Low rates of homologous recombination have broadly encumbered genetic studies in the fungal pathogen *Aspergillus fumigatus*. The CRISPR/Cas9 system of bacteria has recently been developed for targeted mutagenesis of eukaryotic genomes with high efficiency and, importantly, through a mechanism independent of homologous repair machinery. As this new technology has not been developed for use in *A. fumigatus*, we sought to test its feasibility for targeted gene disruption in this organism. As a proof of principle, we first demonstrated that CRISPR/Cas9 can indeed be used for high-efficiency (25 to 53%) targeting of the *A. fumigatus* polyketide synthase gene (pksP), as evidenced by the generation of colorless (albino) mutants harboring the expected genomic alteration. We further demonstrated that the constitutive expression of the Cas9 nuclease by itself is not deleterious to *A. fumigatus* growth or virulence, thus making the CRISPR system compatible with studies involved in pathogenesis. Taken together, these data demonstrate that CRISPR can be utilized for loss-of-function studies in *A. fumigatus* and has the potential to bolster the genetic toolbox for this important pathogen.

*A. fumigatus* is a ubiquitous saprophytic fungus that plays an essential role in carbon and nitrogen recycling within ecosystems but is also a major cause of disease in immunocompromised individuals (1, 2). Over the past several decades, the incidence of invasive aspergillosis has risen along with the susceptible patient population, thus highlighting the need for a better understanding of virulence determinants in the fungus (3–5). The most powerful approach in this regard remains the generation and analysis of mutants. Randomized UV or insertional mutagenesis screens have been used on a limited basis to identify essential genes (6) as well as novel pigmentation (7) and drug-resistant mutants (8). However, due to the growing comparative genomic and biological data from other fungi, the major approach in recent years has by far been that of reverse genetics, involving the targeted disruption of candidate genes.

The major limitation for gene deletion in *A. fumigatus*, and indeed most filamentous fungi, has been the relatively low rates of homologous recombination, reported as lower than 5% depending on the gene (9). This is due largely to the presence of nonhomologous end joining (NHEJ) systems that preferentially promote the ectopic insertion of the targeting DNA. The high false-negative ratios among transformants, coupled with laborious and often low-yield transformation techniques (e.g., chemical transformation of protoplasts), may necessitate the screening of hundreds of transformants across multiple transformation attempts before the desired mutant is isolated. Consequently, several groups have developed *A. fumigatus* recipient strains in which the NHEJ pathway (∆ku70 or ∆ku80) is disabled, allowing homologous recombination to occur at a higher frequency among transformants (9, 10). Newer and more elegant methods, such as the hygromycin resistance split-marker approach, where fragments of a selectable marker must recombine homologously in order to confer drug resistance, further increase the probability of obtaining the desired mutant (11).

Another limiting factor for mutagenesis in *A. fumigatus* stems from the restricted number of dominant selectable markers, of which there are three predominantly employed: the hygromycin phosphotransferase gene (*hph*) for hygromycin selection, *ble* for phleomycin resistance, and *ptrA* for pyrithiamine selection. Therefore, work in wild-type (i.e., prototrophic) backgrounds permits the deletions of only three genes, which are performed in successive transformations. Several auxotrophic strains have been employed by the community, including *adeA*, *adeB*, *argB*, *niaD*, *pyrG*, *pyrE*, *sc*, and *trpC*. A limitation of using such strains, however, is the loss of virulence observed in some of the strains, e.g., *pyrG* strains (12); thus, proper isogenic controls for virulence experiments may be lacking.

Finally, the need for homologous genomic targeting may be bypassed by gene silencing with RNA interference (RNAi). Whereas this method provides an advantage of targeting multiple gene products, a complete loss of target gene expression has never been achieved in this system (13, 14).

