The *Beauveria bassiana* Gas3 β -Glucanosyltransferase Contributes to Fungal Adaptation to Extreme Alkaline Conditions

Zhibing Luo,^{a,b} Tongbing Zhang,^{a,b} Pengfei Liu,^{a,b} Yuting Bai,^{a,b} Qiyan Chen,^{a,b} Yongjun Zhang,^{a,b} Nemat O. Keyhani^c

^aAcademy of Agricultural Sciences, Southwest University, Chongqing, People's Republic of China

^bBiotechnology Research Center, Southwest University, Chongqing, People's Republic of China

^cDepartment of Microbiology and Cell Science, University of Florida, Gainesville, Florida, USA

ABSTRACT Fungal β -1,3-glucanosyltransferases are cell wall-remodeling enzymes implicated in stress response, cell wall integrity, and virulence, with most fungal genomes containing multiple members. The insect-pathogenic fungus *Beauveria bassiana* displays robust growth over a wide pH range (pH 4 to 10). A random insertion mutant library screening for increased sensitivity to alkaline (pH 10) growth conditions resulted in the identification and mapping of a mutant to a β -1,3-glucanosyltransferase gene (*Bbgas3*). *Bbgas3* expression was pH dependent and regulated by the PacC transcription factor, which activates genes in response to neutral/alkaline growth conditions. Targeted gene knockout of *Bbgas3* resulted in reduced growth under alkaline conditions, with only minor effects of increased sensitivity to cell wall stress (Congo red and calcofluor white) and no significant effects on fungal sensitivity to oxidative or osmotic stress. The cell walls of Δ *Bbgas3* aerial conidia were thinner than those of the wild-type and complemented strains in response to alkaline conditions, and β -1,3-glucan antibody and lectin staining revealed alterations in cell surface carbohydrate epitopes. The Δ *Bbgas3* mutant displayed alterations in cell wall chitin and carbohydrate content in response to alkaline pH. Insect bioassays revealed impaired virulence for the Δ *Bbgas3* mutant depending upon the pH of the media on which the conidia were grown and harvested. Unexpectedly, a decreased median lethal time to kill (LT₅₀, i.e., increased virulence) was seen for the mutant using intrahemocoel injection assays using conidia grown at acidic pH (5.6). These data show that BbGas3 acts as a pH-responsive cell wall-remodeling enzyme involved in resistance to extreme pH (>9).

IMPORTANCE Little is known about adaptations required for growth at high (>9) pH. Here, we show that a specific fungal membrane-remodeling β -1,3-glucanosyltransferase gene (*Bbgas3*) regulated by the pH-responsive PacC transcription factor forms a critical aspect of the ability of the insect-pathogenic fungus *Beauveria bassiana* to grow at extreme pH. The loss of *Bbgas3* resulted in a unique decreased ability to grow at high pH, with little to no effects seen with respect to other stress conditions, i.e., cell wall integrity and osmotic and oxidative stress. However, pH-dependent alternations in cell wall properties and virulence were noted for the Δ *Bbgas3* mutant. These data provide a mechanistic insight into the importance of the specific cell wall structure required to stabilize the cell at high pH and link it to the PacC/Pal/Rim pH-sensing and regulatory system.

KEYWORDS β -1,3-glycanosyltransferase, cell wall integrity, filamentous fungi, *Beauveria bassiana*, alkaline tolerance, high pH

Known for their remarkable bioremediation, pathogenic, and/or secondary metabolite production capabilities, fungi have evolved the ability to thrive on a wide variety of substrates under diverse environmental conditions (1). Filamentous fungi (Ascomycota) typically prefer neutral-to-acidic growth conditions and, indeed, often

Received 7 May 2018 Accepted 17 May 2018

Accepted manuscript posted online 25 May 2018

Citation Luo Z, Zhang T, Liu P, Bai Y, Chen Q, Zhang Y, Keyhani NO. 2018. The *Beauveria bassiana* Gas3 β -glucanosyltransferase contributes to fungal adaptation to extreme alkaline conditions. Appl Environ Microbiol 84:e01086-18. <https://doi.org/10.1128/AEM.01086-18>.

Editor Emma R. Master, University of Toronto

Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Zhibing Luo, luozb1@163.com, or Nemat O. Keyhani, keyhani@ufl.edu.

acidify their surrounding media/environment during growth; however, many of these fungi are quite capable of robust growth under alkaline conditions. Environmental pH can affect nutrient and mineral availability, fungal enzyme activities, and fungal membrane integrity and function. The fungal cell wall has evolved specific structural features to protect the cell against adverse stresses resulting from fluctuating environmental conditions. Polymers of β -1,3-glucans form the major cell wall carbohydrate component of yeasts and filamentous fungi, constituting 40 to 70% of the cell wall mass, although cell walls (and particularly septae) contain more small amounts of chitin (long chains of β -1,4-linked *N*-acetylglucosamine) (2). In the process of cell wall synthesis, chitin and glucan synthase complexes on the plasma membrane assemble UDP-*N*-acetylglucosamine and UDP-glucose into linear chitin and β -1,3-glucan chains, respectively, that extrude into and out of the cell wall space (3). For the β -glucans, the linear chains are often branched, elongated, modified, and/or cross-linked to other cell wall components, forming an integrated and flexible steric structure. Moreover, the organized fungal cell wall is dynamic and constantly remodeled both as a function of growth and due to responses to fluctuating environmental conditions, including changes in ambient pH, temperature, and osmotic and oxidative stress (4–6). For fungal pathogens, cell wall epitopes can also change in response to host cues, in particular in order to evade host defense and immune responses (7, 8).

In yeasts and various fungi, several different nomenclature systems have been used to categorize the enzymes involved in β -1,3-glucan synthesis, i.e., β -1,3-glucanosyltransferases, including glycolipid anchored surface (Gas), glucan elongation (Gel) proteins, and pH response (PHR) proteins (6, 9, 10). Characterization of the five GAS proteins found in *Saccharomyces cerevisiae* has revealed differential, but sometimes overlapping, contributions of these proteins in cell morphogenesis, growth, cell wall integrity, and even transcriptional silencing (11–13). In the human yeast pathogen, *Candida albicans*, which also contains five β -1,3-glucanosyltransferases (*PHR*) genes, two, *PHR1* and *PHR2*, are regulated in response to ambient pH (14). The expression of *PHR1* is induced at neutral to alkaline pH (7 to 8) and repressed at pH <5.5, whereas *PHR2* expression is induced under acidic conditions and repressed at neutral/alkaline pH (15). Both of these genes appear to be regulated by PacC/Rim101 that acts as an activator and repressor for *PHR1* and *PHR2* expression, respectively, by binding to the specific sites (GCCARG) in their promoter regions (16, 17). In the opportunistic fungal pathogen *Pneumocystis jirovecii*, *PHR1* is also regulated in response to environmental pH and can rescue the *S. cerevisiae* Δ *gas1* mutant phenotype of lethality under conditions of cell wall stress (18). In the filamentous fungus *Neurospora crassa*, disruption of any of the five *gel* genes did not affect pH sensitivity (19). In terms of functional similarity, both *gel1* and *gel2* of the human pathogen *Aspergillus fumigatus* (containing seven *gel* genes) could rescue the growth and the morphological defects of the *S. cerevisiae* Δ *gas1* mutant (20). In *A. fumigatus*, Gel7 contributes to cell wall integrity under stress conditions, while Gel4 appears to be essential (21, 22). To the best of our knowledge, the contributions of β -1,3-glucanosyltransferases to alkaline adaptation have been examined exclusively in yeast and only under moderate alkaline conditions (up to pH 8), with no reports examining the functions of these enzymes in mediating growth under extreme alkaline conditions (pH 10).

