

Tenebrionid secretions and a fungal benzoquinone oxidoreductase form competing components of an arms race between a host and pathogen

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Entomopathogenic fungi and their insect hosts represent a model system for examining invertebrate-pathogen coevolutionary selection processes. Here we report the characterization of competing components of an arms race consisting of insect protective antimicrobial compounds and evolving fungal mechanisms of detoxification. The insect pathogenic fungus *Beauveria bassiana* has a remarkably wide host range; however, some insects are resistant to fungal infection. Among resistant insects is the tenebrionid beetle *Tribolium castaneum* that produces benzoquinone-containing defensive secretions. Reduced fungal germination and growth was seen in media containing *T. castaneum* dichloromethane extracts or synthetic benzoquinone. In response to benzoquinone exposure, the fungus expresses a 1,4-benzoquinone oxidoreductase, *BbbqrA*, induced >40-fold. Gene knockout mutants ($\Delta BbbqrA$) showed increased growth inhibition, whereas *B. bassiana* overexpressing *BbbqrA* (*Bb::BbbqrA*^O) displayed increased resistance to benzoquinone compared with wild type. Increased benzoquinone reductase activity was detected in wild-type cells exposed to benzoquinone and in the overexpression strain. Heterologous expression and purification of *BbBqrA* in *Escherichia coli* confirmed NAD(P)H-dependent benzoquinone reductase activity. The $\Delta BbbqrA$ strain showed decreased virulence toward *T. castaneum*, whereas overexpression of *BbbqrA* increased mortality versus *T. castaneum*. No change in virulence was seen for the $\Delta BbbqrA$ or *Bb::BbbqrA*^O strains when tested against the greater wax moth *Galleria mellonella* or the beetle *Sitophilus oryzae*, neither of which produce significant amounts of cuticular quinones. The observation that artificial overexpression of *BbbqrA* results in increased virulence only toward quinone-secreting insects implies the lack of strong selection or current failure of *B. bassiana* to counteradapt to this particular host defense throughout evolution.

entomopathogenic fungi | quinone reductase | insect cuticle | *Tribolium castaneum* | evolutionary arms race

Coevolution between hosts and their parasites is considered to be a major driving force of natural selection, and significant literature exists on theoretical and ecological outcomes derived from such interactions. The Red Queen hypothesis, eponymously named after the character in Lewis Carroll's book *Through the Looking Glass*, who says to Alice, "...it takes all the running you can do, to keep in the same place..." serves as a concise metaphor for the selection pressures on hosts to constantly change defense mechanisms against pathogens and the counterselection on the pathogens for continuously developing means for overcoming such evolving defenses, resulting in the appearance of both organisms "running in place" (1). Variations of the Red Queen as well as alternative theories, most unified around the centrality of biotic interactions as a driving force, have been proposed as explanations for (i) taxon extinction [Van Valen's original Red Queen's hypothesis (2)]; (ii) the evolution

and maintenance of sex, genetic recombination, and immune systems (3–5); (iii) as a framework for understanding invasions of exotic species (6); and (iv) as mechanism(s) selecting for host-pathogen coevolution [i.e., broad versus specific host ranges and/or host tolerance (7–9)].

For fungal pathogens, the best-known examples of coevolutionary interactions are the pathogen effector and corresponding plant host target genes that have defined a gene-for-gene disease susceptibility model in which the outcome of infection is based on avirulence (*Avr*) genes in the pathogen and dominant resistance (*R*) genes in the host (10, 11). The fungal protein can trigger immunity due to *R* protein effector recognition, thus limiting or preventing disease. In these systems, certain *Avr* mutations or lack of the appropriate *R* gene product leads to infection. *Avr* systems, however, form only part of the landscape of interactions between plant fungal pathogens and their hosts. Invading microbes must overcome exterior defenses, e.g., the plant epicuticle and cell wall, and interior defenses, e.g., immune-related defenses that are both preformed and induced (11–13). Within a broader context, resistance to host antimicrobial compounds, host-induced oxidative and/or other stress, and host immune evasion can also be considered as points of interactions that can result in an evolutionary arms race. Despite the ubiquity of

Significance

Although entomopathogenic fungi and their invertebrate hosts share a >300 million year co-evolutionary history, little is known concerning the biochemical and genetic basis of insect defensive tactics and the countermeasures evolved and evolving by the pathogen to thwart these defenses. Our results provide a molecular mechanism to help explain why some insects are more resistant to broad host-range entomopathogenic fungi. We identify beetle cuticular secretions and a fungal detoxifying enzyme as components of an arms race between insects and the fungal pathogen, suggesting an evolving role for the quinone reductase enzyme as a specific virulence factor for host quinone detoxification. As races have winners and losers, this paper captures a snapshot where the host is leading the race.

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fungus–arthropod interactions, there are few examples in which the underlying molecular and biochemical mechanism(s) that define the competing aspects of a coevolutionary arms race have been described. Intriguingly, although effector-like sequences have been found in the genome of entomopathogenic fungi, gene-for-gene avirulence mechanisms have yet to be reported for insect pathogenic fungi (14).

Ecologically important as regulators of insect populations, and distributed throughout almost all ecosystems, entomopathogenic fungi are also of significant interest as potential biological control agents against different insect pests, in particular, as more environmentally friendly alternatives to chemical pesticides (15). Several isolates of the Ascomycetes *Beauveria bassiana* and *Metarhizium anisopliae* have been successfully used worldwide to control insects of both agricultural and medical importance (16–19). *B. bassiana* is known as a broad host range arthropod pathogen capable of infecting a wide range of target hosts. Infection occurs via attachment and penetration of the host cuticle, which represents the first and likely most important line of defense against microbial pathogens (20, 21). Mortality typically occurs 3–10 d postinfection, after which the fungus sporulates on the host cadaver. However, for *B. bassiana*, completion of its life cycle does not require parasitism of a host because the fungus is a facultative pathogen that can grow as a saprophyte as well as form intimate associations with plants (22, 23).

Despite its broad host range, an enduring mystery has been the observation that select insect species are recalcitrant to infection by *B. bassiana*, even though other closely related species are susceptible. Tenebrionid beetles including *Tribolium castaneum* (the red flour beetle) and *Ulomoides dermestoides* are two such resistant insect species. The former is of particular importance as a worldwide pest of stored products, resulting in significant agricultural losses per year that often disproportionately affect developing nations (24, 25). The latter, curiously enough, is consumed as an alternative medicine for the treatment of a range of illnesses including diabetes and cancer (26). The genome of *T. castaneum* is available, and it has become an important model system for insect development (27, 28). Both beetle species are known to produce quinone-containing cuticular secretions hypothesized to act as antimicrobial defense compounds (29, 30). These tenebrionids use prothoracic and abdominal glands to produce quinone-containing defensive secretions originally identified as displaying repellent and/or irritant against predators. Secreted quinone derivatives are also involved in cuticle tanning and sclerotization, and these compounds have been shown to possess potent antimicrobial activities (31–33). The chemical structures of the major components of these beetle secretions have been shown to consist of methyl-1,4-benzoquinone and ethyl-1,4-benzoquinone, together with the carrier alkene 1-pentadecene (34, 35).

Biodegradation of quinone derivatives by fungi is a well-known process in the lignin-degrading fungi, where quinones are the product of the oxidation of lignin-related aromatic compounds by ligninases (36). The pathway involves the induction of NAD(P)H:quinone reductases, which catalyze the conversion of cytotoxic benzoquinone to (nontoxic) hydroquinone. Several quinone reductases have been purified and characterized and their genes cloned in both white- and brown-rot fungi (basidiomycetes) as part of lignin degradation pathways (37–40). However, entomopathogenic fungi are incapable of lignin degradation, and no information is available regarding the presence and/or function of these enzymes in this group of fungi. However, entomopathogenic fungi are able to assimilate various hydrocarbon and lipid components of the insect cuticle, responding to surface cues on their hosts to initiate programs for infection (41–43).

