INFECTION AND IMMUNITY, July 2008, p. 3214–3220 Vol. 76, No. 7
0019-9567/08/$08.00+0 doi:10.1128/IAI.00009-08

Defects in Conidiophore Development and Conidium-Macrophage Interactions in a Dioxygenase Mutant of *Aspergillus fumigatus*▽†

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Received 3 January 2008/Returned for modification 10 February 2008/Accepted 19 April 2008

Oxygenated fatty acids, or oxylipins, play an essential role in physiological signaling and developmental processes in animals, plants, and fungi. Previous characterization of three *Aspergillus fumigatus* dioxygenases (PpoA, PpoB, and PpoC), similar in sequence to mammalian cyclooxygenases, showed that PpoA is responsible for the production of the oxylipins 8*R*-hydroperoxyoctadecadienoic acid and 5*S*,8*R*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid and that PpoC is responsible for 10*R*-hydroxy-8*E*,12*Z*-hydroperoxyoctadecadienoic acid. Here, Δ*ppo* mutants were characterized to elucidate the role of fungal dioxygenases in *A. fumigatus* development and host interactions. The Δ*ppoC* strain displayed distinct phenotypes compared to those of other Δ*ppo* mutants and the wild type, including altered conidium size, germination, and tolerance to oxidative stress as well as increased uptake and killing by primary alveolar macrophages. These experiments implicate oxylipins in pathogen development and suggest that Δ*ppoC* represents a useful model for studying the *A. fumigatus*-host interaction.

Aspergillus species are soil-dwelling, saprophytic fungi and opportunistic pathogens that cause a variety of diseases in plants and animals. One of the most devastating pulmonary diseases caused by *Aspergillus fumigatus* is invasive aspergillosis, cases of which have steadily increased over the past several decades due in part to the increased number of immunocompromised people, the use of immunosuppressive agents, and surveillance; associated mortality rates from IA range from 40% to 90% (8, 9, 10, 15). In addition to difficulties associated with diagnosis and treatment, the pathogenesis and development of *A. fumigatus* remain poorly understood.

Several recent studies implicated a role for oxylipins, oxygenated fatty acid derivatives, in host interactions and fungal development (19, 23). Oxylipins are generated by various oxygenases, including linoleate dioxygenases, the related prostaglandin synthases or cyclooxygenases, and lipoxygenases (4). These metabolites are often associated with sporulation processes for a number of fungal genera including *Aspergillus* (2, 26), *Alternaria* (6), *Sclerotinia* (7), and *Neurospora* (14, 17). For example, the deletion of all three *Aspergillus nidulans ppo* genes resulted in alterations in sporulation patterns, primarily in altering the ratio of asexual spore development to sexual spore development in this species. Exogenous exposure to purified oxylipins also engenders similar developmental processes in *A. nidulans*, thus supporting a direct role for oxylipins as sporulation signals (2). In addition, there is strong support for oxylipin-mediated cross-kingdom signaling between interacting organisms (1, 2, 5).

In fungi, the oxylipin biosynthetic pathway is best understood in *A. nidulans*, where three dioxygenases (PpoA, PpoB, and PpoC) with amino acid sequence homology to mammalian cyclooxygenases have been genetically and biochemically defined (22, 24, 25, 26). Biochemical analyses of *A. nidulans* and *A. fumigatus ppo* mutants revealed PpoA to be an 8*R*-dioxygenase generating 8*R*-hydroperoxyoctadecadienoic acid and 5*S*,8*R*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid and PpoC to be a 10*R*-dioxygenase forming 10*R*-hydroxy-8*E*,12*Z*-hydroperoxyoctadecadienoic acid (3). RNA interference (RNAi)-mediated silencing of *ppoA*, *ppoB*, and *ppoC* in *A. fumigatus* led to reduced levels of prostaglandin production in arachidonic acid-fed cultures, increased tolerance to oxidative stress, and hypervirulence in an animal model of IA (21). However, because of the inability to completely "turn off" *ppo* genes by RNAi silencing, those studies did not clearly address the role of specific dioxygenases in *A. fumigatus* biology and pathogenicity.

In this study, individual *A. fumigatus ppo* mutants were examined to determine the contribution of these oxygenases to *A. fumigatus* development and their impact on host-fungus interactions. We reveal that the disruption of *ppoC* led to a unique phenotype among Δ*ppo* mutants, exhibiting morphological and developmental changes and tolerance to oxidative stress. We also observed increased phagocytosis and killing of this mutant by primary alveolar macrophages, although no difference in virulence was observed in murine models of IA. These experiments implicate PpoC in the regulation of *A. fumigatus* fungal development and host cell interactions.

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† Supplemental material for this article may be found at http://iai.asm.org/.

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▽ Published ahead of print on 28 April 2008.