The fungus-specific Pal/Rim signaling and response pathway is known to play an important role in the ability of fungi to cope with variation and changes in the ambient pH of the surrounding environment. Depending upon the fungus, the Pal/Rim pathway encodes sensor and transduction factors that are activated in response to specific pH signals, ultimately targeting the PacC/Rim101 transcription factor that regulates the expression of various genes, presumably involved in pH adaptation (23, 24). Under neutral or alkaline conditions, the Pal/Rim pathway stimulates the proteolytic cleavage of (inactive) cytoplasmic form of PacC, resulting in its activation and subsequent translocation into the nucleus, where it activates the transcription of “alkaline”-expressed genes, while repressing “acid”-expressed genes (25). In various fungi, PacC activity has also been shown to contribute to/regulate other processes, including

secondary metabolite production, ion homeostasis, pathogenicity (in some, but not all cases), and cell wall synthesis and remodeling (26, 27). In the insect pathogen, *Metarhizium robertsii*, deletion of the *pacC* gene (*MrpacC*) resulted in reduced growth across a wide pH range (4 to 10), decreased sporulation, and decreased virulence (4). In contrast, in the insect pathogen *Beauveria bassiana*, the loss of *pacC* resulted in decreased growth under alkaline conditions, along with increased sensitivity to the cell wall stress, but it had little effect on virulence (28). However, few downstream targets of PacC that can account for the various phenotypes have been identified and characterized.

The insect-pathogenic fungus *Beauveria bassiana* represents a class of environmentally friendly fungal pathogens responsible for epizootic infections of insect populations in nature, and it is currently being used in various agricultural applications for the control of different insect pests (29). *B. bassiana* has also developed into a model system useful for exploring the genetics and evolution of fungal host-pathogen interactions, stress response, and development (30–32). Infection begins as spores attach, germinate, and penetrate the insect cuticle, with cuticle penetration involving the coordinated action of mechanical pressure, cuticle-degrading hydrolases, and other factors (33, 34). Changes in pH during the infection process are critical aspects manipulated by the fungus in order to successfully invade and infect the host. Initial acidification of the media, in part due to the production of acidic metabolites, including oxalate, gives way to alkalinization (via an as-yet-unknown mechanism) as the fungus penetrates the host integument and into the hemocoel, with specialized acid- and alkaline-active hydrolases having been characterized (35–38). *B. bassiana* is capable of robust growth over a wide pH range from 4 to 10.5 (39). The components of the Pal/Rim system, including PacC, have been characterized in *B. bassiana*, where although BbPacC was found to be required for acidification of media, e.g., oxalate production, and for growth at high pH (8 to 10.4), *pacC* mutants showed only minor impairment of virulence (28, 40).

Here, we report on a screen of a *B. bassiana* (random) transfer DNA (T-DNA) insertion library for mutants sensitive to extreme alkaline pH (10). One such mutant (T340) was mapped to a β -1,3-glucanosyltransferase annotated as *gas3*. Targeted gene knockout and complementation strains of *Bbgas3* confirmed the initial phenotype, with the $\Delta Bbgas3$ mutant displaying poor growth at high pH (10). The $\Delta Bbgas3$ mutant showed little to no changes in resistance to most other stress conditions or in virulence, indicating a specific role for *Bbgas3* in uniquely mediating alkaline resistance. Changes in cell wall epitopes were observed in the $\Delta Bbgas3$ mutant, and a regulatory pathway involving PacC was established.

RESULTS

Identification of *gas3* and sequence analysis of Gas family in *B. bassiana*. A previously constructed *B. bassiana* T-DNA insertion library (41) was screened for mutants impaired for growth in potato-dextrose agar (PDA) buffered to pH 10. The insertion site of the mutant, designated T340, was mapped to a region containing two putative open reading frames, one encoding a function-unknown protein (accession no. [KGQ07720](#)) and a β -1,3-glucanosyltransferase (accession no. [EJP67416](#)) annotated as *gas3*. Complementation experiments indicated that only the *gas3* gene was able to rescue the T340 sensitivity to alkaline-growth-condition phenotype (see Fig. S1 and S2 in the supplemental material). Analyses of the available genomes for two *B. bassiana* strains (2860 and D1-5) indicated the presence of the following six putative β -1,3-glucanosyltransferase proteins: Gas1 (accession no. [EJP66700](#)), Gas2 (accession no. [EJP62516](#)), Gas3 (accession no. [EJP67416](#)), Gas4 (accession no. [EJP61263](#)), Gas5 (accession no. [EJP68186](#)), and Gas6 (accession no. [EJP65749](#)). As has been previously noted, phylogenetic analyses revealed the separation of the Gas proteins into two subfamilies (GH72– and GH72+) (Fig. S3). *B. bassiana gas3*, as well as *gas4* and *gas6* (GH72+), grouped with other filamentous fungal enzymes, including *Magnaporthe oryzae gel4*, *A. fumigatus gel3*, *gel4*, *gel5*, and *gel7*, *C. albicans PHR1* and *PHR2*, and the yeast *S.*

FIG 1 Transcriptional profiles of *B. bassiana* β -1,3-glucanase genes. (A) Expression of *Bbgas1* to *Bbgas6* in wild-type *B. bassiana* as a function of external pH. (B) Expression of *Bbgas3* and *PacC* in wild-type *B. bassiana*. (C) Expression of *Bbgas3* in a *B. bassiana* $\Delta BbpacC$ background strain as a function of pH. Error bars are the standard deviation (SD) of the mean of the results from three replicate assays. Asterisks indicate significant differences.

cerevisiae gas1 (*Scgas1*) and *Scgas2*. The GH72⁺ enzymes are characterized by containing an X8 (carbohydrate binding module containing six conserved cysteine residues [42]) domain absent in the GH72[−] proteins (Fig. S3). The *B. bassiana* Gas proteins contain conserved N-terminal signal peptides, a catalytic domain with two glutamic residue (E) catalytic residues, and C-terminal glycosylphosphatidylinositol pathogenicity island (GPI) anchor sites, as well as various Ser-rich and Gsn/Asp-rich regions. Variation in the number of *gas* genes identified in different strains was noted. As indicated above, our analysis of the two available *B. bassiana* genomes (2860 and D1-5) revealed six putative β -1,3-glucanase genes; however, the *gas4* gene was not detectable in genomic DNA of Bb0062 (the strain used for construction and screening of the T-DNA mutant library) using PCR. Analyses of 10 different *B. bassiana* strains revealed the amplification of *gas4* sequences in five of them. These included the *B. bassiana* GHA strain (registered product for insect control) and *B. bassiana* ATCC 90517, with ATCC 90517 being one of the most commonly used strains in the molecular characterization of this fungus and the strain used in this study (30–32) (Fig. S4).