In this work we demonstrate the antifungal properties of tenebrionid cuticular secretions and characterize a counteracting fungal NAD(P)H:1,4-benzoquinone oxidoreductase (BbBqrA) in *B. bassiana*. Gene expression and enzyme activity assays revealed induction of *BbbqrA* expression and increased BbBqrA activity in

the presence of exogenous benzoquinone. Heterologous expression and characterization of the BbbqrA enzyme confirmed NAD(P)H-dependent benzoquinone reductase activity. Fungi containing a targeted disruption of *BbbqrA* showed decreased ability to infect *T. castaneum*, but no changes in virulence when tested against nonquinone-producing beetles (*Sitophilus oryzae*, rice weevil) or the Lepidopteran host, the greater wax moth *Galleria mellonella*. Conversely, a *B. bassiana* fungal strain engineered to overexpress *BbbqrA* displayed increased virulence against *T. castaneum* with, again, no changes in virulence when tested against *G. mellonella* or *S. oryzae*. These results link *BbbqrA* to degradation of tenebrionid quinone-containing defensive secretions, identifying a host-specific virulence factor that is part of a coevolutionary arms race between a pathogen and its host. These data reveal a biochemical mechanism that can act as one factor to limit host range.

Results

Molecular Identification and Expression Analyses of a Benzoquinone Reductase in *B. bassiana*. A gene fragment displaying homology to benzoquinone reductases was identified in a *B. bassiana* expressed sequence tagged (EST) library (44, 45). The gene fragment was used to query the *B. bassiana* genome (14), leading to the identification of the full-length genomic sequence of *BbbqrA* (gene locus ID: XM_008596690). Analyses of the 5'-upstream region of the open reading frame (ORF) revealed the presence of TATA and CCAAT boxes defining a putative promoter beginning at positions –57 and –600 relative to the AUG start site, respectively. In addition to these two elements, an inverted GC box was found at nucleotide –308 and a stress response element (STRE) was found at position –288. A xenobiotic response element (XRE), also present in the promoters of a set of *B. bassiana* cytochrome P450 monooxygenases implicated in insect cuticle degradation (43), was identified at positions –235, –267, –326, –539, –599, –606, –616, –1,079, and –1,181 (Fig. S1). The region surrounding the start codon contained the eukaryotic consensus (G/A)NNATGG sequence, including a purine (A) in position –3, as has been reported for highly expressed genes in *Saccharomyces cerevisiae* (46) and has also been found in *B. bassiana* P450 genes (43). Variants of the tripartite signal [5'-TAG...TA(T/A)GT...TTT], indicating potential transcription termination, were found in the 3'-flanking region, although no consensus polyadenylation formation signal (AATAAA-3' or its common hexameric variants) was present in this region. The gene contained two introns of 17 and 67 bp; beginning at 24 and 446 bp from the start codon, respectively. The exon/intron boundaries conformed to the canonical GT/AG donor/acceptor rule. The deduced polypeptide sequence of BbbqrA contained 201 amino acids with an estimated molecular weight of 21,402 Da and an isoelectric point of 6.05. No potential glycosylation or signal peptide cleavage sites were found, and PSORT analysis suggested cytosolic localization of the protein. A phylogenetic tree was constructed using NADH:benzoquinone reductase and NADPH-dependent flavin mononucleotide (FMN) reductases found in yeasts and filamentous fungi. BLAST analysis showed a single gene encoding for a benzoquinone reductase in *B. bassiana* and other filamentous fungi and yeasts. A distantly related NADPH-dependent FMN reductase (gene locus ID XP_008596471) was identified in *B. bassiana*, which shares 30% similarity with BbbqrA. This gene appears to have true orthologs in other filamentous fungi and yeasts. Phylogenetic analyses of BqrA revealed two distinct clusters (Fig. S2), one composed mainly of yeast sequences and Basidiomycetes (e.g., *Phanerochaete chrysosporium* and *Gloeophyllum trabeum*) and a second cluster containing Ascomycetes sequences. *B. bassiana* BqrA subclustered together with proteins from *Cordyceps militaris* and *Tricoderma reesei*. The BqrA derived from *Metarhizium* species, e.g., *M. album*, *M. robertsii*, and *M. acridum*, fungi that are also entomopathogens, subclustered separately.

The presence of the TATA box and regulatory sequences suggested that *BbbqrA* was an inducible gene. To examine whether its expression was specifically induced by benzoquinone, *BbbqrA* transcript levels were measured by quantitative RT-PCR (qRT-PCR) in *B. bassiana* cultures exposed to benzoquinone using the expression of ribosomal 5.8S RNA as an internal control. *Bbbqr* gene expression levels showed a moderate dose-dependent increase in response to benzoquinone levels, rapidly increasing 20- and 40-fold above control levels in the presence of 9.25 and 18.5 μM benzoquinone (1 and 2 $\mu\text{g}/\mu\text{L}$), respectively, after 6 h of coinubation (Fig. 1). After this initial burst, *BbbqrA* levels decreased within 24–72 h to between three- and fivefold above controls. Little to no induction was detected when fungal cells were grown in either 4.6 μM benzoquinone (0.5 $\mu\text{g}/\mu\text{L}$) or in dichloromethane (solvent) compared with controls.

Generation of *BbbqrA* Deletion Mutant and Overexpression Strains.

To probe the function of *BbbqrA*, a targeted gene deletion strain was constructed. *BbbqrA* was disrupted via homologous recombination, leading to the insertion of the phosphinothricin resistance marker (*bar*) into the ORF of the target gene as detailed in *Materials and Methods*. A 303-bp fragment of the gene was deleted, and the insertion event occurred between -83 bp in the 5'-UTR region and 220 bp in the ORF (Fig. 2A). A *BbbqrA* overexpression strain designated as *Bb::BbbqrA^O* was constructed by cloning of the entire *BbbqrA* ORF from cDNA in front of the constitutive glyceroldehydrogenase 3-phosphate dehydrogenase promoter (*gpdA*) and subsequent transformation of the construct into the wild-type strain. The correct integration events for construction of the targeted gene knockout and overexpression strains were confirmed by PCR (Fig. S3). To confirm *BbbqrA* overexpression, 10 (PCR-verified) transformants were chosen, and *BbbqrA* expression was examined in potato dextrose agar (PDA) media by qRT-PCR. Clones varied in *BbbqrA* expression, with one clone displaying a more than eightfold increase in *BbbqrA* transcript levels compared with the wild-type parent (Fig. 2B and C). This strain was used in all subsequent studies reported below.

Tenebrionid Secretions Have Antifungal Properties. Pure benzoquinone and dichloromethane extracts of *T. castaneum* were used to examine the effects of these compounds on fungal growth of

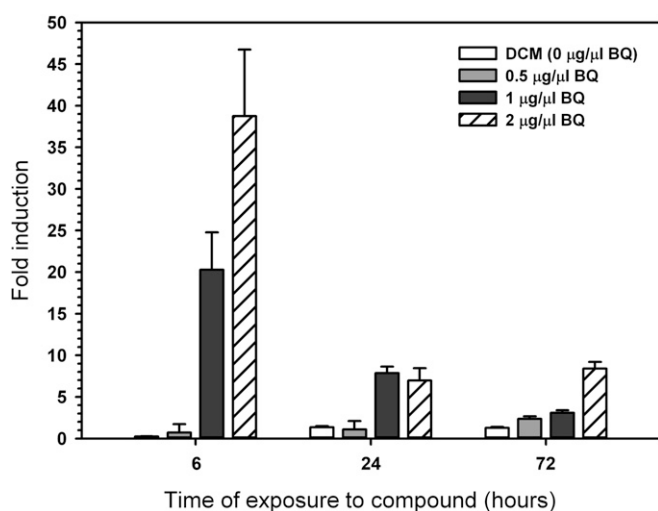


Fig. 1. *B. bassiana* *BbbqrA* gene expression analysis. A time course of *BbbqrA* expression levels was determined by qRT-PCR after growth in the presence (0.5, 1, and 2 $\mu\text{g}/\mu\text{L}$) and absence of benzoquinone (BQ). Expression was normalized to fungal growth on the same media in the absence of BQ. Values are means \pm SD. DCM, dichloromethane.