TABLE 1. *Aspergillus fumigatus* strains used in this study

Fungal strain	Genotype	Source or reference
AF293	Wild type	27
AF293.1	*pyrG1*	27
AF293.6	*pyrG1 argB1*	27
TDWC1.13	Δ*ppoA*::*A. parasiticus pyrG pyrG1*	This study
TDWC2.4	Δ*ppoB*::*A. parasiticus pyrG pyrG1*	This study
TDWC3.4	Δ*ppoC*::*A. nidulans argB argB1 pyrG1*	This study
TDWC4.17	Δ*ppoC*::*A. nidulans argB argB1 A. parasiticus pyrG pyrG1*	This study
TDWC10.5	Δ*ppoC*::*A. nidulans argB argB1 pyrG1 ppoC*::*pyrG*	This study
TDWC10.16	Δ*ppoC*::*A. nidulans argB argB1 pyrG1 ppoC*::*pyrG*	This study
TDWC10.21	Δ*ppoC*::*A. nidulans argB argB1 pyrG1 ppoC*::*pyrG*	This study
TJW62.2	*ppoABC*-RNAi::*A. parasiticus pyrG pyrG1*	21
TJW55.1	*pyrG1 A. parasiticus pyrG*	This study

TABLE 2. Primers used in this study

Oligonucleotide	Sequence
ppoA5FsalI	5′-GATGATGGTTTAAAGTCGACAGCAA-3′
ppoA5RsalI	5′-TAAGTTAAATAGCAGTCGACATGGC-3′
ppoA3FbamHI	5′-CATGAGGCCTGCAGGATCCAATACA-3′
ppoA3RxbaI	5′-CTGAACCAATCTAGAGTGACTCGAC-3′
ppoB5FsalI	5′-TATCATTATTGTCGACCCAGTATTG-3′
ppoB5RecoRI	5′-TAAAAGAATTCGAAATATAGGGGAT-3′
ppoB3FbamHI	5′-CTGGCTTTACCTGGATGATGGATTC-3′
ppoB3RxbaI	5′-TAGGATCTAGAGGATACAGTAACCC-3′
ppoC5FnotI	5′-TCTCACTGCGGCCGCTCCAATTG-3′
ppoC5RsphI	5′-GAGCGAAGAGCATGCCAAAAACTAA-3′
ppoC3FapaI	5′-AAAGCTCTCGGGGCCCATGTCAAGG-3′
ppoC3RkpnI	5′-GCTGGCTTAGGTACCGTACTGGACA-3′
ppoCextF	5′-TCCGAACCTCTCACTGCTCCCA-3′
ppoCextR	5′-GCTGGCTTAGGCATCGTACTGG-3′

MATERIALS AND METHODS

Fungal strains, culture conditions, and transformation conditions. The strains used in the study are listed in Table 1. All strains were propagated at 25°C or 37°C on glucose minimum medium (GMM) (20) or RPMI medium (Sigma, St. Louis, MO) with appropriate supplements and maintained as glycerol stocks.

Nucleic acid analysis and molecular genetic manipulations. Construction, maintenance, and isolation of recombinant plasmids were performed according to standard methods (18). Primers are listed in Table 2. Total fungal genomic DNAs were isolated from lyophilized mycelia as described previously (11). *A. fumigatus ppoA*, *ppoB*, and *ppoC* genes were identified previously (21) and obtained from the database at TIGR (http://www.tigr.org/tdb/e2k1/afu1).

A *ppoA* replacement plasmid, pDWC4.2, carrying *Aspergillus parasiticus pyrG* as a marker gene was created as follows. PCR primers were used to amplify a 1.0-kb 5′-flanking region and a 1.1-kb 3′-flanking region of the *A. fumigatus ppoA* open reading frame (ORF). These primers introduced SalI-SalI and BamHI-XbaI restriction sites (primers ppoA5FsalI and ppoA5RsalI and primers ppoA3FbamHI and ppoA3RxbaI, respectively) (Table 2) at either site of the PCR fragment. These amplified gene fragments were ligated into the upstream and downstream sites of *A. parasiticus pyrG* in vector pJW24 [derived from pBluescript SK(−)]. This resulting vector, pDWC4.2, was used to create the *ppoA* knockout mutant TDWC1.13 by homologous recombination into the *pyrG1* auxotrophic strain AF293.1.

A *ppoB* deletion vector, pDWC6.4, carrying *A. parasiticus pyrG* as a marker gene was designed as follows. PCR primers were used to amplify a 1.0-kb 5′-flanking region and a 950-bp 3′-flanking region of the *A. fumigatus ppoB* ORF. These primers introduced SalI-EcoRI and BamHI-XbaI restriction sites (primers ppoB5FsalI and ppoB5RecoRI and primers ppoB3FbamHI and ppoB3RxbaI, respectively) (Table 2) at either site of the PCR fragment. These amplified gene fragments were ligated into the upstream and downstream sites of *A. parasiticus pyrG* in vector pJW24. The resulting vector, pDWC6.4, was used to create the *ppoB* knockout mutant TDWC2.4.

For *ppoC* disruption vector pDWC7.5, the 1-kb NotI-SphI site of the 5′-flanking region and the 930-bp ApaI-KpnI site of the 3′-flanking region (primers ppoC5FnotI and ppoC5RsphI and primers ppoC3FapaI and ppoC3RkpnI, respectively) (Table 2) were ligated into pUCH2-8 carrying the *A. nidulans argB* cassette. The *pyrG1* and *argB1* double-auxotrophic fungal strain AF293.6 was transformed with pDWC7.5 to create the *ppoC* knockout mutant TDWC3.4. Finally, *A. parasiticus pyrG* was used to transform TDWC3.4 to prototrophic TDWC4.17.