A more recent alternative to homologous repair-based techniques is the clustered, regularly interspaced, short palindromic repeat (CRISPR) technology utilizing an RNA-guided nuclease, CRISPR-associated (Cas9) protein. CRISPR/Cas9 originates as a prokaryotic adaptive defense system against invading viruses and DNA (15, 16). Remarkably, and in just the past 2 years, the system derived from *Streptococcus pyogenes* (17) has been simplified as a genomic engineering tool in diverse eukaryotes, such as mice and...
rats (18–21), plants (22, 23), and even fungi, including *Saccharomyces cerevisiae* (24), *Candida albicans* (25), *Trichoderma reesei* (26), and other *Aspergillus* spp. (27). Briefly, a synthetic single-guide RNA (gRNA), which is a fusion of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), associates with endonuclease Cas9 and guides it to a 20-nucleotide genomic target. When the genomic target is adjacent to a Cas9-specific protospacer-adjacent motif (PAM), 5′-NGG, Cas9 will induce a double-strand DNA break (17, 28, 29). Indel mutagenesis occurs through NHEJ in genomic targets, inducing frameshifts and resulting in nonsense mutations or otherwise nonfunctional proteins. For the CRISPR/Cas9 system in yeast it has been reported that through short NHEJ-mediated indels, there is a 469-fold increase in mutation frequency for target gene disruption after cas9 gene induction (24). Moreover, the simultaneous targeting of multiple loci can be achieved by introducing multiple and unique gRNA sequences (18, 26).

In summary, the CRISPR/Cas9 system provides the benefits of RNAi in that it bypasses the need for homologous recombination and allows for the targeting of multiple genes in a single transformation; however, it is perhaps more attractive than RNAi in that it works at the genomic level, making gene silencing complete and stable. In this report, we demonstrate that CRISPR/Cas9 can be used for high-efficiency gene disruption in *A. fumigatus*, using the pksP gene (essential for melanin production) as a case study (30, 31). We also demonstrate that constitutive expression of Cas9 alone does not alter growth rate, development, resistance to oxidative stress, cell wall homeostasis, antifungal sensitivity, or virulence in a murine model. This, importantly, establishes that *A. fumigatus* mutants generated with CRISPR/Cas9 are compatible for pathogenesis studies. In addition, we describe an NHEJ-mediated integration of transforming DNA to the Cas9 cut site, a phenomenon that, to our knowledge, has not been described in other fungal CRISPR systems.

**MATERIALS AND METHODS**

**Vector construction and transformation into *A. fumigatus*.** All PCR for vector construction were performed with Phusion Flash DNA polymerase (Thermo Fisher) unless otherwise noted. All primer sequences are listed in Table S1 in the supplemental material.

(i) *p-hph-Ptef1-cas9-pksP-gRNA for introduction into wild-type A. fumigatus.* The hygromycin phosphotransferase (*hph*) cassette was amplified from pAN7-1 using primers 1 and 2. The *Ptef1-cas9* cassette was amplified from p414-TEF1-Cas9-CYC1t (Addgene plasmid 43802) with primers 3 and 4. The pksP-gRNA cassette was generated across three PCRs: (i) the SNR52 promoter was amplified from p426-SNR52p-gRNA.CAN1.Y-SUP4t with primers 5 and 6, (ii) the pksP-gRNA was amplified from p426-SNR52p-gRNA.CAN1.Y-SUP4t using primers 7 and 8, and (iii) the SNR52 promoter and pksP-gRNA fragments were fused in an overlap PCR using primers 21 and 8. Finally, the *hph* and pksP-gRNA fragments were assembled and cloned into pRS426 by homologous recombination within *S. cerevisiae* cells. The resulting *p-hph-pksP-gRNA* plasmid was used as a template for PCR using primers 1 and 8. The resulting product was introduced into Af293 wild-type protoplasts as described above.

**Chemical transformation of *A. fumigatus* protoplasts.** Transformation of *A. fumigatus* protoplasts was performed as described previously (34) with some modification. Briefly, conidia were incubated in glucose minimal medium (GMM) (1% glucose, 10 mM ammonium tartrate, salts, pH 6.5) supplemented with 0.5% yeast extract for 10 h at 30°C. Following incubation, the cell wall of germlings was digested with 3.5 mg/ml lysing enzymes (Sigma, L1412) for 5 h at 28°C. Protoplasts were transformed with ~10 µg DNA using 60% polyethyleneglycol (PEG) 3350 (Sigma-Aldrich) and plated onto GMM supplemented with 1.2 M sorbitol. Following 24 h of incubation at room temperature, plates were overlaid with top agar containing hygromycin or phleomycin and subsequently incubated at 37°C.