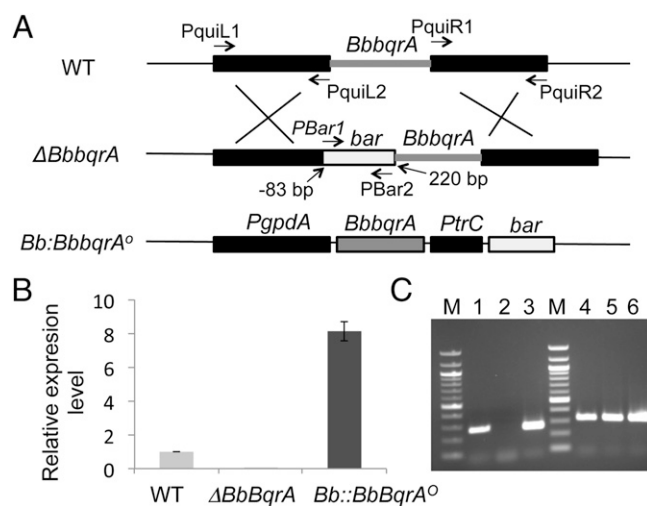


Fig. 2. Construction and verification of *B. bassiana* *BbbqrA* deletion and overexpression strains. (A) Schematic diagram of the $\text{LB}_{\text{BQR}}\text{-bar-RB}_{\text{BQR}}$ and pUC-bar-PgpdA:BQR constructs used to construct the $\Delta BbbqrA$ and the *Bb::BbbqrA^O* strains, respectively. (B) Relative transcript abundance levels in the $\Delta BbbqrA$ and *Bb::BbbqrA^O* strains normalized to the wild type as determined by qRT-PCR. The *actin* and/or *5.8S rRNA* gene products were used as internal reference genes. (C) Agarose gel electrophoresis of RT-PCR products of *BbbqrA* transcripts in wild-type (lanes 1 and 4) and mutant strains (lanes 2 and 5, deletion mutant, and lanes 3 and 6, overexpression strain, respectively). M, 10-kb ladder; lanes 1–3, *BbbqrA*-specific primers; lanes 4–6, *actin* primers. Primers used in the study are listed in Table S1.

B. bassiana wild-type, $\Delta BbbqrA$ (deletion mutant), and *Bb::BbbqrA^O* (overexpression) strains. Addition of benzoquinone or tenebrionid extracts resulted in concentration-dependent inhibition of wild-type *B. bassiana* growth compared with growth on control media (Fig. 3 and Table 1). Fungal growth media (Sabouraud dextrose agar) supplemented with insect extracts resulted in zones of growth inhibition, i.e., radii of 18.9 ± 1.2 mm for 0.3 insect equivalents/ μL and 24.1 ± 1.8 mm for 0.6 insect equivalents/ μL , respectively. Similar results were obtained with dichloromethane extracts derived from the tenebrionid beetle, *U. dermestoides* (Fig. S4). Zones of growth inhibition for wild type in the presence of benzoquinone were 7.6 ± 1.3 mm (37 μM or 4 $\mu\text{g}/\mu\text{L}$), 9.7 ± 1.5 mm (111 μM or 12 $\mu\text{g}/\mu\text{L}$), and 12.1 ± 2.3 mm (231.2 μM or 25 $\mu\text{g}/\mu\text{L}$), respectively (Table 1). Neither hydroquinone nor pentadecene (C15:1) inhibited fungal growth at similar concentrations. A significant decrease in growth in the presence of benzoquinone or *T. castaneum* extracts was seen for the $\Delta BbbqrA$ strain, and, conversely, greater resistance to these compounds was seen for the *Bb::BbbqrA^O* overexpression strain. The $\Delta BbbqrA$ strain showed a relative inhibition (R_i) ratio [(radii of inhibition of test strain)/(radii of inhibition of wild type)] between 1.3–1.7 ($P < 0.01$) for the range of benzoquinone concentrations tested and $R_i = 1.4$ ($P < 0.01$) versus the tenebrionid extracts. In contrast, the *Bb::BbbqrA^O* overexpression strain resulted in $R_i = 0.4$ –0.7 ($P < 0.01$) for benzoquinone and 0.7–0.8 ($P < 0.05$) for the insect extracts. Fungal germination was assayed in the presence of 9.25 μM benzoquinone (1 $\mu\text{g}/\mu\text{L}$) (Fig. 4). The presence of benzoquinone significantly delayed germination in the wild-type, $\Delta BbbqrA$, and *Bb::BbbqrA^O* strains. Germination rates were calculated after 12, 16, 24, and 48 h of exposure to benzoquinone. After 12 h of exposure to benzoquinone, germination was dramatically affected for the mutant ($\Delta BbbqrA$) and the wild-type strains, which exhibited germination rates equal to $1.7 \pm 0.2\%$ and $19.9 \pm 0.5\%$, respectively. Twelve hours of exposure to benzoquinone, however, did not impair germination in the overexpression strain (*Bb::BbbqrA^O*) to the same extent as in the knockout and wild-type strains

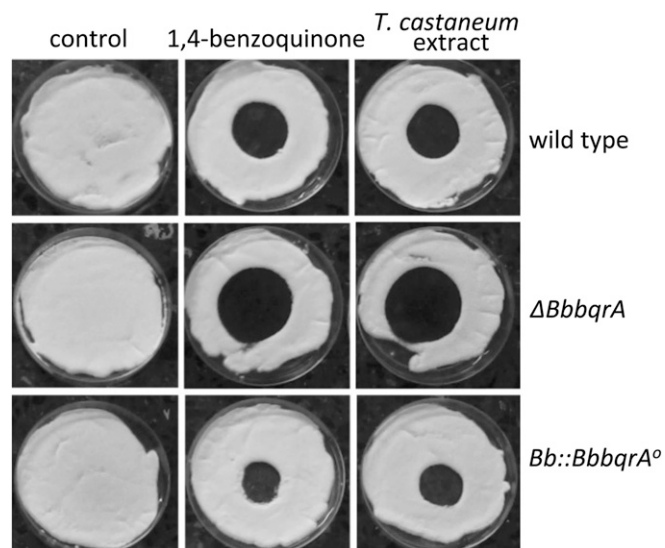


Fig. 3. Quinone and beetle cuticular extract inhibition of *B. bassiana* growth. Lawns of wild type, $\Delta Bbbqra$, and $Bb::Bbbqra^O$ were spotted with different concentrations of either pure benzoquinone or *T. castaneum* cuticular extracts. Zones of growth inhibition were quantified as described in *Materials and Methods* and *Results*.

(germination rate $46.4 \pm 4.0\%$). At 16 h, the respective germination rates for wild-type, $\Delta Bbbqra$, and $Bb::Bbbqra^O$ strains were $74.5 \pm 0.5\%$, $31.5 \pm 3.8\%$, and $87.0 \pm 1.9\%$, respectively. At 24 h, the germination rate reached 100% for the wild-type and the overexpression strains, whereas for the $\Delta Bbbqra$ mutant germination was only $73.1 \pm 2.2\%$. Finally, at 48 h, germination rates were 100% for all of the strains.

NAD(P)H-1,4-Benzoquinone Reductase Activity in Wild-Type, $\Delta Bbbqra$, and $Bb::Bbbqra^O$ Strains. Benzoquinone reductase activity was measured in fungal extracts derived from the wild-type, $\Delta Bbbqra$, and $Bb::Bbbqra^O$ strains grown in the presence and absence of benzoquinone (Fig. 5). Enzyme activity was measured in fungal extracts after 6 h of exposure to $18.5 \mu\text{M}$ ($2 \mu\text{g}/\mu\text{L}$) benzoquinone or dichloromethane as a control using either NADH or NADPH as the electron donor as described in *Materials and Methods*. For the wild type, BqrA activity increased six- to sevenfold after exposure to benzoquinone reaching $\sim 3 \text{ U}/\text{mg}$ total protein (Fig. 5). As expected for a FMN reductase, enzyme activity was seen in the presence of either NADPH or NADH. No increase/induction of BqrA activity above background levels ($0.5\text{--}1 \text{ U}/\text{mg}$) was seen in the $\Delta Bbbqra$ mutant strain. Constitutive BqrA activity was seen in the $Bb::Bbbqra^O$ strain ($2\text{--}3 \text{ U}/\text{mg}$), although no further induction of activity was seen in the presence of benzoquinone.

