A fungal homologue of neuronal calcium sensor-1, Bbcsa1, regulates extracellular acidification and contributes to virulence in the entomopathogenic fungus Beauveria bassiana

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Neuronal calcium sensor proteins and their homologues participate in transducing extracellular signals that affect intracellular Ca\(^{2+}\) levels, which in turn regulate enzyme activities, secretion, gene expression and other biological processes. The filamentous fungus Beauveria bassiana is a broad-host-range pathogen of insects that acidifies the extracellular milieu during growth and pathogenesis towards target hosts. A collection of B. bassiana random insertion mutants were screened on pH indicator plates and one mutant was isolated that displayed reduced acidification. The random insertion site was mapped to a gene that displayed homology to the neuronal calcium sensor/frequenin protein family and was designated Bbcsa1. To validate the role of Bbcsa1 in B. bassiana, a targeted gene-knockout was constructed. Data confirmed that Bbcsa1 was not an essential gene and the ΔBbcsa1 strain displayed delayed acidification of the medium when grown in Czapek–Dox medium, as compared with the wild-type parent. HPLC profiling of secreted metabolites did not detect any major changes in the production of organic acids, although downregulation of the membrane H\(^{+}\) pump/ATPase was noted in the mutant. A slight growth-deficient phenotype was observed for the ΔBbcsa1 strain on Czapek–Dox and potato dextrose media, which was accentuated at high calcium concentrations (500 mM) and 1.5 M sorbitol, but was unaffected by EDTA or SDS. Perturbations in vacuole morphology were also noted for the mutant. Insect bioassays using Galleria mellonella as the target host revealed decreased virulence in the ΔBbcsa1 mutant when applied topically, representing the natural route of infection, but no significant effect was observed when fungal cells were directly injected into target hosts. These results suggest that Bbcsa1 participates in pre-penetration or early penetration events, but is dispensable once the insect cuticle has been breached.

INTRODUCTION

Intracellular Ca\(^{2+}\) levels affect many signal transduction cascades that transduce stimuli to appropriate cellular responses. Cellular Ca\(^{2+}\) levels are carefully controlled by a diverse set of Ca\(^{2+}\) channels, pumps, transporters and calcium-binding proteins (Cunningham, 2011). Members of the last-named group are often divided into two classes: calcium buffers and calcium sensors. Neuronal calcium sensor-1 (NCS-1)/frequenin (the yeast homologue) represents a subfamily of the calcium sensors that contain three functional calcium-binding EF-hand motifs and show high-affinity calcium binding at concentrations below that which would activate other calcium transducers such as calmodulin (Burgoyne, 2004). Upon calcium binding, these proteins are thought to undergo a conformational change allowing for interactions with target proteins and/or receptors. Many NCS proteins are N-terminally myristoylated, with the solvent accessibility of this lipid moiety dependent upon Ca\(^{2+}\) binding. This ‘switch’ is considered to act as a reversible mechanism for association of NCS proteins with membranes and their components, although in some systems (e.g. yeast) the myristoyl tail is permanently extruded, resulting in the constitutive

Abbreviations: CDCFDA, 5,6-carboxy-2',7'-dichlorofluorescein; EST, expressed sequence tag; NCS-1, neuronal calcium sensor-1; PIK1, phosphatidylinositol-4-OH kinase.

The GenBank/EMBL/DDBJ accession numbers for the B. bassiana Bbcsa1 genomic and EST DNA are JQ317159 and DT368258, respectively.

One supplementary table and three supplementary figures are available with the online version of this paper.
association of yeast NCS-1 with membranes (O’Callaghan & Burgoyne, 2004). Myristoylation may also have a structural role, functioning as a hydrophobic centre stabilizing surrounding domains within the larger structure of a protein (Haynes & Burgoyne, 2008).

