.

integrated ectopically had many blastospores, while blastospores were absent in $\Delta cag8$ cultures. Furthermore, $\Delta cag8$ cultures were relatively unpigmented in YPD broth while wild-type cultures were typically dark green (Fig. 3c).

Addition of cAMP (100 μ M) in PDA did not restore conidiation in $\Delta cag8$ strains. However, when PDA was supplemented with 0.8 M NaCl, $\Delta cag8$ strains did produce some conidia, but the yield was significantly less than the wild-type strain or transformants with *cag8-5:bar:cag8-3* integrated ectopically (Table 2). Therefore, high osmolarity in PDA agar can partially restore conidiation in a $\Delta cag8$ strain, similar to $\Delta flbA$ in *A. nidulans* (Lee & Adams, 1994).

Since $\Delta cag8$ strains did not produce conidia on agar media, blastospores were used for bioassays. The $\Delta cag8$ strains showed a significant decrease in virulence compared to the wild-type strain when insects were infected by immersion in a blastospore suspension (Table 2). However, $\Delta cag8$ strains had similar virulence to the wild-type strain when

blastospores were injected into insects (Table 2). Cadavers infected by the wild-type strain were firm and showed emergent mycelia with conidia, while insect cadavers infected by $\Delta cag8$ strains showed no conidiation, sparse mycelial growth and the cadavers were soft (Fig. 3D).

Relationship between *cag8*, *ssgA* and *pr1*

Transcript levels of the hydrophobin-encoding gene *ssgA* and the subtilisin-like protease-encoding gene *pr1A* were analysed by real-time RT-PCR. In the wild-type strain, *ssgA* transcript levels were relatively greater during the late stages of conidiation and *pr1A* transcripts were relatively greater during early and late stages of pathogenesis. Real-time PCR showed that the transcript level of *ssgA* was markedly reduced (>200-fold) in the $\Delta cag8$ strains (Fig. 4). Using RNA derived from mycelia from insect cadavers as the template, *pr1A* transcript levels were similar in the wild-type and $\Delta cag8$ strains (data not shown).

Fig. 2. Disruption of *cag8* in *M. anisopliae* using the *bar* gene. (a) Construct (pPKGFP-*cag8*-5:bar:*cag8*-3) used for creating Δ *cag8*. The grey arrow shows the position of the *cag8* ORF in the fragment of the *cag8* gene. (b) Southern analysis of *cag8* disruptant strains (D1–D5 and D7). Genomic DNA was digested with *Xho*I and *Not*I. The PCR product obtained with primers Hcag8-3 and Hcag8-4 was used as probe. Sizes of expected bands are indicated in kb. (c, d) Verification of the Δ *cag8* in strains D4 and D7 (identical results found for the other Δ *cag8* strains), a transformant with ectopic integration (Bar) and the wild-type strain was performed with (c) primers Dbar and Dcag8-2 and (d) primers Dcag8-1 and Dcag8-2.

Complementation of *A. nidulans* Δ *flbA* with *cag8* from *M. anisopliae*

A 5 kb DNA fragment containing *cag8* as well as upstream and downstream regulatory sequences was used to complement *A. nidulans* Δ *flbA*. Sixty meth⁺ colonies transformed with pUG11-41-*cag8* and 75 meth⁺ colonies transformed with pUG11-41 were selected on minimal medium lacking methionine. All pUG11-41 transformant colonies were similar to *A. nidulans* Δ *flbA* in colony development and conidiation. Transformants, with pUG11-41-*cag8*, were found to conidiate similar to *A. nidulans* wild-type. Insertion of *cag8* into the genome of the transformants was confirmed by PCR and Southern hybridization in five transformants with identical results (data not shown). RT-PCR showed that *cag8* was expressed in the transformant (data not shown). On complete medium, conidiation of the transformant was similar to the wild-type, but, starting in the centre, the colony underwent autolysis after approximately 14 days incubation at 37°C, but autolysis ceased

thereafter (Fig. 5). The *A. nidulans* Δ *flbA* strain never produced conidia and the colony underwent autolysis in the centre after 3.5 days growth; by day 7, the entire colony underwent autolysis. We concluded that *cag8* was able to restore conidiation of *A. nidulans* Δ *flbA*, but was unable to completely halt colony autolysis.