***B. bassiana gas3* is expressed in response to pH and is controlled by *PacC*.** The expression pattern of the six *B. bassiana gas* genes was examined in response to environmental pH by quantitative real-time PCR (Fig. 1A). *Bbgas1* expression was similar under acidic (pH 4) and neutral (pH 7) conditions, with a slight decrease in expression seen at alkaline pH (10). *Bbgas2* expression was similar to that of *Bbgas1*, although its pattern gradually decreased from acidic to alkaline conditions. In contrast, *Bbgas3* expression was low under acidic conditions but was induced 4-fold and 19-fold at pH 7 and 10, respectively. *Bbgas5* and *Bbgas6* expression levels were the reverse of *Bbgas3*, with high expression seen under acidic pH conditions, which was significantly decreased under neutral and alkaline pH conditions. *BbpacC* expression mirrored that of *Bbgas3*, with low expression at pH 4 and 7 and 6-fold induction at pH 10 (Fig. 1B). In

FIG 2 Growth of wild type (WT), $\Delta Bbgas3$ mutant, and complemented ($\Delta Bbgas3::Bbgas3$) strains on PDA buffered to indicated starting pH values, as described Materials and Methods. Plates were spot inoculated with a conidial suspension of each strain and incubated at 26°C for 7 days. (B) Quantitative real-time PCR (qRT-PCR) expression profile of *Bbgas* genes (1–6) in the *B. bassiana* $\Delta Bbgas3$ mutant as a function of the pH of the growth media. Fungal cultures were grown RNA extracted as detailed in Materials and Methods. Error bars are the SD of the mean of the results from three replicate assays; asterisks indicate significant differences (Tukey's HSD, $P < 0.01$).

order to examine whether *Bbgas3* expression was dependent upon PacC, *Bbgas3* and *BbpacC* expression was examined in response to pH (Fig. 1B), and *Bbgas3* expression was examined in the wild type and a $\Delta BbpacC$ mutant background (Fig. 1C). These data showed coordinated expression of *Bbgas3* and *BbpacC* and a failure of *Bbgas3* induction in response to pH 7 and 10 in the $\Delta BbpacC$ mutant. These results are consistent with analyses of the *Bbgas3* promoter region that was found to contain six putative PacC binding sites (GCCARG), as well as putative self-regulation of PacC, as has been previously reported (28).

Construction and analysis of *Bbgas3* targeted gene deletion and complementation strains. In order to verify the initial T-DNA mutant phenotype and to further probe the functions of *Bbgas3*, targeted gene deletion ($\Delta Bbgas3$ mutant) and complementation ($\Delta Bbgas3::Bbgas3$ mutant) strains were constructed as detailed in Materials and Methods (Fig. S5). The phenotype of the $\Delta Bbgas3$ mutant strain was identical to the original T-DNA mutant in sensitivity to alkaline pH (Fig. 2A). Further phenotypic examination revealed small effects with regard to increased sensitivity to Congo red and calcofluor white (Fig. S6), but no apparent consequence of a loss of *Bbgas3* on osmotic (salt and sorbitol), oxidative (H_2O_2 and menadione), or temperature (32°C) stress was noted (Fig. S7). As expected, the targeted gene deletion of *Bbgas3* resulted in a loss of *Bbgas3* transcript, with only minor effects on the expression of the remaining *Bbgas* genes (Fig. 2B). Expression of the *Bbgas* genes in the $\Delta Bbgas3$ mutant continued to be pH dependent (as seen in Fig. 1), with analyses of the ratio of $\Delta Bbgas3$ or wild-type *Bbgas* gene expression (which would be 1 if no change occurred) indicating a slight decrease in *Bbgas1* expression at pH 10, which was found not to be statistically significant, a 1- to 2-fold decrease in *Bbgas2* expression at pH 10, which was statistically significant ($P < 0.05$), as was *Bbgas5* expression (decreased by 1- to 2-fold) at all pH values. In addition, *Bbgas6* expression at pH 10 also appeared to be decreased (at pH 10), but the effect was not statistically significant.

In order to evaluate any effects of loss of *Bbgas3* on cell wall composition, the concentrations of chitin, the alkali-soluble fraction (containing β -1,3/1,6-glucans), and the alkali-insoluble fraction (β -1,3/1,6-glucan covalently linked to chitin) were quantified in the various strains (Fig. 3A). Compared to the wild-type strain, the $\Delta Bbgas3$ mutant showed comparable levels of total sugar content as well as chitin and alkaline-soluble alkaline-insoluble at pH 7, but increases in the alkali-insoluble fraction and chitin content were observed when cells were responding to alkaline conditions (pH 10), with a decrease in the alkali-soluble component at pH 10. Calcofluor white staining of chitin revealed increased labeling of growing germling/hyphal tips in the $\Delta Bbgas3$

FIG 3 Effect of *Bbgas3* deletion on *B. bassiana* cell wall components. (A) Total carbohydrate, alkaline-soluble and -insoluble, and chitin content of *B. bassiana* wild-type and $\Delta Bbgas3$ mutant cells grown in media buffered to pH 7 and pH 10, and analyzed as detailed in Materials and Methods. Error bars are the SD of the mean of the results from three replicate assays, and asterisks on column bars indicate significant difference (Tukey's HSD, $P < 0.01$). (B) Calcofluor white staining of wild-type and $\Delta Bbgas3$ mutant cells. Inset and arrow indicate enhanced chitin deposition in the hyphal tip of the mutant at pH. Scale bar = 500 μm .

mutant strain under neutral and alkaline conditions compared to those of the wild-type control (Fig. 3B).

Transmission electron microscopic visualization of the fungal cell wall revealed a significant ($P < 0.05$) reduction in the cell wall thickness of $\Delta Bbgas3$ aerial conidia grown at pH 10 but no effect on conidia harvested from plates buffered to pH 7 (Fig. 4A and B and S8). In addition, a significant difference ($P < 0.05$) in aberrant conidia, with apparent distorted and/or partially collapsed shapes and poor cytoplasmic staining, was seen for $\Delta Bbgas3$ mutant conidia at pH 7 (5% versus $<0.1\%$ for the wild type) that increased to $\sim 12\%$ of the harvested conidia grown at pH 10 for the mutant, compared

FIG 4 Analysis of conidial cell walls and morphology of the *B. bassiana* wild type (WT) and $\Delta Bbgas3$ mutant. (A and B) TEM images and quantification of conidial cell wall thickness. Scale bar = 0.2 μm . (C and D) Representative images and quantification of distorted conidial morphologies seen in the $\Delta Bbgas3$ mutant. Arrows indicate measurement of cell wall thickness. Scale bar = 0.5 μm . Asterisks on column bars indicate significant difference (Tukey's HSD, $P < 0.01$).

FIG 5 Flow cytometry distribution and quantification of lectin and β -glucan antibody staining in *B. bassiana* wild-type (black) and $\Delta Bbgas3$ mutant (green) aerial conidia. Error bars are the SD of the mean of the results from three replicate assays. ConA and WGA are the lectins of wheat germ agglutinin and concanavalin A, respectively. Stars on column bars indicate significant differences (Tukey's HSD, $P < 0.01$).

FIG 6 (A) Growth of *B. bassiana* wild-type, $\Delta Bbgas3$ mutant, and complemented ($\Delta Bbgas3::Bbgas3$) strains on CZA containing 0.05% bromocresol purple indicating ambient acidification. (B) Determination of the pH of growth media (CZB) inoculated with each respective fungal strain and measured at the indicated time course. (C) Growth of the wild-type, $\Delta BbpacC$ mutant, and $\Delta BbpacC::Bbgas3$ complemented strains are shown on buffered PDA at indicated starting pH values, as described in Materials and Methods. Plates were inoculated with conidial suspensions and incubated at 26°C for 7 days.

to ~2% abnormal cells seen in the wild-type strain (Fig. 4C and D). In order to determine any effects of the loss of *Bbgas3* on cell surface carbohydrate epitopes, lectin (wheat germ agglutinin [WGA] and concanavalin A [ConA]) and β -1,3-glucan antibody mapping of aerial conidia was performed using confocal microscopy and flow cytometry. Conidia derived from pH 7 growth conditions displayed a moderate ($P < 0.05$) decrease in cell surface β -1,3-glucan, as well as a large increase in β -N-acetylglucosamine (β -GlcNAc) and/or sialic acid epitopes (WGA reactive), with no change in α -glucose and/or α -N-GlcNAc epitopes (ConA reactive) (Fig. 5A). Similar to cells at pH 7, conidia derived from pH 10 growth conditions displayed a moderate ($P < 0.05$) decrease in cell surface β -1,3-glucan; however, decreases ($P < 0.05$) in both WGA-reactive and ConA-reactive cell surface epitopes were seen for these cells (Fig. 5B).