NCS-1 was originally described in Drosophila, where its involvement with neurotransmitter release was established (Pongs et al., 1993). Studies in Caenorhabditis elegans implicated the gene in affecting associative learning and memory and subsequent work has validated the importance of this protein in modulating synaptic transmission (Gomez et al., 2004). NCS-1 homologues, however, have been found in lower eukaryotes, implying a more general role of Ca2+ in modulating synaptic transmission. The secretion of acidic metabolites, an essential protein, where it regulates phosphatidylinositol-4-OH kinase (PIK1) activity, and is involved in membrane trafficking and secretion (Hendricks et al., 1999). In contrast with the Saccharomyces cerevisiae homologue, frequentin, appears to be an essential protein, where it regulates phosphatidylinositol-4-OH kinase (PIK1) activity, and is involved in membrane trafficking and secretion (Hendricks et al., 1999). In contrast with the Saccharomyces cerevisiae homologues in Aspergillus fumigatus (NcsA), Magnaporthe grisea (MgNCS-1) and Schizosaccharomyces pombe (ncs1) are not essential (Hamasaki-Katagiri et al., 2004; Mota Júnior et al., 2008; Saitoh et al., 2003). A. fumigatus ΔncsA mutants display decreased growth in the presence of EGTA and SDS, resistance to calcium chloride, but no loss of virulence in a murine model. In contrast, M. grisea ΔMg-NCS-1 mutants display decreased resistance to calcium chloride and were not affected by EGTA, although similar to A. fumigatus, the M. grisea mutant did not display any virulence defects. In Schizosaccharomyces pombe, the ncsA strain showed nutrition-insensitive sexual development and calcium-sensitivity phenotypes. Thus far, however, the role of Ca2+ signalling in Beauveria bassiana development and virulence has not been investigated and the contribution of Ca2+-binding proteins to these processes remains unexplored.

The entomopathogenic fungus B. bassiana is well known as a potential alternative to chemical pesticides for the control of insect pests. As a broad-host-range insect pathogen, strains of this fungus have been exploited for use against crop and invasive pests as well as for insects that act as human and animal disease vectors such as mosquitoes and ticks (de Faria & Wraith, 2007; Fan et al., 2012; Kirkland et al., 2004). Aside from its interest as a pest biological control agent, B. bassiana is also an emerging model organism that can be used to examine unique aspects of fungal growth and development, including host–pathogen interactions (Jin et al., 2010; Wanchoo et al., 2009). Infection of insects does not require any specialized mode of entry and begins with attachment of fungal spores to the target hosts. In response to cuticle surface cues, the fungus germinates, and the emerging germ tubes produce a variety of enzymes that, combined with mechanical pressure, begin the process of cuticle penetration. Similar to some plant pathogens, acidic secretion of host tissues during the necrotrophic interaction forms an important aspect of the disease progression. The secretion of acidic metabolites, oxalic acid in particular, appears to be a shared feature between plant-pathogenic fungi such as Sclerotinia sclerotiorum and B. bassiana (Bidochka & Khachatourians, 1991; Kirkland et al., 2005; Rollins & Dickman, 2001).

Here, we report the characterization of a frequentin/NCS-1 orthologue from B. bassiana, isolated during a screen for acidification-altered mutants from a random insertion library. The insertion site of one such acidification-altered mutant was mapped, and the gene was labelled as calcium sensor and acidification (csa1). Based upon the isolated sequence, a targeted gene-knockout strain was constructed and its phenotype was characterized. Bbcsa1 was not an essential gene and only slight growth and developmental defects were observed, but ablation of the gene reduced virulence in insect bioassays when the conidial spores were applied topically but no effect was seen when mutant conidia were directly injected into the host haemocoel. Analysis of the secreted metabolites did not reveal any major differences between the wild-type and ΔBbcsa1 strains; however, downregulation of the plasma membrane H+ pump/ATPase and perturbations in vacuole morphology were noted in the mutant strain.

METHODS

Cultivation of fungi. B. bassiana (ATCC 90517) was routinely grown on potato dextrose agar (PDA) or Czapek–Dox plates. Plates were incubated at 26 °C for 10–14 days and aerial conidia were harvested by flooding the plate with sterile distilled H2O. Conidial suspensions were filtered through a single layer of Miracloth and final spore concentrations were determined by direct counting using a haemocytometer. Blastospores were produced in Sabouraud dextrose supplemented with 0.5–1 % yeast extract liquid broth cultures (SDY) using conidia harvested from plates to a final concentration of 5–8 × 106 conidia ml⁻¹ as the inoculum. Cultures were grown for 3–4 days at 26 °C with aeration. Cultures were filtered (twice) through glass wool to remove mycelia, and the concentration of blastospores was determined by direct count. In vivo haemolymph-derived hyphal bodies were isolated as described by Lewis et al. (2009). Briefly, the tobacco hornworm, Manduca sexta, was used as the host to isolate the hyphal bodies. Third- to fifth-instar larvae were injected (5–8 × 106 cells per larva) with aerial conidia and incubated for 68–72 h at 26 °C. Individual larvae were then injected with 0.5 ml anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 26 mM citric acid, 30 mM trisodium citrate, 10 mM EDTA, pH 4.6) and bled. In vivo fungal cells were separated from insect haemocytes by using a 25–50 % step Centricoll gradient as a pellet at the bottom of the tube. Pellets were washed free of the gradient material into the desired buffer and used immediately.