Heterologous Expression and Characterization of BbBqrA. To confirm the biochemical activity of the *bqrA* protein product, the ORF of the gene was cloned into an *Escherichia coli* recombinant expression plasmid vector (pET28a) as detailed in *Materials and Methods* that included an N-terminal His-tag on the protein. The protein was purified by Ni^{2+} metal-ion affinity chromatography, and the molecular weight of the protein was estimated to be 24 kDa (Fig. 6A, theoretical 22.92 kDa). The optimum pH for enzyme activity was determined to be pH = 7.8 (Fig. 6B). The enzyme was functional using either NADH or NADPH as a co-factor, and activity was linear with respect to time and protein concentration under the conditions examined. BbBqrA was able to use both 1,4-benzoquinone and 2-methyl-1,4-benzoquinone as substrates. Kinetic analyses of the purified enzyme showed that it exhibited a $K_m = 270.3 \mu\text{M}$ with a $V_{\text{max}} = 26 \mu\text{M}/\text{min}$ ($0.026 \text{ U}/\text{mL}$)

and a $K_{\text{CAT}} = 5.99 \text{ min}^{-1}$ (Fig. 7, *Upper*). The overall catalytic efficiency ($\text{CAT} = K_{\text{CAT}}/K_m$) for the enzyme with respect to 1,4-benzoquinone was $0.01 \mu\text{M}^{-1}\cdot\text{min}^{-1}$. Kinetic analyses using 2-methyl-1,4-benzoquinone indicated a $K_m = 897 \mu\text{M}$, $V_{\text{max}} = 21 \mu\text{M}/\text{min}$ ($0.021 \text{ U}/\text{mL}$), $K_{\text{CAT}} = 4.5 \text{ min}^{-1}$, and $\text{CAT} = 0.005 \mu\text{M}^{-1}\cdot\text{min}^{-1}$ (Fig. 7, *Lower*). The overall catalytic activity of the enzyme toward conversion of 1,4-benzoquinone was two times greater than that for 2-methyl-1,4-benzoquinone.

Bbbqra Contributes to Virulence Against Quinone-Secreting Tenebrionids.

T. castaneum, a benzoquinone-secreting beetle species, was a poor host for wild-type *B. bassiana*, which was able to kill only 15–20% of the target insects over a 22-d time course, with infection leveling off 8 d postinoculation (Fig. 8A). Loss of *Bbbqra* decreased the ability of *B. bassiana* to successfully infect *T. castaneum* below that of wild type, resulting in $\sim 9\text{--}10\%$ ($P < 0.001$) mortality against this host. In contrast, overexpression of *Bbbqra* ($Bb::Bbbqra^O$) increased virulence, resulting in 35–40% mortality of the treated beetles ($P < 0.001$). In all instances, however, mortality reached a plateau 7–8 d postinoculation, and due to overall mortality rates $< 50\%$, accurate mean lethal time to kill 50% of target host (LT_{50}) values could not be calculated. In contrast, insect bioassays using the nonbenzoquinone-producing rice weevil, *S. oryzae* (also an important stored-grain pest), revealed susceptibility of the insect to the wild-type *B. bassiana* parental strain, showing a cumulative mortality of $87.4 \pm 5.4\%$ after 4 wk ($\text{LT}_{50} = 10.2 \pm 2.6 \text{ d}$). No significant differences in virulence were seen between wild type and infections using either the $\Delta Bbbqra$ or $Bb::Bbbqra^O$ strains ($F = 1.289$, $P = 0.2835$), with $\text{LT}_{50} = 8.9 \pm 2.7 \text{ d}$ for $\Delta Bbbqra$ and $7.6 \pm 1.7 \text{ d}$ for $Bb::Bbbqra^O$ (Fig. 8B). Similar results were obtained using the greater wax moth, *G. mellonella* (Lepidoptera), in which neither deletion nor overexpression of *Bbbqra* affected fungal virulence compared with the wild type. In this instance, infection with all strains tested reached $> 80\%$ mortality within 10 d (Fig. 8C), and no significant differences were noted between the wild-type, $\Delta Bbbqra$, and $Bb::Bbbqra^O$ strains. LT_{50} values were calculated to be 5.4 ± 0.3 , 5.5 ± 0.1 , and $6.1 \pm 0.1 \text{ d}$ for the wild-type, $\Delta Bbbqra$, and $Bb::Bbbqra^O$ strains, respectively.

Discussion

Fungal pathogens have evolved a variety of strategies for successful infection of target hosts (47, 48). Fungal spores (conidia) of the insect pathogenic fungus, *B. bassiana*, will attempt to attach and initiate infection essentially anywhere on host cuticle, although preferential infection sites on some hosts have been observed (21, 49, 50). The infection program is initiated by surface cues, and the fungus is capable of assimilating long-chain alkanes and other lipids that are often found as major constituents of the insect epicuticle (41). The subsequent production of cuticle-degrading enzymes and penetrating hyphae results in growth of the fungus through the integument into the hemocoel. Once in the hemocoel, *B. bassiana* undergoes a dimorphic transition elaborating cells that are able to evade the host immune system (51, 52). In almost all cases, if the infection progresses to the hemocoel,

Table 1. Inhibition of *B. bassiana* growth by 1,4-benzoquinone

BQ (μM)	Inhibition radius (mm \pm SE)		
	Wild type	$\Delta Bbbqra$	$Bb::Bbbqra^O$
0	0	0	0
37 (4 $\mu\text{g}/\mu\text{l}$)	$6.6 \pm 0.8a^*$	$8.3 \pm 0.7a$	$2.7 \pm 0.3b$
111 (12 $\mu\text{g}/\mu\text{l}$)	$9.7 \pm 0.7a$	$13.2 \pm 0.2b$	$4.3 \pm 0.4c$
231 (25 $\mu\text{g}/\mu\text{l}$)	$12.2 \pm 1.3a$	$20.3 \pm 0.7b$	$8.2 \pm 0.4c$

*For each dose, different lowercase letters indicate significant differences between columns ($P < 0.05$, Tukey's test).

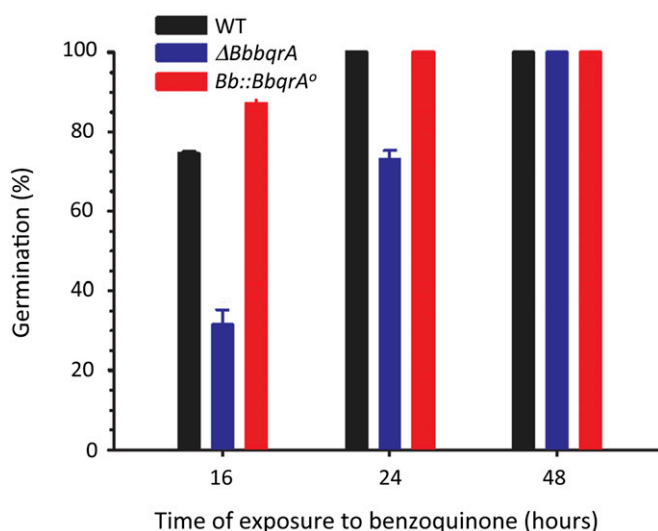


Fig. 4. Time course of *B. bassiana* germination in the presence of 1,4-benzoquinone. Conidia from wild-type, $\Delta BbbqrA$, and $Bb::BbbqrA^{\circ}$ strains were incubated in the presence of media containing 1 $\mu\text{g}/\mu\text{L}$ (9.25 μM) 1,4-benzoquinone, and the percentage of germination was calculated at 16, 24, and 48 h postincubation. The bars represent percentages \pm SD.

mortality of the host will ensue. Thus, the major “battlefield” for thwarting infection has been hypothesized to be the host cuticle (20). A significant puzzle in insect pathology is the observation that some insect species, even if closely related, show greater resistance to broad host range fungal pathogens than others. The underlying biochemical mechanism(s) behind such resistance and counterattempts by the fungus to overcome such defenses has remained obscure. For some insects, resistance has been attributed to the production of antimicrobial cuticular compounds (53–55). More recently there is growing evidence that some of these compounds are not produced by the insects themselves but by endogenous microbial flora, adding an interesting twist to coevolutionary scenarios (56, 57). In other insects, specific behaviors, including heat seeking (behavioral fever), aggregation, and, in the case of social insects, grooming, have also been shown to be strategies for impeding or minimizing microbial infections (58–60).