Deletion of *Bbgas3* does not affect acidification and has minor effect on virulence depending upon the pH of the growth media. Previous work in *B. bassiana* has shown a link between PacC and extracellular acidification, oxalate production, and increased sensitivity to alkaline pH (28). Acidification of the external media was assessed qualitatively via incorporation of the pH indicator dye bromocresol purple in agar plates, and quantitatively in the supernatant derived from liquid cultures (Fig. 6A and B). No differences in acidification were noted between any of the strains. In addition, the expression of *Bbgas3* in the $\Delta BbpacC$ mutant background did not rescue the alkaline sensitivity phenotype of this mutant (Fig. 6C).

The effect of *Bbgas3* deletion on *B. bassiana* virulence was probed via insect bioassays using *Galleria mellonella* larvae. Suspensions of aerial conidia (1×10^7 conidia $\cdot$ ml $^{-1}$) derived from the wild-type, $\Delta Bbgas3$ mutant, and $\Delta Bbgas3::Bbgas3$ complemented strains were isolated from three different growth conditions; namely, PDA buffer to pH values of 5.6 (acidic), 7.0 (neutral), and 10 (alkaline) were either (i) applied topically to the larvae, representing the natural route of infection, or (ii) injected into hemocoel cavity, thus bypassing penetration. Mortality was determined over a 12-day period, and the median lethal time (LT₅₀) was estimated as detailed in Materials and Methods. Using conidia produced on acidic PDA media (pH 5.6), the deletion of *Bbgas3* did not result in any significant changes of LT₅₀ values using topical infections; however, a decrease in LT₅₀ (i.e., increased mortality, $P < 0.01$) was seen in intrahemocoel injection assays (Fig. 7A and B and Table 1). Conidia produced on neutral PDA

FIG 7 Insect bioassays. *G. mellonella* larvae were treated either topically (A, C, and E) or via intrahemocoel injection (B, D, and F) with conidia from wild-type *B. bassiana* (■), $\Delta Bbgas3$ mutant (●), $\Delta Bbgas3::Bbgas3$ complemented (◆) strains, or mock-treated controls (○), as described in Materials and Methods. (A to F) Conidia derived from growth on PDA at pH 5.6 (A and B), 7.0 (C and D), and pH 10 (E and F). The percent survival $\pm$ SD over the indicated time course is presented.

media showed no significant changes in mortality in either the topical or intrahemocoel injection bioassay (Fig. 7C and D and Table 1); however, the conidia derived from the $\Delta Bbgas3$ mutant harvested from alkaline PDA medium showed significantly increased (i.e., decreased virulence; $P < 0.01$) LT₅₀ values, by ~22% and 70%, in topical and intrahemocoel injection assays, respectively (Fig. 7C and D and Table 1). Insect cadavers appeared identical to the wild type, and no differences in sporulation on the cadavers were noted between the wild type and the $\Delta Bbgas3$ mutant (Fig. S9).

DISCUSSION

Remodeling of cell wall β -1,3-glucan via the activities of GPI-anchored β -1,3-glucanotransferases (Gas/Gel proteins) belonging to the glycoside hydrolase family 72 (GH72) is a critical process seen on all fungi (21). These proteins exist as gene families whose functions have diversified. For example, the previously characterized *B. bassiana* *gas1* gene as well as the *F. oxysporum* *gas1* gene (*Fogas1*) were unable to complement

TABLE 1 LT₅₀ values for *B. bassiana* wild-type, $\Delta Bbgas3$ mutant, and complemented ($\Delta Bbgas3::Bbgas3$) strains in topical and intrahemocoel injection bioassays using *G. mellonella* larvae as the host

PDA pH value ^a	<i>B. bassiana</i> strain	LT ₅₀ (days)	
		Topical infection	Injection
5.6	WT	5.09 ± 0.04	4.62 ± 0.02
	$\Delta Bbgas3$ mutant	5.46 ± 0.24	3.87 ± 0.17
	$\Delta Bbgas3::Bbgas3$ complement	5.47 ± 0.37	4.53 ± 0.40
7	WT	5.31 ± 0.20	3.36 ± 0.03
	$\Delta Bbgas3$ mutant	4.89 ± 0.16	3.33 ± 0.08
	$\Delta Bbgas3::Bbgas3$ complement	4.87 ± 0.19	3.30 ± 0.03
10	WT	5.65 ± 0.18	3.34 ± 0.09
	$\Delta Bbgas3$ mutant	6.94 ± 0.35	5.71 ± 0.11
	$\Delta Bbgas3::Bbgas3$ complement	5.84 ± 0.02	3.40 ± 0.02

^aFungal conidia derived from growth on PDA were amended to the indicated pH values and used in the bioassays.

the yeast *gas1* mutant, indicating divergence either in processing/targeting of the enzymes and/or in their enzymatic activities (5, 43). In the yeast *C. albicans*, two β -1,3-glucanotransferase genes, *phr1* and *phr2*, have been shown to contribute to fungal adaptation to neutral/mildly alkaline (6 to 8) and acidic conditions, respectively (17, 44). Germ tubes produced by *Phr1* Δ mutants displayed abnormalities in elongation, accumulate chitin, and show constitutive activation of the Mkc1, Cek1, and Hog1 mitogen-activated protein (MAP) kinase signaling pathways. However, no functions for β -1,3-glycanotransferases in response to pH have yet been reported in filamentous fungi, and the effects at extreme alkaline pH values (10) have yet to be reported for any fungus. In *M. oryzae*, individual β -1,3-glycanotransferases (Gel protein) have been shown to be dispensable for pathogenicity, with none of the mutants affected in terms of fungal sensitivity to pH (2). However, *M. oryzae* $\Delta gel1 \Delta gel3 \Delta gel4$ triple mutants could not infect or sporulate on intact rice leaf. These and other studies indicate that some Gas/Gel proteins appear to show partially overlapping or redundant biological roles, with few reports of specific defined functions for these proteins (2, 19, 45). Previous characterization of β -1,3-glycanotransferases in *B. bassiana* (*Bbgas1*) did not show any effects with respect to pH, with *Bbgas1* instead contributing to conidial thermotolerance and virulence (5).

Here, we report that *B. bassiana gas3*, encoding a putative β -1,3-glycanotransferase, is a key factor for fungal adaptation to extreme alkaline conditions by modulating cell wall integrity. The biological role of *Bbgas3* was very specific, with no effects seen in the $\Delta Bbgas3$ mutant with respect to oxidative, osmotic, or high-temperature stress. Our prior work has documented that both the Msn2 and PacC transcription factors modulate pH effects, with PacC being the target of the Pal pH sensor/response pathway (28). Our data show that *Bbgas3* shares a similar transcriptional pattern with and is positively regulated by PacC in response to ambient pH, with the *Bbgas3* promoter sequence containing multiple putative PacC binding sites. These data are in contrast to observations in *M. robertsii*, where PacC has been shown to negatively regulate the transcription of several glycosyltransferase genes involved in cell wall synthesis/remodeling, although the regulation of β -glucanotransferases in *M. robertsii* has not yet been examined (4). In *B. bassiana*, PacC is also known to control the production or degradation of organic acids (particularly oxalate acid) and environmental acidification (28, 46), with oxalate implicated as an important virulence factor (47). However, the deletion of *Bbgas3* did not affect environmental acidification, a not-too-surprising result, as the genes responsible for these phenotypes are other targets for PacC. In addition, overexpression of *Bbgas3* in a $\Delta BbpacC$ mutant background failed to rescue the pH sensitivity of the parent mutant. This result implies that a loss of PacC results in pleiotropic effects on a loss of cell wall integrity, consistent with