Screening of random insertion mutants and transformant gene tagging. Construction of the B. bassiana random insertion library has already been reported (Fan et al., 2011). Transformants from Czapek–Dox plates were cultured in microtitre plates and used as inocula for phenotypic screening of acidification defects using Czapek–Dox plates containing 0.01 % bromoresol purple and adjusted to pH 7.0. Colonies displaying reduced acidification phenotypes after 7–10 days of growth were rescreened twice using the same conditions to verify the reproducibility and stability of the phenotype. Mapping of the integration site of the mutants derived from the acidification screen was performed as described previously (Fan et al., 2011). The PCR products generated from the mapping were cloned in the T/A pDrive cloning vector and sequenced as described above.
cloning vector (Qiagen) and the insert was sequenced using primers to the vector (US-ICBR DNA sequencing CORE).

Nucleic acid manipulations and construction of Bbcsa1 strains. A list of the primers used in this study is given in Table S1 (available with the online version of this paper). Approximately 714 bp of upstream (LB) and 744 bp of downstream (RB) nucleotide sequence for Bbcsa1 was obtained by PCR using primer pairs PcsaL1/ L2 and PcsaR1/R2. Primers PbcR1 and PbcR2 were used to amplify the phosphinothricin resistance marker (bar gene cassette). PcsaL2 and PcsaR1 were chimeric primers; PcsaL2 and PcsaR1 contained bar sequences at their 5’-ends and Bbcsa1 sequences at their 3’-ends. PCR product fragments, LB, RB and bar, were mixed together in a PCR primer-less assembly reaction with the following cycling conditions: 98 °C (2 min), followed by 22 cycles of 98 °C (15 s), 55 °C (30 s) and 72 °C (1.5 min). An aliquot (1 µl) of the reaction mixture was then used as the template to amplify the full-length construct (LB-bar-RB) with primers L1 and R2. Phusion DNA polymerase (New England Biolabs) was used in all PCRs related to construction of the knockout vector. The PCR fragment (LB-bar-RB) was cloned into the pDrive vector (Invitrogen) resulting in plasmid pDrive-LB-bar-RB. Plasmid pDrive-LB-bar-RB was linearized using HindIII and transformed into B. bassiana for targeted disruption of the Bbcsa1 gene. Preparation of competent cells, transformation and screening of recombinant clones were performed as described previously (Fan et al., 2011). The transformation mixture (0.25–0.5 ml) was plated onto Czapek–Dox medium containing 200 µg phosphinothricin ml⁻¹ and 0.01 % bromocresol purple, pH 6.3, in 150 mm diameter Petri dishes overlaid with a sheet of sterilized cellophane. Genomic DNA was isolated as described by Liu et al. (2000). PCR analysis was performed using primers PcsaT1 and PcsaT2 to the 5’ and 3’ flanking regions of the Bbcsa1 gene using the following protocol: 95 °C (3 min), followed by 35 cycles of denaturation at 95 °C (30 s), annealing at 58 °C (30 s) and extension at 72 °C (40 s), Southern blotting was performed with 20 µg genomic DNA digested with HindIII. The digested DNA was separated on a 1.0 % agarose gel and subsequently transferred to a Biodyne B nylon membrane (Gelman Laboratory) using standard protocols. Blots were probed with a 480 bp PCR-amplified product corresponding to nucleotides upstream of LB, generated using the primer pair PcsaL1 and PcsaS. Probe preparation, membrane hybridization and visualization were performed using a DIG High Prime DNA Labelling and Detection Starter kit I (Roche). The complementation vector was constructed by amplifying the entire Bbcsa1 gene including 1987 bp of upstream sequence using primers Pcsa-cl and Pcsa-c2. The PCR fragment was cloned into the pDrive vector yielding p-D-Bbcsa1, and the integrity of the insert was verified by sequencing. The Bbcsa1 gene was then subcloned from p-D-Bbcsa1 into pCB1536 containing the sulfonylurea resistance marker for selection in B. bassiana using BamHI and XbaI restriction sites. The resultant construct, pSUR-Bbcsa1, was linearized using Xhol and transformed into the ΔBbcsa1 strain as described above.