**A. fumigatus strains used for this study.** The wild-type *A. fumigatus* strain Af293 was obtained from Judith C. Rhodes at the University of Cincinnati, and the wild-type CEA10 and Δ*ksP* mutant (Af293 background) were obtained from Robert A. Cramer, Jr., at Dartmouth. For construction of the cas9-*hph* strains, the *hph-cas9* fragment was amplified from the *p-hph-Ptef1-cas9-pksP-gRNA* plasmid (see above) using primers 9 and 10. The PCR product was then introduced into wild-type Af293 or CEA10 protoplasts by chemical transformation as described above. Integration of the cas9 gene into transformants was initially determined by PCR of gDNA with primers 3 and 4, followed by Southern blotting (described below).

**Southern blotting.** The number of sites into which the cas9 gene was integrated in Af293 or CEA10 recipient strains was analyzed by Southern blotting using the digoxigenin (DIG) system (Roche). Briefly, 40 µg of genomic DNA was digested in a BamHI and NcoI double digestion in buffer, 0.1 M EDTA, 1 mM NaCl, and 3.1 (New England BioLabs) overnight at 37°C and then electrophoresed in a 1% Tris-acetate-EDTA (TAE) agarose gel at 50 V for 2 h. The gel was transferred onto a positively charged nylon membrane (Roche) via capillary transfer overnight in 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membrane was hybridized overnight at 42°C with 200 ng/ml of a 2.4-kb DIG probe (Roche) targeted to the cas9 gene (see Fig. S1A in the supplemental material). The probe was stained with anti-DIG-alkaline phosphatase (AP) antibody (Roche) and visualized with chemiluminescent CDP-Star (Roche) according to the manufacturer’s instructions. The membrane was exposed to a chemiluminescent X-ray film (GE Healthcare) for 1 to 2 min to visualize bands.

**Western blotting.** The expression of Cas9 in the *cas9-hph* strains was analyzed by Western blotting. Briefly, overnight 37°C liquid cultures of recipient strains were collected, lyophilized, and mechanically broken using 3.5-mm steel beads in protein extraction buffer (50 mM Tris-HCl [pH 7.5], 0.1 M EDTA, 1 mM β-mercaptoethanol) and protease inhibitor (Pierce) for 2 min. The slurry was incubated on ice for 10 min, and then the supernatant was collected after centrifugation at 4°C. The protein amount was determined by the Bradford method using protein assay dye reagent concentrate (Bio-Rad). Twenty micrograms of protein was loaded into a 3 to 8% Tris-acetate polyacrylamide gel and wet transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was washed in 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, followed by incubation for 1 h at room temperature in blocking buffer (5% nonfat dry milk in 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20). The membrane was incubated overnight at 4°C with a 1:2000 dilution of rabbit anti-Cas9 antibody (Bethyl). After washing, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) at 1:20 000. Detection was performed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). After detection, the membrane was washed and rehybridized with a probe specific for the 6× His-tag.
membrane (Millipore). The membrane was stained with a 1:500 dilution of anti-Cas9 monoclonal antibody (Epigentek), washed, and then stained with a 1:3,000 dilution of anti-mouse IgG horseradish peroxidase (HRP)-conjugated antibody (Bio-Rad). The membrane was visualized with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) according to the manufacturer’s instructions and exposed to a chemiluminescence-sensitive X-ray film (GE Healthcare) for 1 to 2 min.

Reverse transcription-PCR (RT-PCR) analysis. Conidia of the wild-type and cas9-hph strains were incubated in GMM (supplemented with 0.5% yeast extract) overnight at 37°C. Tissue was harvested by brief vacuum filtration and then frozen with liquid nitrogen. RNA was isolated by crushing the frozen tissue with a glass rod and processing the powder with an RNeasy kit (Qiagen). Two micrograms of RNA was used for first-strand synthesis (Superscript III; Invitrogen), and 1 μl of cDNA was used in a 20-μl PCR mixture using primers 15 and 16.