To reconstitute the *ppoC* gene in the *ppoC* deletion strain TDWC3.4, an ca. 5-kb *ppoC* fragment containing 1 kb of the promoter region, 3 kb of the ORF, and 1 kb past the poly(A) tail was amplified from wild-type genomic DNA by PCR and ligated into pJW24. The resulting vector, pDWC10.5, was used to transform TDWC3.4 to create *ppoC*-complemented strains TDWC10.5, TDWC10.16, and TDWC10.21.

Confirmation of gene disruption and ectopic gene complementation of *ppoC* was achieved by both PCR and Southern hybridization for all transformants.

Physiological studies. Sporulation and radial growth rate tests for all strains were performed based on previously reported methods (25). For conidial counts, top glucose minimum agar (0.7%) containing 10^6 conidia of each strain was spread onto GMM agar plates. All strains were grown for 3 days at 37°C. Conidia were counted from three agar cores (diameter, 0.8 cm) for each strain. Radial growth was assayed by measuring the diameter of point-inoculated colonies every 24 h for 4 days. Both experiments were performed in triplicate. To compare spore sizes, fungal strains were cultured on solid GMM at 37°C, and spores were collected in water containing 0.001% Tween 80. One hundred conidia of all strains were measured by a micrometer eyepiece.

Germination tests were conducted in both shaking (300 rpm) and stationary liquid GMM cultures containing 0.1% yeast extract (YE) at 37°C. A total of 10^6 conidia/ml (shaking) or 5×10^4 conidia/ml (stationary) were incubated for various times and examined under a microscope for germination. Different inoculum concentrations were used, as a concentration of 10^6 conidia/ml was too concentrated to observe individual spores in stationary conditions, whereas a concentration of 5×10^4 conidia/ml was not concentrated enough to observe individual spores under shaking conditions. A conidium was considered to be germinated when the germ tube was the same length as the spore. One hundred spores were observed for each strain, time point, and condition. To verify statistical differences from the wild type, the Student *t* test at a 95% confidence interval ($P < 0.05$) was performed.

Oxidative stress tolerance assays. For studying the relative sensitivity to oxidative stress, conidia and hyphae were treated with hydrogen peroxide (H_2O_2) under different concentrations based on previously described methods (21). Here, 10^6 conidia of each strain were incubated in 1 ml of liquid GMM with 0 and 50 mM H_2O_2 for 1 h at room temperature or 37°C, and dilutions were plated onto solid GMM. After 2 days, surviving fungal colonies were counted and calculated as a percentage of the control. Data were considered to be significantly different from those for the wild type at a P value of <0.05 by the Student *t* test.

Primary alveolar macrophage isolation. Bronchoalveolar fluid containing >95% macrophages was collected from female C57BL/6 mice, aged 6 to 12 weeks, following several intratracheal washes with warm phosphate-buffered saline (PBS) containing 0.5 mM EDTA. Macrophages were collected on ice, washed with RPMI medium (Gibco), and resuspended in RPMI medium supplemented with 10% fetal bovine serum and antibiotics (RP10). Viability exceeded 99% as determined by trypan blue exclusion.

Phagocytosis assay. Conidia were labeled with Alexa 594 dye (Invitrogen, Carlsbad, CA) according the manufacturer's instructions for protein labeling. Conidia were washed three times in PBS following labeling and prior to the addition to macrophages. Macrophages (10^5 macrophages/well) were added to culture slides and allowed to adhere for 1 to 2 h at 37°C in 5% CO_2 prior to the addition of Alexa 594-labeled conidia (3×10^5 conidia/well). Unbound conidia were removed after 1 h, and macrophage-conidium cocultures were incubated for an additional 2 h. Wells were then washed with PBS and stained with calcofluor white (CW) to label extracellular conidia. This method permitted discrimination between ingested conidia (Alexa 594 positive and CW negative) and extracellular conidia (Alexa 594 positive and CW positive). Slides were washed in PBS and examined under a Zeiss Axioplan2 microscope fitted with an AxioCam MRm digital camera (Carl Zeiss, Thornwood, NY) using Axiovision 4.5 software (Carl Zeiss). At least four random fields containing approximately 50 macrophages/field were used to calculate phagocytosis and the phagocytic