DISCUSSION

Here we report an RGS protein gene, *cag8*, from *M. anisopliae* that plays a critical role in the regulation of conidiation, virulence and hydrophobin synthesis. CAG8 showed significant homology to FlbA found in aspergilli (Lafon *et al.*, 2006). Similar to FlbA, CAG8 also featured two DEP domains. The DEP domain was initially identified in dishevelled (*Drosophila melanogaster*), Egl-10 (*Caenorhabditis elegans*) and human pleckstrin (Rosen *et al.*, 1999), which are all involved in G-protein signalling. DEP domains have been shown to be necessary for membrane localization (Martemyanov *et al.*, 2003) and are also involved in regulating the expression of stress-responsive genes through interaction with other proteins (Burchett *et al.*, 2002). Several putative STREs were identified in the upstream regulatory sequence of *cag8*, the DEP-domain-containing RGS protein gene itself.

Despite the similarities of RGS proteins from various fungi, the knockout phenotypes can sometimes be quite different. In *M. anisopliae*, Δ *cag8* resulted in reduced growth and the presence of aerial mycelia without conidia, which is similar to the phenotype of Δ *cprgs-1* in the plant-pathogenic fungus *Cryphonectria parasitica* (Segers *et al.*, 2004). Δ *flbA* in *A. nidulans* results in a fluffy colony phenotype with abundant mycelial growth, reduced conidiation and autolysis as the colony ages (Lee & Adams, 1994).

From the gene complementation experiments we found that *cag8* can restore conidiation, but cannot completely halt autolysis in *A. nidulans* Δ *flbA*. This could possibly be due to the different flanking sequences of the RGS and DEP domains in these two RGS proteins. FlbA is also 278 aa longer than CAG8, mainly in the flanking sequences of the RGS and DEP domains. Functional domains and motifs in the flanking sequence of the RGS domain are considered to contribute to specific activity, subcellular localization or regulation, resulting in different RGS proteins contributing to different signalling pathways (Ross & Wilkie, 2000). The unique flanking sequence in FlbA affords control over mycelial proliferation as well as conidiation and, thus, RGS protein genes from different fungi may not be fully complementary.

The association between RGS proteins and hydrophobin synthesis was shown in Δ *cag8* where real-time RT-PCR showed significantly reduced *ssgA* gene expression. Similarly, Δ *thn-1* *Schizophyllum commune* and Δ *cprgs-1* *Cryphonectria parasitica* were deficient in expression of their hydrophobin-encoding genes *Sc3* and *cryparin*, respectively (Fowler & Mitton, 2000; Segers *et al.*, 2004).

Fig. 3. Phenotype of $\Delta cag8$ strains (D4 and D7), transformants with ectopic integration (Bar) and the wild-type strain (WT). (a) Colony after 10 days growth on M100 agar. The $\Delta cag8$ strains showed slower growth and no conidial production. (b) The blastospores (stained with Lacto-phenol Cotton Blue) of D4, D7, wild-type and Bar. (c) Pigmentation of strains in YPD broth. The mycelia of the wild-type and Bar are dark green after 8 days growth in YPD broth, while $cag8$ -deficient strains D4 and D7 are relatively unpigmented. (d) Surface mycelial growth and conidiation in infected insects. Note the dark-coloured conidia as well as fluffy aerial mycelia in WT and Bar, but only surface white mycelia in $\Delta cag8$ strains. Similar results were found for the other $\Delta cag8$ strains.

Mutations in RGS genes have diverse effects on fungal pathogens of insects, plants or humans. In this study, $\Delta cag8$ mutants had virulence similar to that of the wild-type strain when blastospores were injected into insects. However, when insects were infected by immersion into a blastospore suspension, the virulence of $\Delta cag8$ mutants was significantly lower than that of the wild-type strain. The disruption of the RGS gene *cprgs-1* resulted in complete loss in virulence in

the phytopathogenic fungus *Cryphonectria parasitica* (Segers *et al.*, 2004). On the other hand, mutations in *crg1* increased virulence in *Cryptococcus neoformans*, a human-pathogenic fungus (Wang *et al.*, 2004).