Growth and phenotype assays. Conidia (0.5 µl, 10⁷ ml⁻¹) were inoculated onto PDA plates supplemented with KCl (1.2 M), CaCl₂ (10–500 mM), Congo Red (100 mg ml⁻¹), sorbitol (1.5 M) or SDS (0.01 %), and growth was observed daily over 3–10 days. Growth and acidification in agar media was examined on Czapek–Dox agar containing 0–100 mM CaCl₂, with or without the pH indicator bromocresol purple (0.01 %). For liquid cultures, 100 µl of a suspension of 10⁸ conidia ml⁻¹ was inoculated into 100 ml Czapek–Dox broth and the culture was grown at 26 °C with aeration (120 r.p.m.) over 15 days. Culture aliquots (1–2 ml) were removed daily and the cells were removed by centrifugation (10 000 g, 10 min). The pH of the resulting supernatant was measured before analysis for carbohydrates and organic acids by HPLC (HP Series II 1100; Hewlett Packard/Agilent) using an AmideX HPX-87H column (Bio-Rad) run isocratically in 4 mM H₂SO₄ and coupled to both UV and refractive index detectors. All experiments were performed three times.

Gene expression analyses. Total RNA was isolated using the Aurum Total RNA Mini kit (Bio-Rad) and first-strand cDNA was synthesized using the Affinityscript multiple temperature cDNA synthesis kit (Stratagene). Expression of Bbcsa1 and Bbpmp (plasma membrane pump, H⁻/ATPase, accession no. DT368427.1) was investigated during growth under various conditions. These included a time course of growth on SDY agar (3–15 days), in SDY broth (4 days), in Czapek–Dox broth (7–10 days) and in PD broth (PDB, 4 days). RT-PCR analyses were performed using expression levels of Bbgpd (DT378065) and/or Bb-β-tubulin (DT370071) as references. Primer sequences are given in Table S1. RT-PCR analyses were performed as described by Cho et al. (2007). Each PCR was performed at least in duplicate with duplicate biological samples.

Fluorescence microscopy. Fungal cells, either germinated conidia or haemolymph-isolated hyphal bodies, were harvested by centrifugation (6000 g, 5 min, 4 °C) and resuspended in 50 mM sodium phosphate buffer, pH 7.3. The vacuolar dye 5,6-carboxy-2’,7’-dichlorofluorescein (CDCFDA) was added to a final concentration of 3 µM and the cells were allowed to stand at room temperature for 15 min prior to visualization. Mounted slides were observed using a PASCAL LM55 confocal microscope fitted with Nomarski differential interference contrast optics and an LP505 filter (Ex480/Em515).

Insect bioassays. Fungal virulence bioassays were performed using greater wax moth (Galleria mellonella) larvae (Pet Solutions). Aerial conidia were harvested from PDA plates directly in sterile distilled H₂O + 0.02 % Tween 80, counted using a haemocytometer, and the spore concentration was adjusted to 10⁷ cells ml⁻¹. Two different infection protocols were used. (1) Insects were treated topically by immersion in the spore suspension for 3–5 s and the excess liquid on the insect body was removed with a dry paper towel; control larvae were treated with sterile H₂O. (2) Larvae were injected with 8 µl of 10⁷ fungal conidia ml⁻¹ into the haemocoeal cavity; controls were injected with 5 µl sterile H₂O + 0.02 % Tween 80. Experimental and control larvae were placed in plastic chambers or large (150 mm) Petri dishes and incubated at 26 °C. The number of dead insects was recorded daily. For each experimental condition, 20–30 larvae were used, and all experiments were repeated three times. Data were analysed by PROC MIXED in SAS using a linear mixed model. The least significant difference test was conducted for comparisons between treatments.