Within this context, some tenebrionid beetles, e.g., *T. castaneum*, are resistant to *B. bassiana* infection under most conditions. Therefore, dose-mortality response curves, i.e., LT_{50} and mean lethal dose (LD_{50}) values, cannot accurately be determined (30, 61, 62). A comparative study of the chemical defense system of tenebrionids, examining 147 different species, revealed that all such beetles contain abundant amounts of soluble quinones, i.e., not cross-linked to other macromolecules as can occur to quinones during cuticle sclerotization, that are produced and stored within the organism and secreted via both prothoracic and abdominal glands (63). Such insect quinone-containing secretions are typically sprayed from the various glands toward predators and intruders and have been extensively studied due to their role in deterring insects and other animals, with their participation in other chemical communication processes also reported (64–67). *T. castaneum* is a particularly prolific soluble quinone producer, secreting a quinone mixture that, in addition to acting as a noxious discharge to repel threats, displays antimicrobial activities against a number of bacteria and yeasts (33, 68). Our data extend the antimicrobial activities of these secretions to filamentous fungi, including insect pathogens, demonstrating inhibition of *B. bassiana* germination and growth. This observation suggests that production of cuticular quinones represents an adaptation that can inhibit microbial infections, even

of specialized pathogens, by blocking their ability to gain a foothold on the organism and initiate infection. As rapid fungal growth and germination has been shown to correlate with virulence (69), likely due to an ensuing increased rate of fungal penetration of the insect cuticle, limiting germination on the cuticle via production of toxic compounds by the insect would be an effective strategy for resisting infection. However, quinones appeared to be fungistatic toward *B. bassiana*, and over time, in growth media containing quinones, the fungus is able to germinate to a similar extent as the wild type, bearing in mind that the chemical oxidation of quinones to a nontoxic derivative also occurs over time. On the insect, however, defensive/cuticular quinones are constantly being replenished. No differences were observed in terms of sporulation on the cadavers of those insects killed by any of the fungal strains examined, i.e., wild type, $\Delta BbbqrA$, and $Bb::BbbqrA^{\circ}$. As quinones are oxidized over time, the concentrations of quinones on a dead insect appear to drop to below fungal inhibitory levels within several days. Sporulation takes between 2 and 4 d, a time period in which quinone levels are likely to have decreased substantially on the cadaver.

In response to benzoquinone, the fungus produces a benzoquinone reductase (BbBqrA) that acts to detoxify the compound. The enzyme is widely distributed in fungi, and the *B. bassiana* *BbbqrA* gene product showed high sequence identity to quinone reductases found, but not yet characterized, in other filamentous fungi including *C. militaris* and *T. reesei*, as well as in *M. roberstii*, the latter another broad host range insect pathogen. BbBqrA clustered relatively far from orthologous proteins in the brown- and white-rot basidiomycetes, *G. trabeum* and *P. chrysosporium*, whose quinone reductases have been implicated in lignin biodegradation processes. Unlike these enzymes, however, *B. bassiana* BbBqrA does not contain a 5'-putative leader sequence for protein secretion (37, 39). The secreted enzymes target cross-linked quinones present in extracellular lignin substrates (70). In contrast, soluble quinones can readily enter cells, where they are cytotoxic in part because they rapidly undergo intracellular one-electron reduction to semiquinones, which react rapidly with O_2 to produce superoxide (71). In contrast, hydroquinones are relatively nontoxic because they undergo one-electron oxidation to semiquinones only in the presence of transition metal oxidants, such as Fe^{3+} , which are

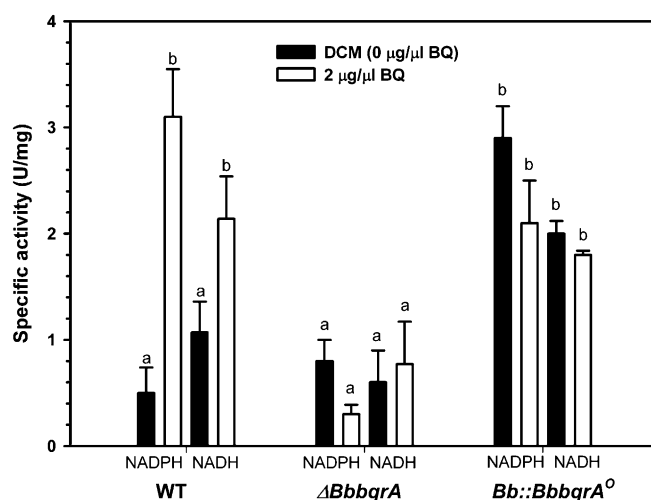


Fig. 5. Determination of 1,4-benzoquinone reductase activity in *B. bassiana*. Enzyme activity was determined by measuring either NADH or NADPH consumption in *B. bassiana* partially purified fractions derived from fungal cells exposed to 2 $\mu\text{g}/\mu\text{L}$ (18.5 μM) benzoquinone for 6 h before harvesting. Bars represent means of 5–10 replicates \pm SD. Different lowercase letters indicate significant differences ($P < 0.05$, Tukey's test).

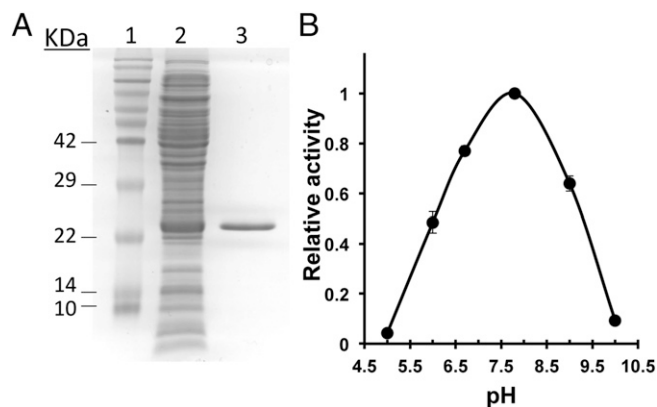


Fig. 6. Heterologous expression and purification of BbBqrA. (A) Sixteen-percent SDS-polyacrylamide gel of recombinant BbBqrA: lane 1, molecular weight standards; lane 2, *E. coli* BbBqrA expression crude extract; lane 3, purified recombinant BbBqrA (3 µg). (B) Relative benzoquinone reductase activity at different pH values using 1,4-benzoquinone as the substrate in the presence of NADPH.

generally unavailable inside cells because they are sequestered in redox-inactive complexes.