the broad phenotypic effects of the mutant (as opposed to the narrow phenotype seen in for the $\Delta Bbgas3$ mutant). The $\Delta Bbgas3$ mutant showed little to no defects in virulence via either topical infection, which represents the natural route of infection, or via injection of the fungal spores directly into the insect hemocoel cavity, thus bypassing the need for cuticle penetration when using conidia derived from PDA at neutral pH (7). A slight increase in virulence was noted for the $\Delta Bbgas3$ mutant in intrahemocoel injection assays when using conidia harvested from acidic (pH 5.6) PDA, whereas decreased virulence was seen for the $\Delta Bbgas3$ mutant harvested from alkaline (pH 10) PDA plates in both topical and intrahemocoel injection (particularly intrahemocoel injection) bioassays. Although at present there is little indication as to why we observed the increase in virulence in the mutant in injection assays using acid-condition derived conidia, the decreased virulence under alkaline conditions likely reflects the decreased cell wall robustness/integrity seen in the mutant. These results identify pH- and cell wall-dependent effects on virulence attributable to a cell wall-remodeling enzyme. When successful in infecting the host, sporulation on cadavers was normal, indicating that the mutant could successfully complete all stages of the infection cycle. Although tangential to the main results of this report, our data indicate some strain variation in the number of β -glucanoyltransferase genes identified, with approximately half (5/10) of the strains examined appearing to lack a *gas4* gene homolog. As complete genomes for the strains lacking a *gas4* homolog are not yet available, it cannot presently be ruled out that such a gene may still exist but has diverged enough from the test primers used to detect the gene that PCR amplification was not possible. Alternatively, redundancy or other factors may have made this gene expendable in some lineages.

The specific contribution of *Bbgas3* to mediating resistance to extreme alkaline pH is, to our knowledge, the only example of such a strictly defined role for a fungal β -1,3-glycanosyltransferase. Further work determining the enzymatic mode of its action, i.e., what glucan cross-links are made by the enzyme that helps confer alkaline resistance to a membrane, is warranted. Our data show a decrease in cell wall thickness in the $\Delta Bbgas3$ mutant (at pH 10 but not pH 7), as well as global changes in cell wall carbohydrate content, including increased alkaline-insoluble glucan and chitin content and decreased alkaline-soluble content (again all only at pH 10), factors that affect cell wall integrity. In addition, increased deposition of chitin was seen at hyphal tips, and alterations in cell wall carbohydrate epitopes were seen that can help account for the alkaline sensitivity of the *gas3* mutant. However, it should also be noted that aside from the role in glucan remodeling, at least one of Gas proteins, namely, *S. cerevisiae* GAS1, has been shown to affect transcriptional silencing via carbohydrate modification of chromatin and/or via maintenance of ribosomal DNA (rDNA) integrity (12, 48). Thus, it cannot be ruled out that the effects of *Bbgas3* may also involve such mechanisms. In conclusion, we report the function of *gas3* in the insect-pathogenic fungus *B. bassiana* as a specific modulator of fungal adaptation to extreme alkaline conditions. *Bbgas3* is a downstream target of PacC and acts as a cell wall glucan-remodeling enzyme.

MATERIALS AND METHODS

Fungal strains and culture conditions. *B. bassiana* wild-type strains Bb0062 and ATCC 90517 and various mutants were routinely grown/maintained on potato dextrose broth/agar (PDB/PDA) or Czapek-dox broth/agar (CZB/CZA) (BD Difco, Sparks, MD, USA) at 26°C for 7 to 14 days. Conidia harvested from agar plates were suspended in sterile 0.05% Tween 80 solution and filtered through a single sheet of lens-wiping paper to remove any hyphae and/or mycelia. The final conidial concentration was determined using a hemocytometer. For pH adaptation determination, the PDA medium was buffered with $\text{NaH}_2\text{PO}_4 \cdot \text{NaH}_2\text{PO}_4$ or citric acid-glycine, as described previously (39).

Gene deletion, complementation, and expression. Targeted gene deletion of *B. bassiana* was performed by homologous recombination via *Agrobacterium*-mediated fungal transformation, as described previously (39, 49). In brief, the coding region of *Bbgas3* was replaced by the *bar* cassette conferring resistance to phosphinothricin to generate the $\Delta Bbgas3$ knockout mutant. The 3'-flanking (1,030 bp) and 5'-flanking (601 bp) sequences of *Bbgas3* were amplified from *B. bassiana* genomic DNA via PCR using primer pairs *gas3*-LB-F/*gas3*-LB-R and *gas3*-RB-F/*gas3*-RB-R, respectively. The PCR products were inserted into the *EcoRI* and *XbaI* sites of pK2bar to form pK2bar-*gas3*-ko for fungal transformation. For $\Delta Bbgas3$ complementation, a fragment containing the complete open reading frame (ORF) (1,694 bp), plus promoter (2.1 kb upstream) and terminator (0.6 kb downstream) sequences, was amplified

using the gas3-C-F/gas3-C-R primer pair. The product was inserted into pK2sur (39) at the XbaI restriction site, forming pK2sur-gas3 (*sur* marker conferring resistance to chlorimuron-ethyl). A constitutive *Bbgas3* expression vector, pK2sur-pb3::gas3, was constructed as follows: a fragment containing the transcriptional terminator *trpC* from *Aspergillus nidulans* and the multiple-cloning site (MCS) from the vector pBARGPE1 was amplified using the MCS-F/TrpC-R primer pair. The constitutive promoter derived from the glyceraldehyde phosphate dehydrogenase gene (*Bbgpd_g*) was amplified from *B. bassiana* genomic DNA using the Pb-F/Pb-R primer pair. The above-mentioned two PCR fragments were digested with BglII/BamHI and XbaI/BamHI, respectively, and subsequently ligated into the vector pK2sur at the BamHI/XbaI site to produce pK2sur-pb3. Subsequently, the *Bbgas3* coding region was amplified from *B. bassiana* cDNA using the OE-gas3-F/OE-gas3-R primer pair and inserted into the BamHI/EcoRV site of pK2sur-pb3 to form pK2sur-pb3::gas3.

RNA isolation and real-time PCR analysis. *B. bassiana* conidia were inoculated in PDB for 3 days, and mycelia were harvested by filtration, transferred to PDB buffered to pH values of 4, 7, and 10, and incubated with aeration for 2 h. Cells were harvested by centrifugation, and samples were ground in liquid nitrogen. Total RNA was isolated using the TRIzol reagent (Invitrogen) and treated with DNase I (TaKaRa, Dalian, China). The cDNA of each sample was synthesized using the RevertAid first-strand cDNA synthesis kit (MBI Fermentas). Quantitative real-time PCR analysis was performed using the CFX Connect real-time PCR detection system (Bio-Rad, USA) using SYBR Premix (Bio-Rad). PCRs were performed as follows: a 10-min denaturation at 95°C was followed by 40 cycles of 30 s at 95°C, 30 s at 56°C, and 30 s at 72°C. The relative expression of each gene was normalized against the expression of the β -tubulin gene (GenBank accession no. [AJ312228](#)) using the TubF/TubR primer pair.