RESULTS

Screening of random insertion mutants, sequence analysis and generation of Bbcsa1 disruption mutants

A collection of random insertion transformants was screened for loss of acid metabolite production using a pH indicator plate assay. Approximately 4000 clones were screened on Czapek–Dox agar plates adjusted to pH 7.0 and containing the pH indicator bromocresol purple (0.01 %). Five clones displaying decreased acidification were isolated from the initial screen, of which three appeared to display a stable phenotype upon rescreening (on at least two additional occasions). The insertion site of one such mutant was mapped, yielding 433 bp of B. bassiana genomic sequence flanking the gene marker insertion site. Additional nucleotide sequence for the
mapped clone was identified in a previously described B. bassiana EST library dataset (Cho et al., 2006a, b) and the full-length genomic sequence including 5' and 3' flanking sequences was obtained from the B. bassiana genome sequencing effort (kindly provided by S. H. Ying and M. G. Feng, unpublished data). Analysis of the open reading frame (ORF) indicated a 1103 bp long gene with a 485 bp intron(s) encoding a protein of 190 aa (molecular mass, 22 kDa; pl, 4.2). The predicted protein displayed high homology to neuronal calcium sensor proteins. NCBI BLAST and phylogenetic analyses revealed the best matches to known proteins to be Cordyceps militaris (99% identity) and Verticillium albo-atrum (96% identity), with 60% identity to the Saccharomyces cerevisiae frequenin gene (Fig. S1). The predicted B. bassiana protein contained four putative EF-hand calcium-binding domains. As seen in Schizosaccharomyces pombe and other NCS proteins, the first EF-hand (EF-1) contains substitutions (Lys51, Cys53 and Pro54) that are expected to disrupt the binding of Ca^{2+} to this site (Hamasaki-Katagiri et al., 2004). The remaining three EF-hand loops contained a subset of putative 12 Ca^{2+}-binding sites (Fig. S1). NCS family members contain consensus motifs for N-myristoylation (Ames et al., 1997). Intriguingly, although the Gly^{2} myristoylation site could be identified, a 15 aa insertion at the N terminus (aa 3–17, see Fig. S1) disrupted identification of the consensus N-myristoylation site (in silico removal of this sequence resulted in positive identification of a putative N-myristoylation site using Myristoylator; ExPASy Tools). Both genomic and cDNA sequencing of the B. bassiana gene confirmed the presence of the additional 45 nt that would result in the added 15 aa to the N terminus of the protein. This sequence appeared to be unique and none of the other orthologues used in the construction of the phylogenetic tree (Fig. S1) contained this sequence. Due to the predicted calcium binding and screen used to isolate the mutant, the gene was designated Bbcsa1 (B. bassiana calcium sensor and acidification regulator).

To verify the role of Bbcsa1 and further characterize its phenotype, a targeted insertion mutation was constructed based upon the identified sequence. Gene disruption via homologous recombination was achieved using a 600 bp region of the Bbcsa1 coding sequence replaced by the (942 bp) bar gene cassette conferring resistance to phosphinothricin (Fig. S2). Approximately 150 transformants were initially screened for the correct integration event by PCR, of which five appeared to represent targeted disruptions of Bbcsa1, with the rest representing ectopic integration events. Southern blotting and RT-PCR using primers designed for Bbcsa1 were used to verify the integrity of the targeted gene deletion clones. These data showed genomic disruption and loss of Bbcsa1 transcripts, respectively (Fig. S2). Complementation vectors were constructed using 3.2 kb of genomic sequence (1.1 kb ORF plus 1.9 and 0.2 kb of upstream and downstream flanking sequences, respectively). The complemented strain using the entire Bbcsa1 ORF, designated strain Bbcsa1::Bbcsa1, was indistinguishable from the wild-type strain in all phenotypic aspects examined. A C-terminal green fluorescent protein (Bbcsa1-gfp) fusion vector was also constructed; however, although transformation and stable integration of the plasmid in B. bassiana could be verified, no consistent GFP signal could be detected in the transformants under any of the growth conditions examined, including on/in Czapek–Dox media supplemented with the pH indicator dye bromocresol purple (0.01%). Plates were incubated for the indicated times (d, days) at 26 °C. A slight growth impairment on PDA was noted for the mutant (~15–17 days) was delayed by several days compared with the wild-type parent (~12–15 days). Delayed acidification of media during growth of ΔBbcsa1 on solid media. Wild-type (WT) and ΔBbcsa1 conidia were inoculated (2 µl of 10^7 conidia ml^-1) in the centre of plates containing Czapek–Dox agar supplemented with the indicated concentrations of CaCl₂. The bottom four rows show Czapek–Dox media supplemented with the pH indicator dye bromocresol purple (0.01%). Plates were incubated for the indicated times (d, days) at 26 °C.