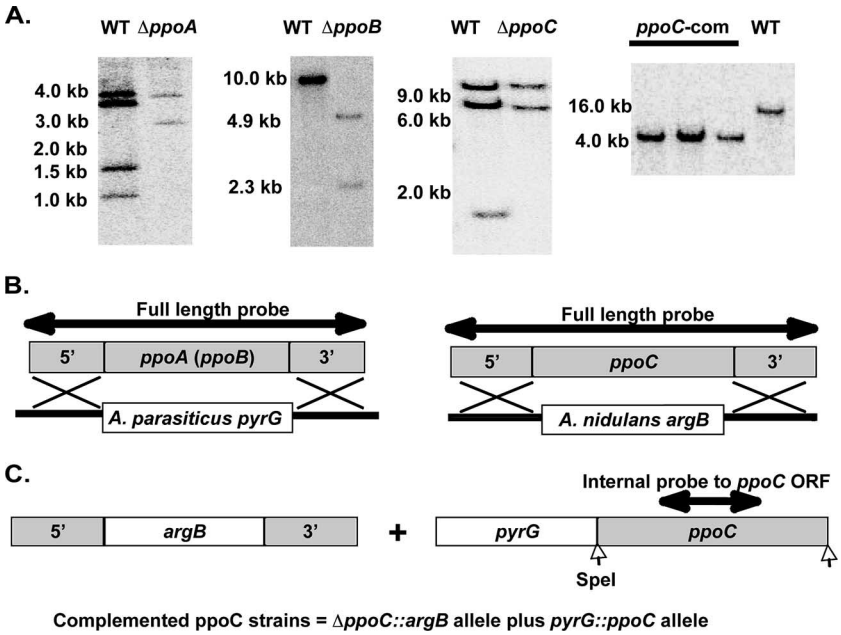

FIG. 1. (A) Southern blots showing digests used to identify correct transformants. A HindIII digest of the wild type (WT) gives 3,718-, 3,111-, 1,365-, and 1,005-bp fragments, and that of the Δ*ppoA* mutant gives 3,493- and 2,691-bp fragments. An EcoRV digest of the wild type gives a 10,425-bp fragment, and that of the Δ*ppoB* mutant gives 4,886- and 2,314-bp fragments. An EcoRV digest of the wild type gives 10,265-, 7,043-, and 1,861-bp fragments, and that of the Δ*ppoC* mutant gives 10,762- and 7,043-bp fragments. A double digest with NotI and SpeI presents a 16,000-bp fragment for the wild type but a 4,900-bp fragment for the complemented Δ*ppoC* strains. The complemented Δ*ppoC* strains contain both the Δ*ppoC*::*argB* allele and a full-length *ppoC* gene inserted ectopically into the genome as described in Materials and Methods. (B and C) Schematic of double-crossover events to obtain Δ*ppoA*, Δ*ppoB*, and Δ*ppoC* transformants (B) and the *pyrG*::*ppoC* vector used to complement the Δ*ppoC* transformant (C). The probes in B and C were used for the Southern analyses shown in A.

index for each sample. Phagocytosis was calculated as the percentage of macrophages containing one or more ingested conidia, and the phagocytic index was calculated as the average number of ingested conidia per phagocytosing macrophage.

Alveolar macrophage killing of conidia. Macrophages (10^5 macrophages/well) were added to 96-well tissue culture plates and incubated for 1 to 2 h at 37°C in 5% CO_2. Wild-type or Δ*ppoC* conidia (5×10^4 conidia/well) were added for 1 h, wells were washed with RP10 to remove unbound conidia, and cocultures were incubated for an additional 8 h. Wells were washed several times with PBS, and macrophages were lysed with water. An equal amount of 2× liquid GMM–0.5% YE was added to each well, and the conidial solutions were transferred onto culture slides for a 10-h incubation to discriminate live (germinated) and dead (nongerminated) conidia. Germinating conidia were not detected in the conidial solutions prior to this incubation. Killing was assayed as the ratio of the number nongerminated conidia to the numbers of total germinated and nongerminated conidia. Data from phagocytosis and killing assays were considered to be significantly different between groups at a P value of <0.001 by the Student t test.

Virulence assays. All animal experiments were approved by the University of Wisconsin animal care committee and followed standard protocols. The virulences of Δ*ppoC*, *ppoC*-complemented, and wild-type strains were compared in vivo using neutropenic and nonneutropenic murine models of IA (21). Six-week-old outbred Swiss ICR mice (Harlan Sprague-Dawley, Indianapolis, IN) were used for these experiments. To induce neutropenia, mice were given cyclophosphamide (100 mg/kg of body weight) through intraperitoneal injection on days −4, 1, and 3 with a single dose of cortisone acetate (200 mg/kg) on day 0. Nonneutropenic mice were given cortisone acetate only (10 mg/mouse) by intraperitoneal injection on days −3, 0, 2, and 4. For both models, conidial suspensions (2.5×10^6 conidia/ml) were prepared in 0.85% saline, and 50 μl of the suspension was inoculated into 10 mice/strain on day 0 through nasal instillation. Mice were monitored three times a day for mortality, and moribund animals were sacrificed. The cumulative number of surviving mice was recorded.

RESULTS

Gene replacements of *ppoA*, *ppoB*, and *ppoC*. The three dioxygenase loci were identified in a previous study (25). Disruption of the *ppo* loci was obtained by the replacement of *ppoA* and *ppoB* by *pyrG* and *ppoC* by *argB* as schematically represented in Fig. 1. Several correct gene replacement transformants, as determined by DNA fragment patterns in Southern analysis (Fig. 1; see Fig. S1 in the supplemental material), were identified for all three genes. There was no difference in the radial growth rates among all Δ*ppo* mutants and the wild type (data not shown). One transformant for each gene replacement was selected for in-depth physiological analysis, as listed in Table 1. The Δ*ppoC* strain, showing a pleiotropic phenotype, was complemented by a full-length copy of *ppoC* as confirmed by Southern analysis (Fig. 1). Three complemented strains were retained for the study, with TDWC10.5 being selected for most studies (Table 1).