Phenotypic assays. Fungal growth was assessed by spotting conidial suspensions ($3 \mu\text{l}$ of 1×10^7 conidia/ml in 0.05% Tween 80) on buffered PDA at indicated pH values with or without Congo red (100 $\mu\text{g/ml}$) or calcofluor white (100 $\mu\text{g/ml}$) and incubating at 26°C for 7 days. To determine the effects of oxidative, osmotic, and temperature stress on fungal growth, conidial suspensions ($3 \mu\text{l}$ of 1×10^7 conidia/ml in 0.05% Tween 80) of each strain were spotted on CZA plates or CZA containing menadione (60 μM), H_2O_2 (20 μM), 0.8 M NaCl, 1.0 M KCl, or 1.2 M sorbitol and incubated at 26°C or 32°C over a 10-day time course. The pH indicator dye bromocresol purple (0.01%) was added to the CZA to monitor acidification of the media during fungal growth. Colony morphologies and diameters were determined at the end of each time period.

Chitin and glucan determination. Fungal cells were grown in PDB for 72 h, after which the cells were transferred to fresh PDB buffered to pH 7 or pH 10. The total fungal mass of the cultures was harvested by filtration. Chitin content determination was performed as described previously (50, 51), with a slight modification. Briefly, samples were washed three times using 1 M NaCl and then ground in liquid nitrogen before SDS extraction (0.3 g) via boiling in 5 ml isolation solution (1.35 mM SDS, 2 mM EDTA, and 3% [vol/vol] β -mercaptoethanol) for 5 min. Samples were centrifuged at 12,000 rpm for 3 min and the resultant pellet suspended in 6 N HCl (6 ml), followed by hydrolysis at 100°C for 6 h and evaporation at 65°C. The samples were then dissolved in 1 ml water, and aliquots of equal volume (0.1 ml) of sample and solution A (1.5 N Na_2CO_3 in 4% acetylacetone) were mixed and incubated at 100°C for 20 min, followed by the addition of 0.7 ml of 96% ethanol and then 0.1 ml solution B (1.6 g of *p*-dimethylaminobenzaldehyde in 30 ml of concentrated HCl and 30 ml of ethanol). After 1 h of incubation at room temperature in the dark, the absorbance of the mixture at 520 nm was measured. Chitin content was quantified using a standard curve of 0 to 200 ng glucosamine.

Glucan content was determined as described previously (52), with minor modifications. Briefly, harvested cells were ground in liquid nitrogen, and samples (10 mg) were suspended in 1 ml 98% formic acid and incubated at 100°C for 20 min. After centrifugation at 12,000 rpm for 3 min, the supernatant was collected and dried in a vacuum desiccator. The dried residue was then dissolved in 2 ml water. Neutral carbohydrate content was quantified using the phenol-sulfuric acid method of Dubois et al. (53). For the detection of alkali-soluble and -insoluble fractions, ground samples (10 mg) were suspended in 1 ml water followed by incubation at 100°C for 20 min. The suspension was centrifuged at 12,000 rpm for 2 min and the supernatant removed from the pellet. The pellet was resuspended either in (i) 1 ml KOH (for determination of the alkali-soluble fraction), followed by incubation at 72°C for 30 min, or (ii) 1 ml of 98% formic acid (for determination of the alkali-insoluble fraction), followed by incubation at 100°C for 20 min. Samples were centrifuged at 12,000 rpm for 2 min, and the supernatants of each treatment were used to determine carbohydrate (glucan) content as described above.

Microscopy and β -1,3-antibody/lectin binding assay. Samples for transmission electron microscopy (TEM) were prepared as described previously (5). Sections were examined using a Hitachi H-7000 TEM and digital images acquired using a Veleta camera and the iTEM software. Lectin binding assays were performed as described previously (54). Briefly, conidia of each strain were harvested by centrifugation and suspended in the appropriate lectin binding buffer, as recommended by the manufacturer's protocols. Alexa Fluor 488-labeled concanavalin A (ConA) and wheat germ agglutinin (WGA) lectins were obtained from Molecular Probes (Invitrogen). Cell wall β -1,3-glucan was labeled using a mouse monoclonal antibody (Biosupplies, Parkville, Australia), as described previously (33). Briefly, conidia were fixed in 3% (vol/vol) formaldehyde for 30 min and then washed three times using phosphate-buffered saline (PBS) buffer before being suspended in PBS buffer containing 1% (vol/vol) Tween 20 (PBS-T). Subsequently, the conidial suspensions were incubated with the anti- β -1,3-glucan antibody ($0.1 \text{ mg} \cdot \text{ml}^{-1}$ in PBS buffer) overnight at 4°C. Samples were then washed three times using chilled (4°C) PBS before incubation with a fluorescein isothiocyanate (FITC) goat anti-mouse IgG (H+L) antibody (Proteintech) ($0.1 \text{ mg} \cdot \text{ml}^{-1}$ in PBS-T buffer) as the secondary antibody for another 2 h in the dark. Samples were then rinsed 3 times (PBS) and resuspended in PBS. Fluorescence was observed using a confocal laser scanning

microscope (FV1000; Olympus). Data acquisition and manipulation were performed with FACSCalibur (BD) and FACSDiva (BD), and the fluorescence was measured for $\sim 10^6$ conidia.

Insect bioassays. Fungal virulence bioassays were performed using *Galleria mellonella* larvae as the host. Conidia harvested from PDA (pH 5.6) or PDA buffered to pH values of 7 and 10 after 10 to 14 days of growth were used for topical infection and intrahemocoel injection assays. Briefly, (i) larvae were dipped into conidial suspensions (10^7 conidia $\cdot$ ml $^{-1}$) for about 5 s and placed on dry paper towels to remove the excess liquid on the insect surface. Larvae of the control were treated with 0.05% Tween 80. (ii) Larvae were injected with 5 μ l of 10^5 conidia $\cdot$ ml $^{-1}$ directly into the hemocoel cavity. Control larvae were treated with 0.05% Tween 80. All treated larvae were placed in 150-mm petri dishes and incubated in a manual climatic box at 26°C. Each treatment including the control contained 90 to 100 larvae, and all experiments were repeated three times with independent batches of conidia. The number of dead larvae was recorded daily, and the median lethal time (LT $_{50}$) was calculated using Probit analysis of the SPSS 11 software. Statistical analyses for mortality experiments were performed using one-way analysis of variance (ANOVA) (SPSS).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01086-18>.

SUPPLEMENTAL FILE 1, PDF file, 3.8 MB.

ACKNOWLEDGMENTS

This work was jointly supported by the National Key R&D Program of China (grant 2017YFD0201202), the Natural Science Foundation of Chongqing (grant CSTC2017jcyjAX0342), and the Fundamental Research Funds for the Central Universities (grants XDJK2015B032 and XDJK2017D196) to Z.L. and T.Z., and a U.S. National Science Foundation grant (IOS-1557704) to N.O.K.

We declare no conflicts of interest.