Phenotypic characterization of the ΔBbcsa1 strain

A slight growth impairment on PDA was noted for the ΔBbcsa1 strain compared with the wild-type parent, and the mutant colony was distinctly yellow at 10 mM CaCl₂ whereas a similar phenotype was observed for the wild-type at 50 mM CaCl₂ (Fig. 1). Furthermore, conidiation of the mutant (~15–17 days) was delayed by several days compared with the wild-type parent (~12–15 days). Slight growth impairment was also noticeable in Czapek–Dox media, although supplementation of the media with a pH indicator dye (bromocresol purple) revealed a reduced acidification phenotype (consistent with the original screening
and isolation of the gene from the random insertion mutant library (Figs 1 and S3). Similarly, in Czapek–Dox liquid media, a delayed acidification phenotype was observed (Fig. 2). However, no significant delay in acidification of the media was noted when cells were grown in PD or Sabouraud dextrose broth (data not shown).

To determine whether secretion of any organic acids was impaired or altered in the mutant strain, aliquots from media was noted when cells were grown in PD or Sabouraud dextrose broth (data not shown).

Gene expression profile of **Bbcsa1**

Expression of **Bbcsa1** was investigated during growth under various conditions, including on SDY agar (3–15 days), in SDY broth (4 days) and in Czapek–Dox broth (10 days) (Fig. 4a). These data indicated that **Bbcsa1** was most highly expressed in maturing mycelia, starting at 7 days of growth on SDY agar and continuing until 15 days. Under these conditions, conidial production begins after ~7–10 days of growth. Much lower **Bbcsa1** expression was noted in liquid cultures, even in rich media (SDY, 4 days) (Fig. 4a). As we could find no obvious differences in metabolite production between the wild-type and Δ**Bbcsa1** mutant, expression of the plasma membrane proton pump (**Bbpmp**, **H**⁺-**ATPase**) was examined (Fig. 4b). These data showed decreased **Bbpmp** expression in Δ**Bbcsa1** cells when grown in Czapek–Dox broth as compared with the wild-type, but no significant differences in **Bbpmp** expression were noted when the cells were cultured in PDB.

**Vacuole morphology**

Because calcium-binding and -signalling proteins have been implicated in affecting vesicle and/or vacuole dynamics, a vacuole-specific fluorescent probe, CDCFDA, was used to examine vacuole morphology as described in Methods. After growth, i.e. conidial germination in PDB for 18–20 h, ~40% of Δ**Bbcsa1** cells contained one (often several) enlarged vacuole(s), whereas only ~19% of wild-type cells contained enlarged vacuoles (often just a single one) (Fig. 5). Furthermore, more vacuoles were noted in Δ**Bbcsa1** germ tubes and hyphae per cell than in the wild-type. Hyphal bodies are differentiated cells produced during the infection process that are capable of evading the host immune system, and can be isolated from insect haemolymph as described in Methods. In contrast with the germinated conidia, hyphal bodies derived from Δ**Bbcsa1** infection contained vacuoles that varied more in size and tended to be smaller (rather than larger as seen for Δ**Bbcsa1** in vitro germinated conidia) than those observed for wild-type hyphal bodies, with the latter being more uniform in vacuole size and appearance. Taken together, these results imply some form of impairment of normal vacuole morphology and development in Δ**Bbcsa1** cells.
Effect of \( \Delta Bbcsa1 \) on pathogenicity

The effect on virulence of \( Bbcsa1 \) was probed via insect bioassays using the \( G. mellonella \) larvae as the host and two different infection protocols. In the first type of assay, conidia of \( B. bassiana \) wild-type and the \( \Delta Bbcsa1 \) mutant were adjusted to \( 10^7 \) c.f.u. ml\(^{-1} \) and applied topically, which represents the natural route of infection, to the larvae. Mortality was determined over a 10-day period and the data show a decreased lethal time (LT\(_{50}\)) for the mutant compared with the wild-type strain (Fig. 6a). Mean (± SD) LT\(_{50}\) values of the wild-type, \( \Delta Bbcsa1 \) and \( \Delta Bbcsa1: Bbcsa1 \) were 6.5 ± 0.6, 9.5 ± 0.5 and 6.9 ± 0.5 days, respectively. These data indicate an ~25 % decrease in the virulence of the mutant strain when applied topically. In the second protocol, fungal conidia were injected directly into the host haemocoel, thus bypassing cuticle penetration. In this latter assay, no significant differences in the calculated LT\(_{50}\) values were noted between the strains, which for the wild-type, \( \Delta Bbcsa1 \) and \( \Delta Bbcsa1: Bbcsa1 \) were 3.8 ± 0.1, 3.3 ± 0.2 and 3.3 ± 0.3 days, respectively (Fig. 6b). No significant differences in sporulation were noted between the mutant and wild-type when grown in vitro or on the cadavers of infected insects.