Expression of the *BbbqrA* gene was induced in the presence of benzoquinone, and, as expected, an increase in benzoquinone reductase activity in fungal extracts was noted. Deletion of *BbbqrA* resulted in a fungal strain with increased susceptibility to benzoquinone. Although mortality of the wild-type strain against *T. castaneum* was poor (~18–20%), the mutant strain was even worse, resulting in no more than 10% mortality even after 24 d. In contrast, the $\Delta BbbqrA$ mutant showed no significant alternation in virulence toward the nonquinone-secreting weevil *S. oryzae* or the greater wax moth *G. mellonella*. These data suggest that BbBqrA acts as a host-specific virulence factor for host quinone detoxification. To provide further evidence for such a hypothesis, a fungal strain overexpressing the benzoquinone reductase was produced. Overexpression of the enzyme increased fungal resistance to benzoquinone, and the virulence of the *BqrA*-overexpressing strain (*Bb::BbBqrA^O*) was increased compared with the wild type against *T. castaneum*, reaching from 25 to 40% of the infected population. As seen for the deletion strain, no change in virulence was seen for the *Bb::BbBqrA^O* strain when tested against *S. oryzae* or *G. mellonella*. The observation that mortality still did not reach >50% suggests that *T. castaneum* contains additional barriers to thwart infection by *B. bassiana* and/or that the reductase levels were still insufficient to completely overcome the amounts of toxic quinones present in and/or on the insect. The fact that overexpression did increase virulence suggests that the fungal response to the host defense is not yet optimized, supporting the idea that the host and pathogen are involved in an evolutionary arms race, in which, at present, *T. castaneum* is in the lead. Our results can possibly be viewed as capturing a snapshot of the selective pressures on a specific biochemical enzyme expressed by the pathogen. As the benzoquinone reductase is found in (nonpathogenic) fungi that do not interact with quinone-producing beetles, this indicates the potential for *B. bassiana* to redirect a general (quinone) detoxification enzyme to a specialized function, an adaptation that has not yet occurred.

The fact that expression of the detoxifying enzyme by the pathogen is not yet optimized can be explained by several factors. First, as a broad host range pathogen, the targets of *B. bassiana* are manifold, and therefore selection pressures on any given single target, which, if representing a small fraction of potential hosts, will be diluted by the availability of other more susceptible

targets. As significant concentrations of antimicrobial-acting quinones are rare on most potential hosts, any selective pressure may be small within a larger environmental context. This would be especially true if, as our data show, selection for the trait does not confer a more generalized benefit, i.e., increased virulence toward other targets. Second, although we could not detect any obvious phenotypic differences in addition to BqrA activity, a potential trade-off might exist; i.e., increased BqrA activity may decrease some aspect of fungal fitness that we did not detect. This is often seen because any counteradaptive change entails some costs and most often reduces adaptation to some other factor; e.g., even a small decrease in growth rate can constrain environmental selection for higher enzyme activity. In addition, the selective pressure on *T. castaneum* to avoid fungal infection may be stronger because in most cases infection by the fungus results in death, whereas the fungus only loses one opportunity to grow and disperse in that particular host, a potential example of the “life-dinner principle” (72).

Significant efforts have been made to unveil the molecular mechanisms behind the strategies that entomopathogenic fungi use to overcome host defenses. These findings have led to several genetic engineering approaches to improve mycoinsecticide

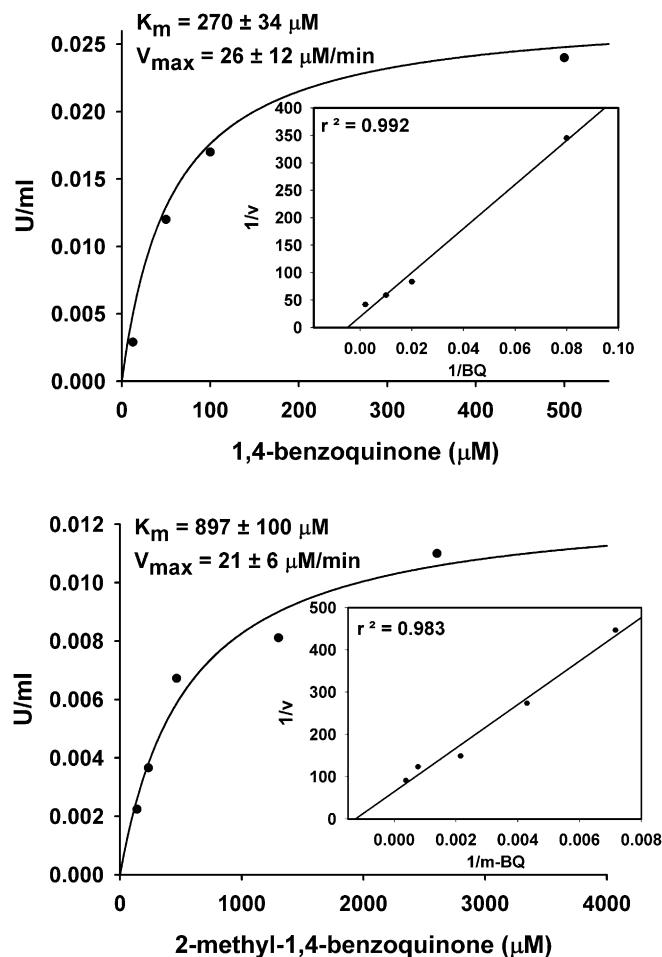


Fig. 7. BbBqrA enzyme kinetics. (Upper) Kinetics of recombinant BbBqrA with 1,4-benzoquinone. K_{CAT} and the catalytic efficiency ($CAT = K_{CAT}/K_m$) were calculated to be 5.99 min^{-1} and $0.01 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$, respectively. (Lower) Kinetics of recombinant BbBqrA with 2-methyl-1,4-benzoquinone. K_{cat} and the catalytic efficiency were calculated to be 4.50 min^{-1} and $0.005 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$, respectively. The ratio of catalytic efficiency of BqrA for 1,4-benzoquinone to 2-methyl-1,4-benzoquinone, $CAT_{bq}/CAT_{mbq} = 2$.

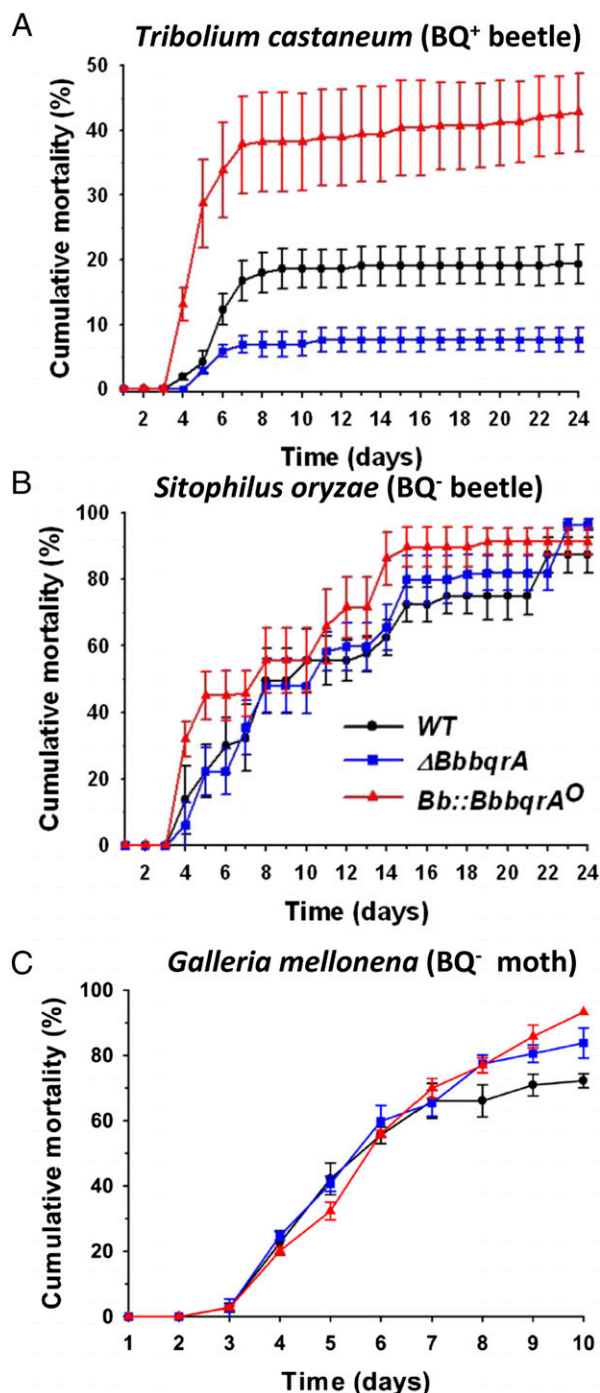


Fig. 8. Insect bioassays using *B. bassiana* wild-type, $\Delta BbbqrA$, and $Bb::BbbqrA^O$ strains. Survival curves of the quinone-producing beetle *T. castaneum* (A), the nonquinone-producing beetle *S. oryzae* (B), and the lepidopteran moth *G. mellonella* (C) infected with spores derived from the wild-type (black lines), $\Delta BbbqrA$ (blue), and $Bb::BbbqrA^O$ (red) strains, respectively. Data represent mean cumulative mortality percentage \pm SE from four independent experiments. Control insects were immersed in water containing 0.01% Tween 80. Mortality data were corrected for control mortality using the Abbott's formula.