PpoC is required for normal conidial development. Given that the disruption of *ppo* genes in *A. nidulans* impacted the ratio of sexual to asexual spore balance, we reasoned that the loss of *ppo* genes could affect the sporulation process of *A. fumigatus*. Figure 2A shows that the Δ*ppoC* strain (TDWC4.17) produced fewer conidia than did the other strains; a similar reduction in conidial numbers in the *A. nidulans* Δ*ppoC* strain was observed previously (24). Δ*ppoA*, Δ*ppoB*, and the previously described *ppoABC*-RNAi mutants did not show significant differences from the wild type with regard to spore production. All three *ppoC*

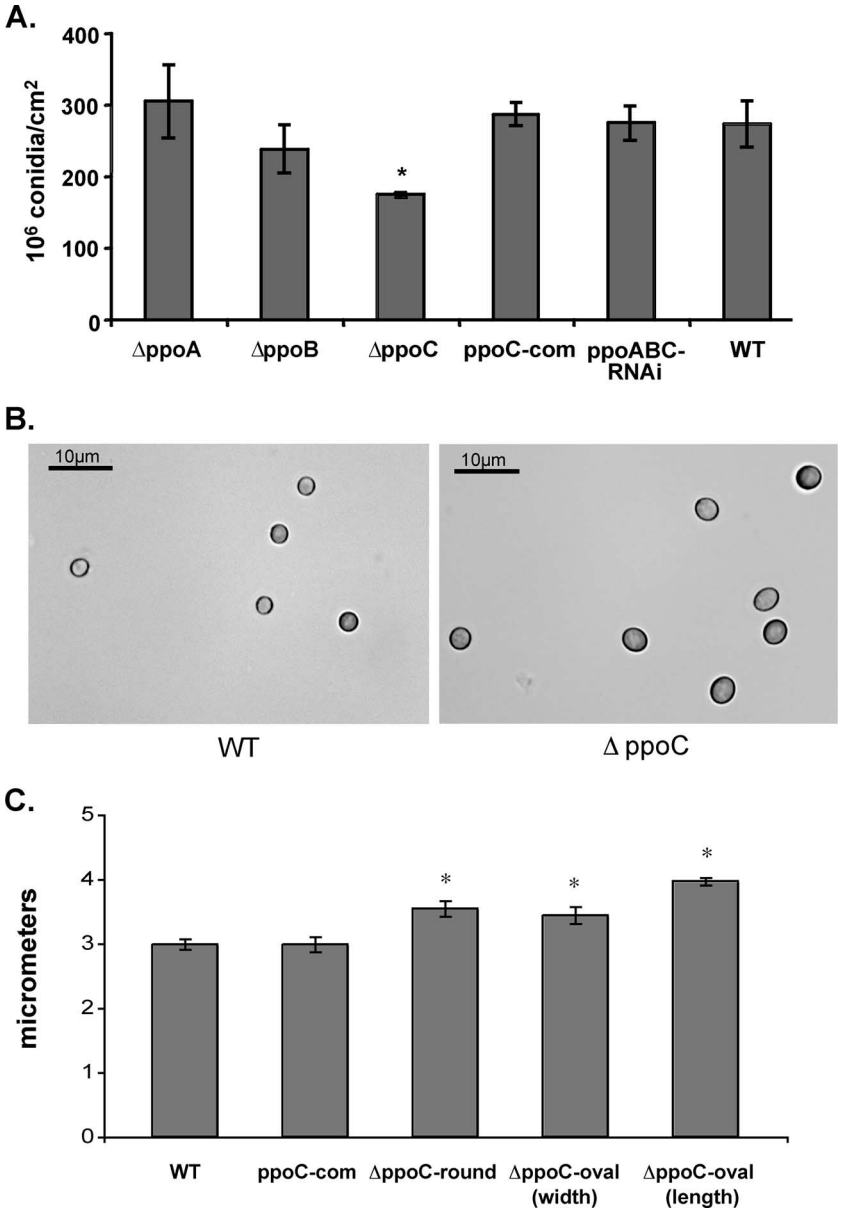

FIG. 2. Conidial size and production in *ppo* mutants and the wild type (WT). (A) Conidia from three agar cores for each strain were counted after 3 days of growth at 37°C. Means ± standard errors are indicated. Asterisks indicate a significant difference from the wild type ($P < 0.05$). (B) Resting wild-type and resting Δ*ppoC* conidia were examined by light microscopy. (C) Averaged measurements of wild-type, *ppoC*-complemented, round Δ*ppoC*, and oval Δ*ppoC* conidia using a micrometer eyepiece. Three replicates of 50 conidia were counted per sample; oval Δ*ppoC* conidium sizes are provided in widths and lengths. Means ± standard errors are indicated. Asterisks indicate a significant difference from the wild type ($P < 0.05$).

complementation transformants (TDWC10.5, TDWC10.16, and TDWC10.21) restored the wild-type phenotype (Fig. 2A and data not shown). Examination of the different strains under a microscope revealed that the majority of Δ*ppoC* conidia displayed an oval shape, and both oval and round Δ*ppoC* conidia were significantly larger than those of wild-type and other *ppo* strains (Fig. 2B and C and data not shown).