REFERENCES

- Selvig K, Alspaugh JA. 2011. pH response pathways in fungi: adapting to host-derived and environmental signals. *Mycobiology* 39:249–256. <https://doi.org/10.5941/MYCO.2011.39.4.249>.
- Samalova M, Melida H, Vilaplana F, Bulone V, Soanes DM, Talbot NJ, Gurr SJ. 2016. The β -1,3-glucanase (Gels) affect the structure of the rice blast fungal cell wall during appressorium-mediated plant infection. *Cell Microbiol* 19:e12659. <https://doi.org/10.1111/cmi.12659>.
- Beauvais A, Drake R, Ng K, Diaquin M, Latge JP. 1993. Characterization of the 1,3-beta-glucan synthase of *Aspergillus fumigatus*. *J Gen Microbiol* 139:3071–3078. <https://doi.org/10.1099/00221287-139-12-3071>.
- Huang W, Shang Y, Chen P, Gao Q, Wang C. 2015. MrpC regulates sporulation, insect cuticle penetration and immune evasion in *Metarhizium robertsii*. *Environ Microbiol* 17:994–1008. <https://doi.org/10.1111/1462-2920.12451>.
- Zhang S, Xia Y, Keyhani NO. 2011. Contribution of the *gas1* gene of the entomopathogenic fungus *Beauveria bassiana*, encoding a putative glycosylphosphatidylinositol-anchored β -1,3-glucanase, to conidial thermotolerance and virulence. *Appl Environ Microbiol* 77:2676–2684. <https://doi.org/10.1128/AEM.02747-10>.
- Mouyna I, Hartl L, Latge JP. 2013. β -1,3-Glucan modifying enzymes in *Aspergillus fumigatus*. *Front Microbiol* 4:81. <https://doi.org/10.3389/fmicb.2013.00081>.
- Brown GD, Gordon S. 2005. Immune recognition of fungal β -glucans. *Cell Microbiol* 7:471–479. <https://doi.org/10.1111/j.1462-5822.2005.00505.x>.
- Wang C, St. Leger RJ. 2006. A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. *Proc Natl Acad Sci U S A* 103:6647–6652. <https://doi.org/10.1073/pnas.0601951103>.
- Kovacova K, Degani G, Stratilova E, Farkas V, Popolo L. 2015. Catalytic properties of Phr family members of cell wall glucan remodeling enzymes: implications for the adaptation of *Candida albicans* to ambient pH. *FEMS Yeast Res* 15:fou011. <https://doi.org/10.1093/femsyr/fou011>.
- de Sousa Lima P, Bailao EF, Silva MG, Castro Nda S, Bao SN, Orlandi I, Vai M, de Almeida Soares CM. 2012. Characterization of the *Paracoccidioides* β -1,3-glucanase family. *FEMS Yeast Res* 12:685–702. <https://doi.org/10.1111/j.1567-1364.2012.00819.x>.
- Ragni E, Fontaine T, Gissi C, Latge JP, Popolo L. 2007. The Gas family of proteins of *Saccharomyces cerevisiae*: characterization and evolutionary analysis. *Yeast* 24:297–308. <https://doi.org/10.1002/yea.1473>.
- Koch MR, Pillus L. 2009. The glucanase Gas1 functions in transcriptional silencing. *Proc Natl Acad Sci U S A* 106:11224–11229. <https://doi.org/10.1073/pnas.090809106>.
- Mouyna I, Fontaine T, Vai M, Monod M, Fonzi WA, Diaquin M, Popolo L, Hartland RP, Latge JP. 2000. Glycosylphosphatidylinositol-anchored glucanase transferases play an active role in the biosynthesis of the fungal cell wall. *J Biol Chem* 275:14882–14889. <https://doi.org/10.1074/jbc.275.20.14882>.
- Saporito-Irwin SM, Birse CE, Sypherd PS, Fonzi WA. 1995. *PHR1*, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. *Mol Cell Biol* 15:601–613. <https://doi.org/10.1128/MCB.15.2.601>.
- Mühlschlegel FA, Fonzi WA. 1997. *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of pH-dependent expression. *Mol Cell Biol* 17:5960–5967. <https://doi.org/10.1128/MCB.17.10.5960>.
- Ramon AM, Fonzi WA. 2003. Diverged binding specificity of Rim101p, the *Candida albicans* ortholog of PacC. *Eukaryot Cell* 2:718–728. <https://doi.org/10.1128/EC.2.4.718-728.2003>.
- Calderon J, Zavrel M, Ragni E, Fonzi WA, Rupp S, Popolo L. 2010. *PHR1*, a pH-regulated gene of *Candida albicans* encoding a glucan-remodelling enzyme, is required for adhesion and invasion. *Microbiology* 156:2484–2494. <https://doi.org/10.1099/mic.0.038000-0>.
- Kottom TJ, Thomas CF, Jr, Limper AH. 2001. Characterization of *Pneumocystis carinii* *PHR1*, a pH-regulated gene important for cell wall integrity. *J Bacteriol* 183:6740–6745. <https://doi.org/10.1128/JB.183.23.6740-6745.2001>.
- Kamei M, Yamashita K, Takahashi M, Fukumori F, Ichiishi A, Fujimura M. 2013. Deletion and expression analysis of β -(1,3)-glucanase transferase genes in *Neurospora crassa*. *Fungal Genet Biol* 52:65–72. <https://doi.org/10.1016/j.fgb.2012.12.001>.
- Mouyna I, Morelle W, Vai M, Monod M, Lechenne B, Fontaine T, Beauvais A, Sarfati J, Prevost MC, Henry C, Latge JP. 2005. Deletion of *GEL2* encoding for a β -(1,3)-glucanase transferase affects morphogenesis and virulence in *Aspergillus fumigatus*. *Mol Microbiol* 56:1675–1688. <https://doi.org/10.1111/j.1365-2958.2005.04654.x>.
- Gastebois A, Fontaine T, Latge JP, Mouyna I. 2010. β -(1,3)-Glucanase transferase Gel4p is essential for *Aspergillus fumigatus*. *Eukaryot Cell* 9:1294–1298. <https://doi.org/10.1128/EC.00107-10>.