Sequencing of the target locus in transformant colonies. The coding sequence of pksP (AfU2g17600) was amplified from each of the strains using Phusion Flash DNA polymerase and primers 17 and 18 and then cleaned using a PCR purification kit (Qiagen). Two micrograms of RNA was used for first-strand synthesis (Superscript III; Invitrogen), and 1 μl of cDNA was used in a 20-μl PCR mixture using primers 15 and 16.

Growth rate and stress resistance assays. To assess the radial growth rate, 2 μl of a 5 × 10⁶ ml⁻¹ conidial suspension of the indicated strains was point inoculated onto the center of a GMM plate. Plates were incubated for 72 h at 37°C.

For the stress tolerance assays, 2 μl of a 5 × 10⁶ ml⁻¹ conidial suspension was plated across 15-mm GMM agar plates and incubated for either two (Af293 background) or three (CEA10 background) days at 37°C. Following incubation, conidia were harvested by swabbing conidia into 10 ml of sterile water, and this was repeated three times (40 ml total). Conidial suspensions were diluted and subsequently enumerated with a hemacytometer. Three plates were analyzed per group.

RESULTS

Targeted disruption of pksP in wild-type A. fumigatus using CRISPR/Cas9. Our overall goal was to assess the feasibility of using the CRISPR/Cas9 system for targeted gene disruption in A. fumigatus. As a proof of principle, we chose to target the pksP gene (AFUA_2G17600), encoding a polyketide synthase essential in the synthesis of dihydroxynaphthalene (DHN) melanin. In contrast to wild-type A. fumigatus colonies, which appear green due to the production of melanized conidia, ΔpksP mutants appear completely white (albino) (30, 31). Accordingly, the successful disruption of pksP could be determined simply by the presence of albino transformants, and the overall targeting efficiency could be discerned from the phenotypic ratios.

The target gRNA sequence was designed based on criteria that had proved successful in S. cerevisiae (24). First, we searched for PAM sites (-NGG) that existed within an exon and approximately 100 to 200 nucleotides downstream of the pksP translational start. The 13-nucleotide sequence directly upstream of each PAM site (i.e., the seed sequence) was used in a BLAST analysis against the A. fumigatus Af293 genome; those that lacked complete identity to another locus were considered candidate gRNA targets. Three sites emerged based on these criteria, the most ATG proximal of which was selected for our studies (Fig. 1).

We first generated a vector that, following random integration into the genome, would allow for pksP targeting in wild-type A. fumigatus (Fig. 2A). The construct consisted of three components: (i) Ptef1-cas9, in which the cas9 nuclease gene, used previously in S. cerevisiae (codon optimized for use in human cells), is driven by

Quantification of conidiation. A 120-μl portion of a 1.0 × 10⁶ ml⁻¹ conidial suspension was plated across 15-mm GMM agar plates and incubated for either two (Af293 background) or three (CEA10 background) days at 37°C. Following incubation, conidia were harvested by swabbing conidia into 10 ml of sterile water, and this was repeated three times (40 ml total). Conidial suspensions were diluted and subsequently enumerated with a hemacytometer. Three plates were analyzed per group.
the teff1 promoter; (ii) pksP-gRNA, consisting of the above-described pksP target sequence fused with the structural gRNA element; and (iii) the hygromycin phosphotransferase gene (hph) for the positive selection of transformants. Following the fusion and cloning of the three cassettes via yeast recombination, the hph-cas9-gRNA insert was PCR amplified and introduced into A. fumigatus (strain AF293) protoplasts by chemical transformation. Two independent transformations yielded a total of 98 hygromycin-resistant colonies, which were subcultured onto GMM plates and incubated for 72 h at 37°C to assess their phenotype. Remarkably, 52 transformants (53%) resembled the ΔpksP control; nonalbino colonies resembled the wild type. (C) Phenotypic ratios of the transformants.