Δ*ppoC* conidia germinate faster in shaking cultures and slower in stationary cultures. Hyphae are the only fungal form found in *Aspergillus* infections, highlighting the importance of conidial germination in the establishment of infection. To examine the role of Ppos in the germination of *A. fumigatus*, *ppo* disruption mutants and wild-type *A. fumigatus* were cultured under two different conditions. First, fungal strains were incubated in GMM–0.1% YE liquid shaking medium for 6 h, and germlings were counted in 2-h time intervals. Under these conditions, the Δ*ppoC* mutant germinated faster than other Δ*ppo* mutants, the wild type, and *ppoC*-complemented strains (Fig. 3A). All strains were also incubated in GMM–0.1% YE liquid stationary medium. In contrast to shaking conditions, the Δ*ppoC* strain was delayed in germination compared to other Δ*ppo* strains and the wild

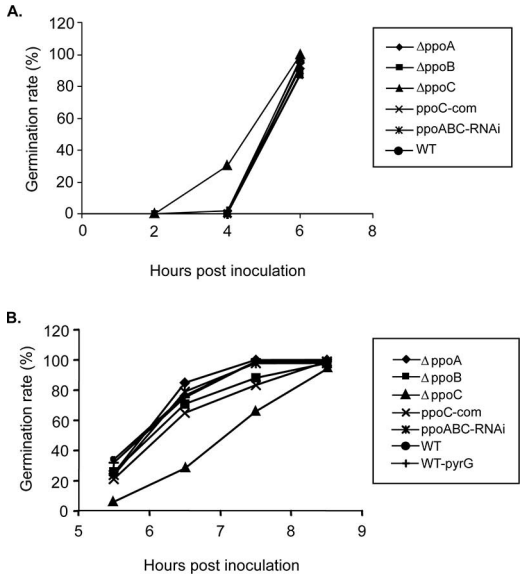

FIG. 3. Germination rates in *ppo* mutants and the wild type (WT). Conidia were incubated in shaking (A) or stationary (B) liquid GMM–0.1% YE at the times indicated. One hundred conidia for each strain were assessed for germination. Data are representative of three independent experiments.

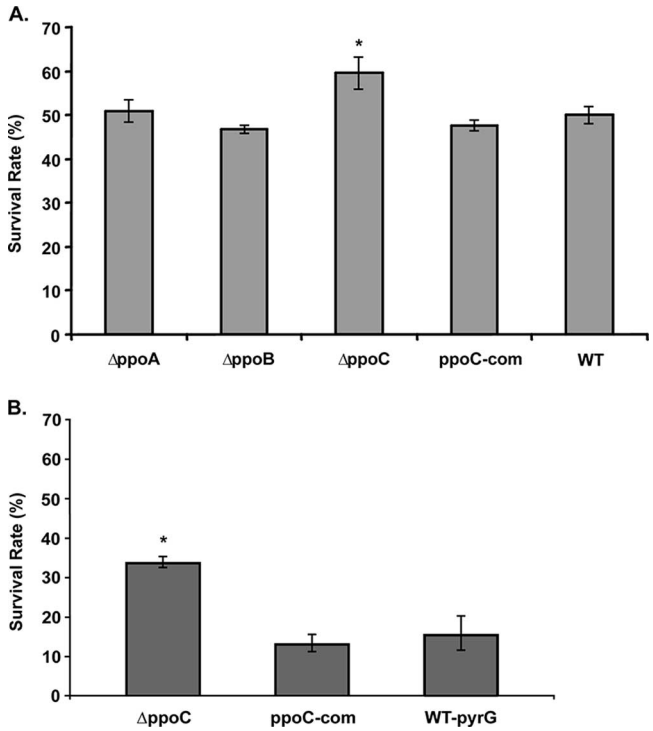

FIG. 4. Survival of *ppo* mutant and wild-type (WT) conidia after H_2O_2 treatment. Conidial suspensions of 10^6 spores were incubated with 0 and 50 mM H_2O_2 for 1 h at room temperature (A) or 37°C (B). Each spore suspension was diluted and transferred onto solid GMM plates. After incubation at 37°C for 36 h, colony numbers were counted and calculated as a percentage of the control (0 mM). The experiments were performed in triplicate. Means ± standard errors are indicated. Asterisks indicate a significant difference from the wild type ($P < 0.05$).

type (Fig. 3B). *ppoC*-complemented strains restored the wild-type germination rate under both conditions.

Oxidative stress tolerance in the *ppoC* null mutant. Treatment with H_2O_2 in vitro is commonly employed to assess the relative resistance of pathogenic fungi to oxidative stress. Previous examination of the *ppoABC*-RNAi mutant demonstrated that conidia were more resistant to H_2O_2 than was the wild type (21). An analysis of conidial tolerance to H_2O_2 as assessed by the survival rate showed an increase in survivability of Δ*ppoC* conidia at both room temperature and 37°C (Fig. 4). The complemented strain restored wild-type susceptibility.