DISCUSSION

Calcium-binding and sensor proteins regulate numerous cell signalling events, presumably via interactions with Ca\(^{2+}\) pools that can be transient and variable in their subcellular distribution. Here, we have characterized an orthologue of the neuronal calcium sensor (\( ncs1 \)) in the entomopathogenic fungus \( B. bassiana \) termed \( Bbcsa1 \). Higher eukaryotes contain a diversity of \( ncs \) genes, with mammalian genomes coding for 14 members of this family...
Fig. 6. Insect bioassays. G. mellonella larvae were treated either topically (a) or via intra-haemocoel injection (b) with conidia from wild-type B. bassiana (○), ΔBbcsa1 (●), ΔBbcsa1:ΔBbcsa1 (⊙) or mock-treated controls (∆), as described in Methods. The percentage survival ± SD over the indicated time course is presented.

(Burgoyn, 2004). As their name implies, NCS proteins are thought to function in neurotransmitter release, vesicle trafficking, and channel and receptor regulation (Amici et al., 2009). Specialized functions for specific NCS proteins have also been observed. The NCS protein recoverin, for example, is expressed only in the retina where it regulates phototransduction (Burgoyn, 2004). In Saccharomyces cerevisiae the essential NCS-1 homologue (FRQ1) regulates PIK1 and overexpression of PIK1 can rescue an FRQ1 temperature-sensitive allele (Hendricks et al., 1999). Intriguingly, however, no consistent phenotype has been observed for gene disruption mutants in fungi. In Schizosaccharomyces pombe, deletion of ncs1 resulted in nutrition-insensitive sexual development and a growth defect at high extracellular Ca²⁺ (0.1 M CaCl₂) (Hamasaki-Katagiri et al., 2004). As similar sexual development does not occur in the filamentous fungi examined thus far, i.e. Aspergillus, Magnaporthe and Beauveria, this phenotype cannot be examined. However, ncsA mutants in A. fumigatus were sensitive to EGTA and SDS but displayed increased resistance to exogenous CaCl₂ concentrations (Mota Júnior et al., 2008). Null ncs-1 mutants in M. grisea displayed normal growth and pathogenicity, but growth was impaired at high Ca²⁺ concentrations as well as at pH 3.5 (Saitoh et al., 2003).

For B. bassiana, conidial spores bind to the surface of the insect, where they germinate and begin the process of penetrating the insect cuticle (Holder & Keyhani, 2005; Holder et al., 2007). When the penetrant hyphae reach the haemocoel, they undergo a dimorphic transition, elaborating free-floating cells termed hyphal bodies (Pendland et al., 1993). Acidification of host tissues is known to contribute to virulence in plant-pathogenic fungi as well as in B. bassiana where the production of oxalic acid correlated with virulence (Kirkland et al., 2005). The B. bassiana ncs-1 orthologue (Bbcsa1, calcium sensor and acidification) was isolated during a screen for acidification-altered (decreased) mutants. Similar to other NCS proteins, analysis of the B. bassiana gene and its translated product protein identified three putatively functional EF-hand motifs that would mediate Ca²⁺ binding and a fourth motif that has apparently degenerated. A unique aspect of the B. bassiana protein, however, appears to be the potential disruption of the N-terminal myristoylation site via a 15 aa insertion (aa 3–17) not found in any other NCS protein examined. Thus, although a putative Gly² myristoyl-linkage site remains, an N-terminal myristoylation site could not be identified in silico unless these amino acids were deleted. Whether the B. bassiana protein contains an N-terminal myristoyl group remains to be determined.

The reconstructed targeted knockout mutant strain of Bbcsa1 and its complement were used to verify the delayed acidification phenotype in Czapek–Dox medium, the initial medium used for screening and which represents a minimal medium with sucrose as the carbon source. However, the delayed acidification phenotype was not observed in rich media, i.e. PDB or Sabouraud dextrose broth. These data imply that at least some of the activities mediated by Bbcsa1 may be linked to nutrient availability or metabolic status. Profiling of the secreted metabolites formed in Czapek–Dox media failed to detect any significant changes in the concentration of the solutes examined, including the major organic acids produced by B. bassiana (e.g. citrate, formate and oxalate), that could account for the delayed acidification phenotype. Our data indicate that one target of Bbcsa1 (whether direct or indirect) may be the plasma membrane ATPase. Only one such gene was identified in a draft of the B. bassiana genome, although a full examination of any such protein(s) awaits publication of the complete B. bassiana genome. Decreased expression of the plasma membrane ATPase was noted in the ΔBbcsa1 strain when grown in Czapek–Dox broth but not when grown in PDB, thus correlating with the delayed acidification. Whether this decrease is sufficient to account for the full phenotype remains unclear and it is likely that other processes are also affected.