FIG 2 Disruption of pksP using CRISPR/Cas. (A) Scheme of the transformation construct used for transformation into AF293 protoplasts. Transcriptional promoter (P) and terminator (T) elements are noted. (B) Hygromycin-resistant transformants were incubated on GMM to assess their pigmentation phenotype. Representative transformants are shown. Albino colonies resemble the ΔpksP control; nonalbino colonies resemble the wild type. (C) Phenotypic ratios of the transformants.

Because the gRNA portion hph-cas9-gRNA cassette contains homology to the genomic target, albeit across a small region (20 bp), we wanted to exclude the possibility that pksP disruption was due to a homologous recombination event. Accordingly, constructs containing the pksP-gRNA alone (gRNA-hph) or the cas9 gene alone (cas9-hph) were generated and introduced into AF293 wild-type protoplasts. Whereas albino transformants were generated from the hph-cas9-gRNA construct, all hygromycin-resistant colonies derived from the gRNA-hph or the cas9-hph transformations resembled the wild type (see Fig. S1 in the supplemental material). This suggested that both the cas9 gene and the pksP-gRNA are essential components for pksP targeting in our system and that the contribution of homologous recombination alone is negligible.

Effect of constitutive cas9 expression on A. fumigatus growth and virulence. If mutants generated with the CRISPR system are to be useful for pathogenesis studies, it is imperative that expression of the cas9 nuclease itself is not deleterious with respect to A. fumigatus growth or virulence. We sought to test this by generating strains that constitutively express cas9 without the presence of a targeting gRNA. To do so, the Pteff1-cas9 cassette (described above) was fused with the hph marker, and the resulting construct (the same used in the control transformation described above) was introduced into the two most commonly employed A. fumigatus strains, AF293 and CEA10 (Fig. 3A). The integration of cas9 into the genome of hygromycin-resistant colonies was first tested by PCR (data not shown) and then confirmed by Southern blotting (see Fig. S2A in the supplemental material). The expression of cas9 was subsequently confirmed by both Western blot (see Fig. S2B in the supplemental material) and RT-PCR (Fig. 3A) analyses. The colonial appearance of all cas9-expressing transformants (called cas9-hph) was identical to that of the respective AF293 or CEA10 recipient strain. A single isolate in either background, chosen on the basis of fewest cas9 integrations by Southern blotting, was chosen for further phenotypic analysis and use.

Finally, the virulence of the strains was tested in a murine model of invasive aspergillosis. Briefly, groups of CD-1 outbred mice were immunosuppressed with the synthetic corticosteroid triamcinolone acetonide and then subsequently inoculated intranasally with the indicated strains. Whereas the animal survival curves are different in the AF293 and CEA10 backgrounds, the cas9-hph strains were statistically indistinguishable from their respective wild-type controls (Fig. 3D).

In summary, constitutive expression of the cas9 endonuclease does not alter growth, development, stress resistance, and, importantly, virulence in either the AF293 or CEA10 background of A. fumigatus under the tested conditions. The cas9-hph strains as recipients for gRNA transformation.
We next assessed whether the cas9-hph strains could serve as recipients for a separate gRNA transformation. A plasmid that contained both the pksP-gRNA (described above) and the bleR cassette (bleomycin/phleomycin resistance) as a selectable marker was constructed (Fig. 4). Linearized plasmid was introduced into either the Af293 or CEA10 cas9-hph strain by chemical transformation, and the phenotypes of the phleomycin-resistant colonies were analyzed following incubation on GMM. In the Af293 background, 19 out of 41 (46.3%) phleomycin-resistant colonies displayed the albino phenotype, whereas 16 out of 63 (25.4%) were albino in the CEA10 background (Fig. 4). Cumulatively, these data suggest that pksP targeting via CRISPR/Cas is efficient in both the Af293 and CEA10 backgrounds. Moreover, the cas9-hph strains can be used as recipient strains for downstream gRNA transformation and in this way may serve as universal strains for CRISPR-mediated mutagenesis.