The Δ*ppoC* mutant is more susceptible to phagocytosis and killing by alveolar macrophages. The phenotypic differences in the Δ*ppoC* mutant led us to investigate the interaction of the Δ*ppoC* mutant with primary murine alveolar macrophages, the first line of defense upon conidial inhalation. Adherent alveolar macrophages were combined with Alexa 594-labeled Δ*ppoC* or wild-type conidia and counterstained with CW to distinguish ingested and extracellular conidia. A significant increase in the number of macrophages ingesting Δ*ppoC* conidia compared to wild-type conidia was observed, suggesting more efficient recognition of Δ*ppoC* conidia within the initial 1 h of incubation (Fig. 5A and C). The phagocytic index, or the average number of conidia per macrophage, was also greater for macrophages containing Δ*ppoC* conidia (Fig. 5B and C). Furthermore, when Δ*ppoC* or wild-type conidia were cultured with macrophages for 9 h, macrophages killed three times the number of Δ*ppoC* conidia compared to wild-type conidia (27.1% versus 8.9%, respectively) (Fig. 5D). These in vitro data suggested a possible reduction in the virulence of Δ*ppoC*. However, we were unable to observe significant differences in virulence between Δ*ppoC* and wild-type strains using either neutropenic (immunosuppression by cyclophosphamide and cortisone acetate) or nonneutropenic (cortisone acetate only) murine models of IA (Fig. 5E and data not shown).

DISCUSSION

Physiological examination of the Δ*ppoC* strain showed distinct phenotypes compared to those of other Δ*ppo* and wild-type strains. The reduced sporulation of the Δ*ppoC* mutant agreed with previous findings with *A. nidulans* that *ppoC* positively regulates conidium production (24). As both *A. fumigatus* and *A. nidulans* Δ*ppoC* mutants share a similar decrease in conidial production, we propose that the major PpoC oxylipin 10*R*-hydroxy-8*E*,12*Z*-hydroperoxyoctadecadienoic acid (3) represents one of the *Aspergillus* sporulation factors first described by Mazur and coworkers nearly 20 years ago (13). The promiscuity of oxygenases may also allow for some production of prostaglandin-like species by this protein, although detailed chemistry is necessary to authenticate this possibility (21).

Considering the multiple alterations in the conidial development of this mutant, we focused on conidium-host interactions by exposing Δ*ppoC* conidia to alveolar macrophages, primary mediators of host defense upon conidial inhalation. Intriguingly, we found that primary murine alveolar macrophages engulf and kill significantly more Δ*ppoC* conidia than wild-type conidia. The greater resistance of Δ*ppoC* spores to