The identification of an RGS gene involved in the regulation of conidiation is a potentially important step in the further development of this fungus as a biocontrol agent. Several

Table 2. Blastospore yield in YPD broth, conidial yield on high osmolarity medium (0.8 M NaCl/PDA) and virulence of $\Delta cag8$ strains (D1–D5 and D7), the wild-type strain and a transformant with pPKGFP-*cag8*-5:bar:*cag8*-3 integrated ectopically (Bar)

Strain	Blastospores (ml ⁻¹)*	Conidial yield (mm ⁻²)†	LT ₅₀ (days) (95 % confidence limits)‡	Mortality (%)§
Wild-type	$5.15 \times 10^7 \pm 0.4 \times 10^7$	$2.21 \times 10^5 \pm 1.9 \times 10^5$	4.22 (3.64–4.89)	80.0 $\pm$ 3.2
Bar	$5.24 \times 10^7 \pm 0.3 \times 10^7$	$2.16 \times 10^5 \pm 2.7 \times 10^5$	3.91 (3.62–4.24)	83.3 $\pm$ 1.2
D1	$2.18 \times 10^6 \pm 0.2 \times 10^6$	$1.45 \times 10^4 \pm 0.3 \times 10^4$	4.11 (3.79–4.45)	26.2 $\pm$ 3.3
D2	$2.82 \times 10^6 \pm 0.6 \times 10^6$	$1.63 \times 10^4 \pm 0.6 \times 10^4$	4.28 (3.83–4.71)	22.2 $\pm$ 1.9
D3	$2.56 \times 10^6 \pm 0.5 \times 10^6$	$1.10 \times 10^4 \pm 0.1 \times 10^4$	4.44 (4.03–4.85)	26.7 $\pm$ 2.7
D4	$2.37 \times 10^6 \pm 0.7 \times 10^6$	$1.35 \times 10^4 \pm 0.2 \times 10^4$	4.01 (3.64–4.42)	27.2 $\pm$ 1.1
D5	$2.77 \times 10^6 \pm 0.9 \times 10^6$	$1.36 \times 10^4 \pm 0.3 \times 10^4$	3.92 (3.59–4.47)	21.7 $\pm$ 4.2
D7	$2.25 \times 10^6 \pm 0.4 \times 10^6$	$1.33 \times 10^4 \pm 0.5 \times 10^4$	4.33 (3.96–4.74)	24.1 $\pm$ 3.6

*Conidia of wild-type and Bar as well as mycelia of six $\Delta cag8$ strains were inoculated into YPD broth and incubated at 27 °C for 4 days. The culture of each strain was filtered through glass wool to harvest blastospores. The blastospores were then inoculated into 100 ml YPD broth at a final concentration of 5×10^4 blastospores ml⁻¹. After 5 days of culture, the concentration of newly produced blastospores was recorded. The experiment was repeated three times with three replicates for each strain.

†One hundred microlitres of blastospore suspension (10^6 blastospores ml⁻¹) from above was spread onto PDA with 0.8 M NaCl. After 20 days, the conidial yield of each strain was recorded. The experiment was repeated three times with three replicates for each strain.

‡Bioassay was conducted by injecting 3 μ l blastospore suspension (10^4 blastospores ml⁻¹) into insects.

§Bioassay was conducted by immersion of insects in blastospore suspension (3×10^6 blastospores ml⁻¹). Mortalities in $\Delta cag8$ strains were low and LT₅₀ could not be calculated, thus the mortality after 15 days is shown.

Fig. 4. Transcript levels of the hydrophobin-encoding gene *ssgA* in a transformant with *cag8-5:bar:cag8-3* integrated ectopically (Bar) and two $\Delta cag8$ strains (D4 and D7), relative to the level in the wild-type (=1.0). Standard errors are shown. Similar results were found for the other $\Delta cag8$ strains.

genetic manipulations have previously been performed to improve the efficacy of *M. anisopliae* (St Leger *et al.*, 1996). However, there are potential negative ecological implications in the dissemination of genetically modified fungal conidia from insect cadavers (Hu & St Leger, 2002). The information generated in this study allows for the control of conidiation and the restriction in the dissemination potential of a genetically modified biocontrol fungus.

Fig. 5. Complementation of *A. nidulans* $\Delta flbA$ with the RGS protein gene *cag8* from *M. anisopliae*. Colony phenotype and conidiation of the transformant, *A. nidulans* $\Delta flbA$ and the wild-type strain. After 14 days growth on complete medium agar supplemented with methionine and pyridoxine the colony phenotype of the wild-type (a), one transformant (b) and *A. nidulans* $\Delta flbA$ (c) are shown. After 7 days of culture, conidiation of the wild-type (d), the transformant (e) and *A. nidulans* $\Delta flbA$ (f) were visualized microscopically. Four other transformants showed similar results.

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