Genotypic analysis of the pksP locus in both albino and non-albino transformants. The Cas9/gRNA complex is known to induce double-strand breaks at the gRNA target sequence, which are then resolved through error-prone NHEJ in the absence of a homologous sequence (24). To determine if the albino mutants obtained in our experiments could be attributed to a similar mechanism, primers spanning the 5′ end of the pksP coding sequence were used to generate products (expected size, 1.5 kb), which were then subjected to Sanger sequencing. The Af293 wild-type and cas9-hph recipient strains were analyzed as controls, and both were identical to the Af293 genomic reference at the pksP locus (Fig. 5).

Two of the albino transformants in our analysis yielded a PCR product of the expected size, the sequencing of which revealed that either a single nucleotide insertion or deletion had occurred three base pairs upstream of the targeted PAM site (Fig. 5, transformants 1 and 2). An in silico translation of the mutant sequences

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**FIG 3** Generation and analysis of constitutive cas9-expressing strains. (A) Top, scheme of the construct used for transformation into Af293 or CEA10 wild-type protoplasts. Bottom, RT-PCR analysis confirming cas9 expression in the transformants. (B) Growth rate analysis. Conidia of the indicated strains were point inoculated on GMM and incubated for 72 h at 37°C. Pictures, shown below the respective growth curve, were taken following the 72 h of incubation. (C) Stress sensitivity. Conidia were point inoculated onto GMM plates containing increasing concentrations of the indicated drugs and incubated for 48 h at 37°C. (D) Virulence. Groups of 10 CD-1 mice were immunosuppressed with triamcinolone acetonide and inoculated intranasally with the indicated strains.
predicted a frameshift that would ultimately lead to a premature translational stop within 14 or 25 amino acids of the insertion or deletion, respectively (data not shown). Thus, these single nucleotide indels are consistent with the CRISPR-mediated mutations described in other systems (24).

Surprisingly, most albino colonies yielded amplicons that were of a higher molecular weight than the 1.5 kb band of wild-type, indicating perhaps that larger insertions had taken place. For the single-construct transformation into wild-type, sequencing of the amplicons indeed revealed that a portion of the transforming PCR fragment had integrated at the expected Cas9 cut site. More completely, the insertions included the 3' end of the PCR fragment, extending variably into the structural Cas9 cut site. More completely, the insertions included the 3' end of the PCR fragment, extending variably into the structural gRNA sequence (Fig. 5, transformants 3 and 4). Because non-integrated (episomal) DNA is typically degraded in A. fumigatus, these data suggested that both cas9 and the pksP-gRNA were rapidly expressed within the protoplasts, allowing for the NHEJ machinery to integrate part of the introduced DNA at the Cas9-mediated double-strand break.

Similarly for the transformation of the cas9-hph recipient strains (described in Fig. 4), four out of eight albino colonies yielded products of up to 6.5 kb larger than the expected size, suggesting that the entire linearized plasmid had been inserted into the pksP locus (see Fig. S4 in the supplemental material). This was confirmed, as sequencing of the high-molecular-weight bands (~8 kb) all revealed plasmid integration at the Cas9 cut site (data not shown).

We also analyzed several of the nonalbino transformants, and all yielded sequences that were identical to the wild-type reference (see Fig. S5 in the supplemental material). This suggested that the nonalbino phenotype is most likely due to a lack of targeting at the pksP locus, rather than an insertion/deletion that yielded no functional alteration to PksP (e.g., a single amino acid insertion).

Taken together, these data indicate that the albino phenotypes observed in our transformants were due to the expected Cas9-mediated cutting at the gRNA targeting sequence. The specific NHEJ-mediated events at the cut site varied, however, ranging from the single-nucleotide insertion/deletions, as observed in other systems, to large insertions of transforming DNA constructs.

FIG 4 pksP targeting in cas9-hph recipient strains. A plasmid containing the pksP-gRNA sequence and the bleomycin resistance gene (bleR) was constructed and introduced into Af293 and CEA10 cas9-hph recipient protoplasts. Phleomycin-resistant colonies were incubated on GMM, and the proportion of albino colonies was determined.