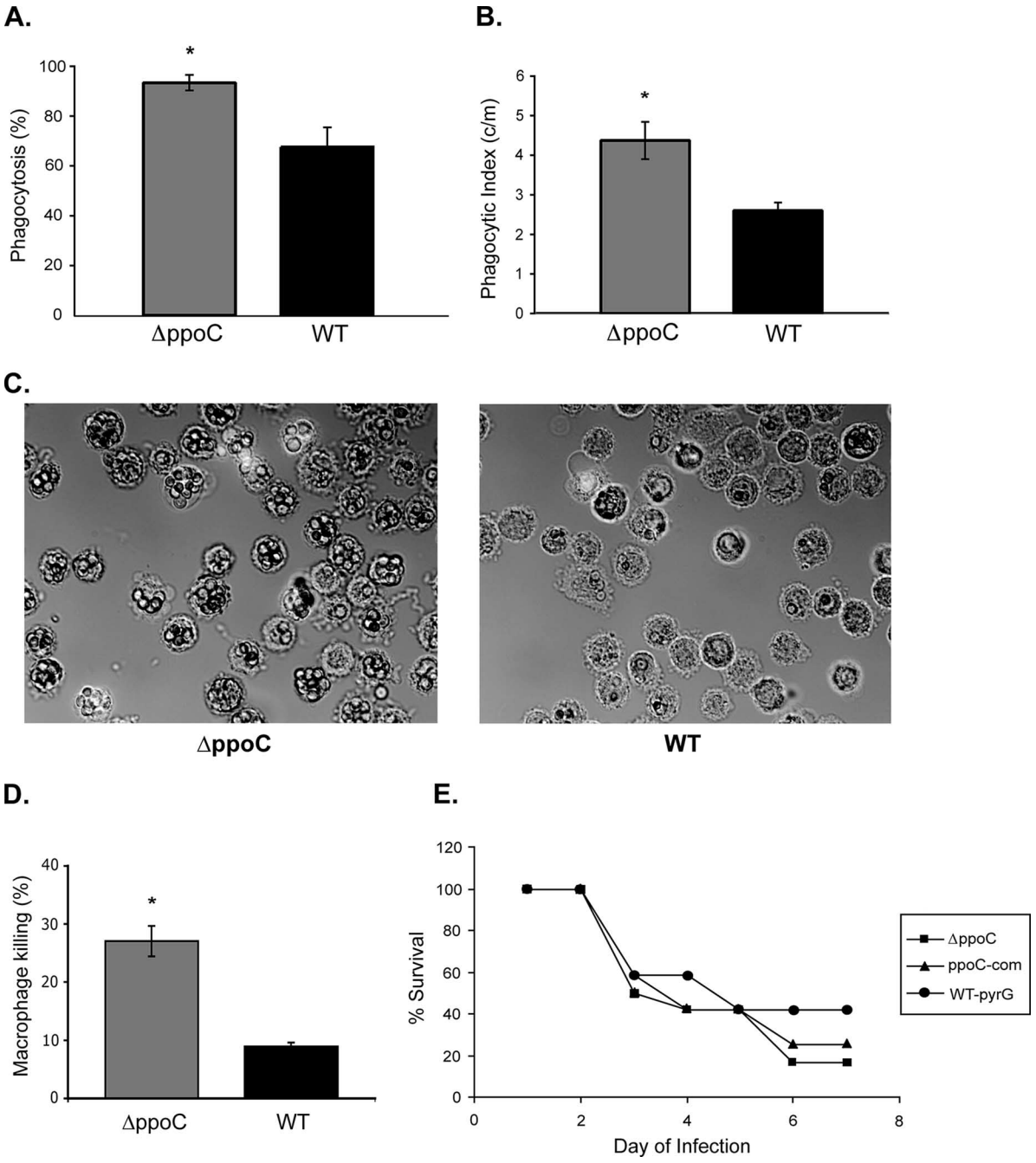

FIG. 5. Primary murine alveolar macrophage responses to wild-type (WT) and Δ*ppoC* conidia. (A, B, and C) Macrophages were combined with Alexa 594-labeled wild-type or Δ*ppoC* conidia for 1 h at 37°C, washed, and incubated an additional 2 h before counterstaining with CW. Phagocytosis (percentage of macrophages containing 1 or more ingested conidia) (A) and the phagocytic index (average number of ingested conidia per phagocytosing macrophage [c/m]) (B) were calculated from at least four replicates per strain. Means ± standard errors are shown. Data represent at least three independent experiments. (C) Light microscopy of adherent macrophages containing Δ*ppoC* or wild-type conidia. (D) Macrophages were cultured with wild-type or Δ*ppoC* conidia for 1 h, washed, and incubated for an additional 8 h. Macrophages were lysed in water, combined with 2× GMM–0.5% YE, and cultured for 10 h to discriminate live (germinated) and dead (nongerminated) conidia. Macrophage killing was calculated as the number of live conidia/total number of conidia × 100. Four replicates with 100 conidia each were counted; means ± standard errors are shown. Results are representative of two independent experiments. Asterisks indicate a significant difference from the wild type ($P < 0.05$). (E) Virulence of the Δ*ppoC* mutant was not statistically significant from that of the wild type in a neutropenic murine model of IA. Outbred Swiss ICR mice immunosuppressed with cyclophosphamide and cortisone acetate were infected with either Δ*ppoC*, *ppoC*-complemented, or wild-type conidia (1.25×10^5 conidia/mouse; 10 mice/strain) by nasal instillation and monitored for mortality for 7 days.

H_2O_2 suggested an increased tolerance to reactive oxygen species (ROS)-mediated antimicrobial host responses. Although macrophages from immunocompetent hosts utilize ROS to kill engulfed *A. fumigatus* conidia, the relevance of ROS-mediated *A. fumigatus* killing in an immunocompromised host has been questioned (12, 16). That fact that Δ*ppoC* conidia were killed at a significantly higher percentage than were wild-type conidia suggests that the H_2O_2 test is not predictive of spore susceptibility to ROS host responses and/or that other defenses such as nitrosative stress or phagolysosomal acidification contribute to the increased killing of Δ*ppoC* conidia.

Despite the increased susceptibility of Δ*ppoC* conidia to macrophage ingestion and killing, we did not observe a reduced virulence of Δ*ppoC* in either neutropenic or nonneutropenic animal models. Reduced alveolar macrophage function and/or number as a result of drug treatment could negate any correlation between host survival and susceptibility to macrophages in vitro. Regardless, our results demonstrate that *ppoC* enhances resistance to alveolar macrophage defenses. The increased recognition and increased uptake of Δ*ppoC* conidia suggest altered cell wall composition or distribution, and the contribution of *ppoC* to normal cell wall development is currently under investigation.

In summary, these studies provide the first biological characterization of Ppo deletions in *A. fumigatus*. PpoC is the only linoleate dioxygenase to play a significant role in the fungal development of this species. The deletion of *ppoC* resulted in a unique phenotype with altered conidial size, an altered germination rate, and increased phagocytosis and killing by alveolar macrophages. An understanding of how PpoC affects development and the interaction with host immune cells will provide further insight into the role of oxylipins and the processes that they regulate in *Aspergillus*.

ACKNOWLEDGMENTS

This research was funded by NIH 1 R01 Al065728-01 to N.P.K. and D.A. and NSF MCB-0196233 to N.P.K.

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Editor: A. Casadevall