performance by targeting specific defenses, e.g., cuticular compounds and insect immune pathways (73–76). This paper adds benzoquinones to the list of insect cuticular defenses and presents BbBqrA as a potential adaptive response of *B. bassiana* to counteract the insect's attempt to escape fungal infection. By

revealing the competing components of an arms race between *B. bassiana* and *T. castaneum*, we have discovered a way to increase the virulence of the fungal biological control agent against a previously recalcitrant target and demonstrated that, although infection of insects by *B. bassiana* is a multifactorial process, in some instances a single host specificity virulence factor can be identified.

Materials and Methods

Cultivation of Fungi. *B. bassiana* (ATCC 90517) was routinely grown on PDA, Czapek–Dox, or complete medium agar (CMA) plates. Plates were incubated at 26 °C. Except for inhibition and germination assays, fungi were grown on the surface of a cellophane sheet in CMA plates with and without addition of dichloromethane extracts of *T. castaneum* adults or synthetic 1,4-benzoquinone, hydroquinone, and 1-pentadecene (C15:1) (Sigma-Aldrich). Fungi were first cultured for 4 d on cellophane/CMA plates until abundant mycelia were observed, after which the sheet was transferred to CMA plates containing the test extracts/compounds. Plates were returned for incubation over the indicated time course (typically up to an additional 4 d). Dichloromethane extracts were prepared from laboratory-reared adults of *T. castaneum*, as previously described (26). The extracts contained mainly ethyl- and methyl-1,4-benzoquinones and 1-pentadecene, as verified by thin-layer chromatography. Solvents and other reagents were obtained from Fisher Scientific and Carlo Erba Reagents SAS.

Antimicrobial Assay. Suspensions of conidia were prepared from PDA plates grown for 6–10 d by rubbing the sporulating surface with a bent needle. After filtering through a 60- μ m mesh sieve to remove debris, sterile distilled water containing 0.01% Tween 80 was added to the suspension to reach desired cell concentrations based upon cell counts using a hemocytometer. Suspensions of 4×10^8 con/mL were homogeneously dispersed with a bent glass rod on CMA plates. After 30 min, a sterile paper dish (20-mm diameter) containing the insect cuticular extract was placed on the center of the plate. Doses assayed ranged from 0.3 to 0.6 insect equivalents/ μ L. Additional assays were performed on agar plates containing solutions of (reagent grade) benzoquinone in dichloromethane, hydroquinone in ethanol, or C15:1 in hexane. Concentrations of the pure chemical compounds tested included the following: (i) 37, 111, and 231.2 μ M (4, 12, and 25 μ g/ μ L) benzoquinone; (ii) 36.3, 108.9, and 227 μ M (4, 12, and 25 μ g/ μ L) hydroquinone; and (iii) 19, 57, and 118.8 μ M (4, 12, and 25 μ g/ μ L) pentadecene, spotted onto the agar plates with corresponding solvents used as controls. The zones of inhibition (diameters in millimeters) around the spotted compounds on plates containing uniformly spread fungal spores were measured after 3 d incubation at 26 °C. Each experiment used four replicate plates and was repeated at least two times with different batches of conidia.

Fungal Germination Assay. A 40- μ L drop of the conidial suspension was inoculated onto the center of CMA plates containing 1 μ g/mL benzoquinone. Controls included spotting of conidial solution on CMA plates containing no additional reagents or containing the solvent dichloromethane. After inoculation, plates were incubated for 12, 16, 24, and 48 h in the dark at 26 °C. Conidial germination was assessed microscopically, and conidia were considered to be germinated when the length of the germ tube was at least half of the length of the diameter of the conidium. For each of three plates inoculated for each concentration, at least 300 conidia per plate were evaluated and the percentage of germination was calculated.

Molecular Manipulations: Construction of *BbbqrA*-Targeted Gene Knockout and Overexpression Strains.

A list of primers used in this study is given in Table S1. A putative benzoquinone oxidoreductase gene was identified in *B. bassiana* EST libraries, and the genomic DNA sequence was obtained from the *B. bassiana* whole genome (14). The sequences were analyzed using programs implemented at the Biology WorkBench website (workbench.sdsc.edu). Introns were detected by comparison between both cDNA and DNA sequences. Coding sequences were translated to amino acid sequences using the ExPASy tools (ExPASy Proteomics Server, www.expasy.org). Phylogenetic analysis was performed with the Phylogeny.fr platform (77), which uses the programs MUSCLE for multiple alignment, Gblocks for automatic alignment curation, PhyML for tree building, and TreeDyn for tree drawing. The protein sequence is annotated in GenBank under accession no. XP_008594912.1.

For construction of a targeted gene knockout strain, the upstream (LB) and downstream (RB) regions of *BbbqrA* were obtained by PCR using primer pairs PquiL1/L2 and PquiR1/R2 (Table S1). Primers PBar1 and PBar2 were used to amplify the *bar* gene expression cassette, conferring resistance to phosphinothricin. PquiL2 and PquiR1 were designed to contain a 20-bp *bar* gene

cassette overlapping sequences. The products derived from individual PCR, i.e., LB, RB, and *bar*, were used to perform primer-less assembly by PCR cycling conditions as follows: 98 °C (2 min), followed by 22 cycles of 98 °C (15 s), 55 °C (30 s), and 72 °C (1.5 min), after which 1 μL of the reaction mixture was used as the template to PCR-amplify the desired full-length homologous recombinant construct (LB_{bqrA}-*bar*-RB_{bqrA}) with primers of L1 and R2. During the whole process of construction, Phusion DNA polymerase (New England Biolabs) was used. The PCR product was introduced into *B. bassiana* competent cells following our method (78). Screening for the correct homologous recombinant mutant was performed using primers Pqui-U and Pqui-D as described (79).

To construct a *B. bassiana BbbqRA* overexpressing strain, the full-length *BbbqRA* gene was obtained via PCR amplification using primers Pquiuv-1 and Pquiuv-2. The resultant PCR product was fused to a PCR fragment containing the P_{gpdA} promoter by primer-less assembly, taking advantage of a 20-bp P_{gpdA} sequence overlap designed in primer Pquiuv-1. The obtained construct, P_{gpdA}::*BbbqRA*, was cloned into pUC-*bar* and transformed into *B. bassiana* competent cells as above. To confirm *BbbqRA* overexpression, qPCR analyses were performed with 10 transformants and the wild type. Fungi were grown in PDA plates with cellophane sheets for 5 d. RNA and cDNA preps were obtained with RNAeasy Plant Mini Kit (Qiagen) and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed with 10 ng of reverse-transcribed RNA/sample under the following conditions (30 s at 95 °C for denaturation, 30 s at 59 °C for annealing, and 30 s at 72 °C for polymerase elongation + melting step). The *BbbqRA*-specific primers, qui-qPCR2-fwd and qui-qPCR2-rev, and primers encoding for the actin gene, *actin-fwd* and *actin-rev*, were used. The experiment was performed in triplicate, and appropriate controls were included.