22. Zhao W, Li C, Liang J, Sun S. 2014. The *Aspergillus fumigatus* β -1,3-glucanotransferase Gel7 plays a compensatory role in maintaining cell wall integrity under stress conditions. *Glycobiology* 24:418–427. <https://doi.org/10.1093/glycob/cwu003>.
23. Tilburn J, Sarkar S, Widdick DA, Espeso EA, Orejas M, Mungroo J, Penalva MA, Arst HN, Jr. 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. *EMBO J* 14:779–790.
24. Peñalva MA, Arst HN, Jr. 2002. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. *Microbiol Mol Biol Rev* 66:426–446. <https://doi.org/10.1128/MMBR.66.3.426-446.2002>.
25. Peñas MM, Hervás-Aguilar A, Munera-Huertas T, Reoyo E, Penalva MA, Arst HN, Jr, Tilburn J. 2007. Further characterization of the signaling proteolysis step in the *Aspergillus nidulans* pH signal transduction pathway. *Eukaryot Cell* 6:960–970. <https://doi.org/10.1128/EC.00047-07>.
26. Cornet M, Gaillardin C. 2014. pH signaling in human fungal pathogens: a new target for antifungal strategies. *Eukaryot Cell* 13:342–352. <https://doi.org/10.1128/EC.00313-13>.
27. Kim YT, Prusky D, Rollins JA. 2007. An activating mutation of the *Sclerotinia sclerotiorum* *pac1* gene increases oxalic acid production at low pH but decreases virulence. *Mol Plant Pathol* 8:611–622. <https://doi.org/10.1111/j.1364-3703.2007.00423.x>.
28. Luo Z, Ren H, Mousa JJ, Rangel D, Zhang Y, Bruner SD, Keyhani NO. 2017. The PacC transcription factor regulates secondary metabolite production, stress response, but has only minor effects on virulence in the insect pathogenic fungus *Beauveria bassiana*. *Environ Microbiol* 19: 788–802. <https://doi.org/10.1111/1462-2920.13648>.
29. Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Kohl J, Marrone P, Morin L, Stewart A. 2012. Have biopesticides come of age? *Trends Biotechnol* 30:250–258. <https://doi.org/10.1016/j.tibtech.2012.01.003>.
30. Ortiz-Urquiza A, Keyhani NO. 2016. Molecular genetics of *Beauveria bassiana* infection of insects, p 165–249. In Lovett B, St. Leger RJ (ed), *Genetics and molecular biology of entomopathogenic fungi* (Advances in genetics), vol 94. Academic Press, Cambridge, MA.
31. Ortiz-Urquiza A, Keyhani NO. 2015. Stress response signaling and virulence: insights from entomopathogenic fungi. *Curr Genet* 61: 239–249. <https://doi.org/10.1007/s00294-014-0439-9>.
32. Pedrini N, Ortiz-Urquiza A, Huarte-Bonnet C, Fan Y, Juárez MP, Keyhani NO. 2015. Tenebrionid secretions and a fungal benzoquinone oxidoreductase form competing components of an arms race between a host and pathogen. *Proc Natl Acad Sci U S A* 112:E3651–E3660. <https://doi.org/10.1073/pnas.1504552112>.
33. Zhang S, Xia YX, Kim B, Keyhani NO. 2011. Two hydrophobins are involved in fungal spore coat rodlet layer assembly and each play distinct roles in surface interactions, development and pathogenesis in the entomopathogenic fungus, *Beauveria bassiana*. *Mol Microbiol* 80: 811–826. <https://doi.org/10.1111/j.1365-2958.2011.07613.x>.
34. Ortiz-Urquiza A, Keyhani NO. 2013. Action on the surface: entomopathogenic fungi versus the insect cuticle. *Insects* 4:357–374. <https://doi.org/10.3390/insects4030357>.
35. Fan Y, Ortiz-Urquiza A, Kudia RA, Keyhani NO. 2012. A fungal homologue of neuronal calcium sensor-1, *Bbsc1*, regulates extracellular acidification and contributes to virulence in the entomopathogenic fungus *Beauveria bassiana*. *Microbiology* 158:1843–1851. <https://doi.org/10.1099/mic.0.058867-0>.
36. St. Leger RJ, Joshi L, Roberts D. 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. *Appl Environ Microbiol* 64:709–713.
37. Shankar S, Saxman RS. 2015. Biophysicochemical characterization of an alkaline protease from *Beauveria* sp. MTCC 5184 with multiple applications. *Appl Biochem Biotechnol* 175:589–602. <https://doi.org/10.1007/s12010-014-1314-3>.
38. St. Leger RJ, Nelson JO, Screen SE. 1999. The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity. *Microbiology* 145:2691–2699. <https://doi.org/10.1099/00221287-145-10-2691>.
39. Luo Z, Li Y, Mousa J, Bruner S, Zhang Y, Pei Y, Keyhani NO. 2015. *Bbmsn2* acts as a pH-dependent negative regulator of secondary metabolite production in the entomopathogenic fungus *Beauveria bassiana*. *Environ Microbiol* 17:1189–1202. <https://doi.org/10.1111/1462-2920.12542>.
40. Zhu J, Ying S-H, Feng M-G. 2016. The Pal pathway required for ambient pH adaptation regulates growth, conidiation, and osmotolerance of *Beauveria bassiana* in a pH-dependent manner. *Appl Microbiol Biotechnol* 100:4423–4433. <https://doi.org/10.1007/s00253-016-7282-5>.
41. Luo Z, Zhang Y, Jin K, Ma J, Wang X, Pei Y. 2009. Construction of *Beauveria bassiana* T-DNA insertion mutant collections and identification of thermosensitive and osmosensitive mutants. *Wei Sheng Wu Xue Bao* 49:1301–1305.
42. Popolo L, Ragni E, Carotti C, Palomares O, Aardema R, Back JW, Dekker HL, de Koning LJ, de Jong L, de Koster CG. 2008. Disulfide bond structure and domain organization of yeast β -(1,3)-glucanotransferases involved in cell wall biogenesis. *J Biol Chem* 283:18553–18565. <https://doi.org/10.1074/jbc.M801562200>.
43. Caracul Z, Martinez-Rocha AL, Di Pietro A, Madrid MP, Roncero ML. 2005. *Fusarium oxysporum* *gas1* encodes a putative β -1,3-glucanotransferase required for virulence on tomato plants. *Mol Plant Microbe Interact* 18:1140–1147. <https://doi.org/10.1094/MPMI-18-1140>.
44. Ragni E, Calderon J, Fascio U, Sipiczki M, Fonzi WA, Popolo L. 2011. Phr1p, a glycosylphosphatidylinositol-anchored β -(1,3)-glucanotransferase critical for hyphal wall formation, localizes to the apical growth sites and septa in *Candida albicans*. *Fungal Genet Biol* 48:793–805. <https://doi.org/10.1016/j.fgb.2011.05.003>.
45. Rolli E, Ragni E, de Medina-Redondo M, Arroyo J, de Aldana CR, Popolo L. 2011. Expression, stability, and replacement of glucan-remodeling enzymes during developmental transitions in *Saccharomyces cerevisiae*. *Mol Biol Cell* 22:1585–1598. <https://doi.org/10.1091/mbc.e10-03-0268>.
46. Lou Y, Han Y, Yang L, Wu M, Zhang J, Cheng J, Wang M, Jiang D, Chen W, Li G. 2015. *CmpacC* regulates mycoparasitism, oxalate degradation and antifungal activity in the mycoparasitic fungus *Coniothyrium minitans*. *Environ Microbiol* 17:4711–4729. <https://doi.org/10.1111/1462-2920.13018>.
47. Kirkland BH, Eisa A, Keyhani NO. 2005. Oxalic acid as a fungal acaricidal virulence factor. *J Med Entomol* 42:346–351. <https://doi.org/10.1093/jmedent/42.3.346>.
48. Ha CW, Kim K, Chang YJ, Kim B, Huh WK. 2014. The β -1,3-glucanotransferase *Gas1* regulates Sir2-mediated rDNA stability in *Saccharomyces cerevisiae*. *Nucleic Acids Res* 42:8486–8499. <https://doi.org/10.1093/nar/gku570>.
49. Fang W, Zhang Y, Yang X, Zheng X, Duan H, Li Y, Pei Y. 2004. *Agrobacterium tumefaciens*-mediated transformation of *Beauveria bassiana* using an herbicide resistance gene as a selection marker. *J Invertebr Pathol* 85:18–24. <https://doi.org/10.1016/j.jip.2003.12.003>.
50. Kapteyn JC, Hoyer LL, Hecht JE, MuE Iler WH, Andel A, Verkleij AJ, Makarow M, Van Den Ende H, Klis FM. 2000. The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. *Mol Microbiol* 35: 601–611. <https://doi.org/10.1046/j.1365-2958.2000.01729.x>.
51. Luo X, Keyhani NO, Yu X, He Z, Luo Z, Pei Y, Zhang Y. 2012. The MAP kinase *Bbslt2* controls growth, conidiation, cell wall integrity, and virulence in the insect pathogenic fungus *Beauveria bassiana*. *Fungal Genet Biol* 49:544–555. <https://doi.org/10.1016/j.fgb.2012.05.002>.
52. Oka T, Hamaguchi T, Sameshima Y, Goto M, Furukawa K. 2004. Molecular characterization of protein O-mannosyltransferase and its involvement in cell-wall synthesis in *Aspergillus nidulans*. *Microbiology* 150: 1973–1982. <https://doi.org/10.1099/mic.0.27005-0>.
53. DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. *Anal Chem* 28:350–356. <https://doi.org/10.1021/ac60111a017>.
54. Wanchoo A, Lewis MW, Keyhani NO. 2009. Lectin mapping reveals stage-specific display of surface carbohydrates in *in vitro* and haemolymph-derived cells of the entomopathogenic fungus *Beauveria bassiana*. *Microbiology* 155:3121–3133. <https://doi.org/10.1099/mic.0.029157-0>.