DISCUSSION

Gene replacement through homologous recombination can be improved by several approaches in A. fumigatus. First, Krappman et al. showed that by simply increasing the size of homologous flanks in the transforming construct (directed toward abr2), targeting efficiency could be increased from 0% (0.1-kb flanks) to 10% (1 kb) to 22% (2 kb) (10). Though targeting could presumably be increased with even larger flanking regions, the cloning efficiency becomes the limiting factor; therefore, standard protocols typically involve flanking regions of approximately 1 kb on either side of the target gene. The second approach involves introduction of DNA into mutant strains of A. fumigatus that are deficient in an essential component of the NHEJ pathway. For example, in the same study, the replacement of abr2 with 0.1-kb flanks was increased to 75%, and with 2-kb flanks it was increased to 95% in a ΔakuA (ku70) mutant (10). Similarly, da Silva Ferreira et al. demonstrated that pksP targeting could be increased from 3% in a wild-type background to 85% in a ΔakuB (ku80) strain (9). Finally, Agrobacterium tumefaciens-mediated transformation (ATMT) of A. fumigatus conidia has been found to yield recombination rates of 35 to 66%, depending on the gene (38, 39).

Unlike all of the above-mentioned strategies, the CRISPR/Cas9 system bypasses the need for homologous recombination altogether, as the Cas9 nuclease is directed to its target through association with a short gRNA. In this study, we have demonstrated the utility of this system in A. fumigatus by disrupting the pksP gene with an efficiency of 25 to 53%. We propose that the system functions in A. fumigatus as it does in other organisms, based on two lines of evidence: (i) mutant phenotypes were observed only upon introduction of both the cas9 gene and the gRNA, and (ii) pksP disruption occurred at the expected Cas9 cut site in all albino mutants tested. What is perhaps unique to our data, however, is the finding that the transforming DNA can be integrated at the Cas9 cut site, presumably through the NHEJ pathway. Indeed, this phenomenon has recently been described in a mammalian cell CRISPR system (40). However, to our knowledge, this is the first report of such construct integrations among fungal CRISPRs. Why such a mechanism might dominate (>50% of albino transformants tested) in A. fumigatus, but not other fungi, remains unclear.

Importantly, we have demonstrated that the CRISPR/Cas9 system works in two commonly employed strains of A. fumigatus, Af293 and CEA10. Although we did find different targeting ratios in the two backgrounds in our experiment (46.3% for Af293 and 25.4% for CEA10), we cannot yet state whether these differences would hold up across multiple transformations or across different loci. Nevertheless, the ability to introduce CRISPR/Cas9 constructs into any A. fumigatus background represents a distinct advantage of the system. In contrast, although targeting rates may be higher in NHEJ mutants (up to 95%), researchers are confined to work with strains in which akuA/akuB mutations have been generated.

The expression of heterologous enzymes has been known to alter fungal physiology in unpredictable ways. In the dimorphic fungal pathogen Histoplasma capsulatum, for example, the expression of the hph selectable marker does not affect growth or development but remarkably enhances virulence. This influence of
PHH on virulence is dependent upon its phosphotransferase activity, although the exact mechanism is unknown (41). Accordingly, we addressed concerns regarding the impact of the Cas9 nuclease on A. fumigatus by generating strains that constitutively express the cas9 gene. Importantly, we report that the cas9-hph strains are identical to their wild-type controls with respect to all tested phenotypes, including virulence in an immunosuppressed murine model. Thus, growth- and pathogenesis-related phenotypes associated with CRISPR/Cas-generating mutants can likely be attributed to the gene under study rather than to unknown effects of the cas9 transgene. We propose that the cas9-hph strains we have generated could be important community resources for all downstream CRISPR-based experiments for two reasons: (i) the large cas9 cassette would not have to be cloned into the transformation constructs, thereby making gRNA construction more efficient and high throughput, and (ii) a strain that expresses cas9 would serve as a more appropriate reference strain (i.e., control) than the standard wild type.

In summary, we have demonstrated that CRISPR/Cas9 can be developed as a tool for high-efficiency gene disruption in A. fumigatus. The further development of this technology (i.e., multigene targeting and recyclable cassettes for the reuse of selectable markers) is under way and holds significant potential for advancing the state of genetic research in this organism and, consequently, an understanding of its pathogenesis.

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