Other phenotypic features of the ΔBbcsa1 strain were similar in some respects, but also distinct from those...
reported in other fungal organisms. Bbcsa1 was not essential, and disruption of Bbcsa1 resulted in a slight growth defect at very high CaCl2 concentrations (0.5 M), where the wild-type strain remained vigorous in growth. As previously mentioned, strikingly different results have been reported in A. fumigatus and M. grisea. Thus, when examined overall – essentiality only in Saccharomyces cerevisiae, resistance to CaCl2 in A. fumigatus, sensitivity to CaCl2 in M. grisea and only a slight CaCl2 effect in B. bassiana – it would appear that widely divergent phenotypic effects are possible regarding this gene and any extrapolation to different organisms should be made with caution. Slight sorbitol sensitivity was noted for B. bassiana ΔBbcsa1, but in contrast with A. fumigatus (Δncs1) no effects were seen with either SDS or EGTA. EGTA also had no effect on the Mg-NCS-1 mutant.

As calcium signalling and/or binding proteins have been implicated in vesicle trafficking and/or vacuole dynamics, the morphology of vacuoles in germinated conidia and in host haemocoel-produced hyphal bodies was examined using the fluorescent dye CDCFDA. Only subtle perturbations in vacuole morphology were noted. Approximately 20% more of the examined B. bassiana ΔBbcsa1-germinated conidia contained at least one large vacuole compared with the parental strain. Furthermore, the mutant-germinated conidia appeared to contain more vacuoles than the parent strain. In contrast, the vacuoles observed in the ΔBbcsa1 hyphal bodies varied more in size, and were smaller than those observed for the parent strain, which were relatively uniform in size and shape. The exact implications of these observations are unclear, although they may be related to vesicle trafficking and/or the (delayed) acidification phenotype. Further experiments exploring these phenomena, particularly as they may relate to transient calcium fluxes, are warranted.

The ncs-null mutants were not impaired in virulence in either A. fumigatus or M. grisea, the former tested in a low-dose murine infection model and the latter in plant pathogenicity assays (presumably against rice, although this is not explicitly stated in the referenced work) (Mota Júnior et al., 2008; Saitoh et al., 2003). In contrast, the ΔBbcsa1 strain displayed decreased virulence when applied topically in insect bioassays, but showed no defect in virulence when directly injected into the insect haemolymph. These results suggest that Bbcsa1 contributes to pre-penetration and/or penetration events. Once the insect cuticle has been breached, however, the contribution of csal to the pathogenic process appears to be minimal. Interestingly, the insect epicuticle is considered to represent a nutrient-poor substratum, whereas the insect haemolymph is nutrient-rich, thus potentially correlating with the phenotypes seen in the minimal Czapek–Dox media as compared with the richer PBD. The lack of any reduction in virulence upon intrahaemocoel injection further suggests that csal is dispensable for any host–immune interactions that may occur during growth in the haemolymph.

The essentiality of NCS-1 in Saccharomyces cerevisiae, but not in the other fungi examined to date (Schizosaccharomyces pombe, A. fumigatus, M. grisea and now B. bassiana, representing a non-pathogen, a mammalian pathogen, a plant pathogen and an insect pathogen, respectively), supports the idea that other proteins in addition to NCS-1 fulfil the role(s) of this protein in all the fungi examined with the exception of yeast. Indeed, genomic analyses of A. fumigatus and M. grisea revealed a wide array of genes encoding EF-hand proteins that may act as calcium binders or sensors (Mota Júnior et al., 2008; Zelter et al., 2004). Although not yet available, the B. bassiana genome is due to be published soon (expected 2012–2013), and it is likely that additional candidate genes and their protein products involved in calcium sensing will be identified. Future work aimed at identifying additional targets of Bbcsa1 is likely to shed light on the mechanism(s) by which this gene affects the regulation of acidification processes and/or virulence.

REFERENCES


Role of Bbcsa1 in B. bassiana


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