Gene Expression Analysis. *B. bassiana* was grown in complete medium and exposed to different concentrations of benzoquinone (0, 0.5, 1, and 2 μg/μL or 0, 4.6, 9.25, and 18.5 μM). Fungal cells were examined over a time course (6, 24, 72 h) of exposure, and total RNA of each culture was extracted using an RNAeasy Plant Mini kit (Qiagen), including an on-column DNA digestion step. RNA was quantified by the Qubit fluorometer using the Quant-iT RNA assay kit (Invitrogen), and the integrity of the RNA was assessed on a 1% (wt/vol) agarose gel. Two-step reverse transcriptase real-time PCR was performed using the iScript cDNA Synthesis kit and iQ SYBR Green Supermix (Bio-Rad). Amplification was performed in a Mx3000P QPCR System (Stratagene) using 20 ng of reverse-transcribed total RNA for each sample. The following amplification program was used: denaturation at 95 °C for 10 min, followed by 40 cycles with three-segment amplification (30 s at 95 °C for denaturation, 30 s at 61 °C for annealing, and 30 s at 72 °C for polymerase elongation). To confirm that only single products were amplified, a temperature melting step was then performed. Relative expression was calculated using 5.8S rRNA as the reference gene amplified with 5.8S-fwd and 5.8S-rev. The gene-specific primers for *BbbqRA* were qui-qPCR1-fwd and qui-qPCR-rev (Table S1). Negative controls included samples generated without reverse transcriptase as templates. Reactions containing primer pairs without template were also included as blank controls. The assay was performed in duplicate with each of the three independent biological replicates.

Subcellular Fractionation and Benzoquinone Reductase Enzyme Activity Measurement. *B. bassiana* was grown on cellophane sheets placed on CMA for 4 d after which the sheets were transferred to fresh CMA plates supplemented with or without 2 μg/μL benzoquinone and incubated for an additional 6-h induction period. Fungal mycelia were harvested from the cellophane sheets and disrupted using a Mini-Bead Beater homogenizer (BioSpec) with glass beads (0.5-mm diameter). Four cycles of 20 s each were performed, with 20-s cooling periods between bursts. Homogenization buffer was 50 mM sodium citrate (pH 6), 300 mM sucrose, 5 mM EDTA, 5 mM DTT, and 1 mM PMSF. The homogenate was centrifuged for 20 min at 10,000 × *g* to remove mitochondria and cell debris, and the supernatant was ultracentrifuged for 60 min at 100,000 × *g*. The soluble fraction (S100,000 × *g*) was used for determination of enzyme activity.

1,4-Benzoquinone reductase activity was measured spectrophotometrically by following the decrease in absorbance at 340 nm due to NADPH or NADH consumption. The amount of enzyme that catalyzed the oxidation of 1 μmol of NAD(P)H/min at 25 °C was defined as 1 U of activity. Standard reaction mixtures (1 mL) consisted of 50 mM sodium citrate buffer (pH 6), 100 μM 1–4 benzoquinone, and 0.1–0.2 mL of the S100,000 × *g* supernatant fraction. Reactions were initiated by the addition of 200 μM NAD(P)H (Sigma-Aldrich). The rate of nonenzymatic oxidation of NAD(P)H by quinones was measured in a blank containing no enzyme, and its value was subtracted from the sample containing the enzyme (80). Protein concentration was measured by the bicinchoninic acid method (Pierce), using BSA as standard.

Heterologous Expression and Characterization of BbBqRA. The *BbbqRA* ORF was amplified from P_{gpdA}::*BbbqRA* pCB1536 vector. The PCR primers quiNcol-fwd and qui-HindIII-rev (Table S1) were designed from the BQR cDNA sequence with the restriction sites NcoI and HindIII appended at the 5' end to facilitate directional cloning into PET28a (AddGene). PCR products were digested with NcoI and HindIII and ligated into the multiple cloning site of the vector, also digested with the same restriction enzymes. The integrity of the construct sequence, which included a six-amino acid histidine tag at the C terminus, was confirmed by sequencing (UF ICBR Molecular Core). The expression plasmid was then transformed in Rosetta (DE3) pLys E. coli competent cells. For protein expression and purification, *E. coli* transformants cells were grown in LB media for 3.5 h using a 1:50 overnight inoculum before being induced with 0.5 mM IPTG and allowed to continue to grow overnight at 18 °C. Cells were harvested by centrifugation (8,000 × *g* for 20 min), resuspended in buffer (20 mM Hepes, 100 mM NaCl, 10 mM imidazole, pH 7.5), and lysed using a French press. The lysate was centrifuged at 15,000 × *g* for 30 min to remove cell debris. The expressed protein in the resulting supernatant was purified using Co²⁺-agarose beads (Gold-Bio). The column was equilibrated and washed with 20 mM Hepes, 100 mM NaCl, 10 mM imidazole (pH 7.5), and BbBqRA was eluted with a stepwise gradient in buffer containing 50, 100, 200, and 300 mM imidazole. Fractions were analyzed by SDS/PAGE and those containing BbBqRA were pooled, dialyzed in 20 mM Hepes and 100 mM NaCl (pH 7.5) and concentrated using a 6- to 8-kDa dialysis membrane (Pierce) via embedding in polyethylene glycol 20000 (Sigma Aldrich). Protein concentration was measured by the bicinchoninic acid method (Pierce) using BSA as standard. 1,4-Benzoquinone and 2-methyl-1,4-benzoquinone reductase activity of the recombinant BbBqRA was measured spectrophotometrically by following the decrease in absorbance at 340 nm due to NADPH consumption. Two protein solutions of 9 and 4.6 μM BbBqRA containing 200 μM NADPH were titrated with 12, 50, 100, and 500 μM 1,4-benzoquinone and 140, 232, 465, 1,300, and 2,600 μM 2-methyl-1,4-benzoquinone, respectively. Data were fit to a Michael-Menten equation to calculate the respective V_{max} and K_m values. The catalytic efficiency for both substrates was calculated as follows: CAT = K_{cat}/K_m, where K_{cat} = V_{max}/[BbbqRA]_{total}. Optimal pH conditions for BbBqRA activity was determined by assaying 1,4-benzoquinone reductase activity as described above using benzoquinone (100 μM) as the substrate and different buffers including 50 mM citrate phosphate buffer (pH 5.0), 50 mM citrate phosphate buffer (pH 6.1), 50 mM citrate buffer (pH 6.6), 50 mM phosphate buffer (pH 7.7), 50 mM Tris-ClH buffer (pH 8.9), and 50 mM glycine-NaOH buffer (pH 10.0).

Insect Bioassays. Fungal insect assays were performed using *G. mellonella* larvae (Pet Solutions) and adults of *T. castaneum* and *S. oryzae* from colonies regularly maintained at El Instituto de Investigaciones Bioquímicas de La Plata (25). For *G. mellonella*, larvae were treated topically by dipping of larvae for 5–10 s in solutions of 1 × 10⁸ conidia/mL harvested in sterile distilled H₂O containing 0.01% Tween 80. Excess liquid on the insect bodies was removed by placement on a dry paper towel. Controls were treated with sterile distilled H₂O/0.01% Tween 80. Experimental and control larvae were placed in large (150-mm diameter) petri dishes and incubated at 26 °C. For each experimental condition, 40–50 larvae were used, and all experiments were repeated three times. For *T. castaneum* and *S. oryzae*, groups of 10 adults were separately immersed for 6 s in a fungal suspension (1 × 10⁹ conidia/mL) and then placed on a paper towel to remove excess water and transferred to different 200-mL glass vials covered with a muslin cloth (treated insects). Control insects were treated similarly with water containing 0.01% Tween 80. Insect diet (wheat flour for *T. castaneum*, durum wheat for *S. oryzae*) was added to the vials 1 d posttreatment. Five replicates of each treatment (fungus-treated and controls) with 10 insects per replicate were performed, and the entire experiment was repeated three more times. Insect mortality was checked daily, and dead insects were removed after each count. Mortality data were corrected for control mortality using the Abbott's equation (81). Cumulative mortality percentage and LT₅₀ values were calculated by Probit analysis, and differences were determined by analysis of variance and by using Tukey's test to separate treatment mean (*P* < 0.05). Instat 3.05 (GraphPad Software Inc.) was used for all statistical analyses.

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