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# Identification and recombinant expression of a cutinase from *Papiliotrema laurentii* that hydrolyzes natural and synthetic polyesters

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**ABSTRACT** Given the multitude of extracellular enzymes at their disposal, many of which are designed to degrade nature's polymers (lignin, cutin, cellulose, etc.), fungi are adept at targeting synthetic polyesters with similar chemical composition. Microbial-influenced deterioration of xenobiotic polymeric surfaces is an area of interest for material scientists as these are important for the conservation of the underlying structural materials. Here, we describe the isolation and characterization of the *Papiliotrema laurentii* 5307AH (*P. laurentii*) cutinase, Plcut1. *P. laurentii* is basidiomycete yeast with the ability to disperse Impranil-DLN (Impranil), a colloidal polyester polyurethane, in agar plates. To test whether the fungal factor involved in this clearing was a secreted enzyme, we screened the ability of *P. laurentii* culture supernatants to disperse Impranil. Using size exclusion chromatography (SEC), we isolated fractions that contained Impranil-clearing activity. These fractions harbored a single ~22 kD band, which was excised and subjected to peptide sequencing. Homology searches using the peptide sequences identified, revealed that the protein Papla1 543643 (Plcut1) displays similarities to serine esterase and cutinase family of proteins. Biochemical assays using recombinant Plcut1 confirmed that this enzyme has the capability to hydrolyze Impranil, soluble esterase substrates, and apple cutin. Finally, we confirmed the presence of the Plcut1 in culture supernatants using a custom antibody that specifically recognizes this protein. The work shown here supports a major role for the Plcut1 in the fungal degradation of natural polyesters and xenobiotic polymer surfaces.

**IMPORTANCE** Fungi play a vital role in the execution of a broad range of biological processes that drive ecosystem function through production of a diverse arsenal of enzymes. However, the universal reactivity of these enzymes is a current problem for the built environment and the undesired degradation of polymeric materials in protective coatings. Here, we report the identification and characterization of a hydrolase from *Papiliotrema laurentii* 5307AH, an aircraft-derived fungal isolate found colonizing a biodeteriorated polymer-coated surface. We show that *P. laurentii* secretes a cutinase capable of hydrolyzing soluble esters as well as ester-based compounds forming solid surface coatings. These findings indicate that this fungus plays a significant role in biodeterioration through the production of a cutinase adept at degrading ester-based polymers, some of which form the backbone of protective surface coatings. The work shown here provides insights into the mechanisms employed by fungi to degrade xenobiotic polymers.

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Fungi are crucial to a variety of biological processes occurring in the environment. Fungi are well known for the degradation of organic matter in soil ecosystems, especially wood, from which these organisms can obtain nutrients and energy (1, 2). Through the expression of a diverse arsenal of extracellular enzymes, naturally designed to degrade natural polymeric compounds (lignin, cellulose, cutin, etc.), fungi are adept at targeting xenobiotic compounds with similar chemical composition (3, 4). Due to the universal action of these fungal enzymes (lipases, esterases, cutinases, etc.), fungi are candidates for plastic waste bioremediation and other pollutants, including hazardous chemicals (5–7). Furthermore, fungal extracellular enzymes represent a significant problem in the undesired colonization and deterioration of the built environment, such as polymeric materials with ester or urethane linkages in their backbone (8–11).

Polyester and polyether polyurethane-based polymers are commonly applied as physical barriers between underlying structural materials (e.g., wood, steel, and aluminum) and the environment, and are used widely to prevent deterioration by physical, chemical, and biological processes. Several studies have examined the enzymatic basis for polyester polyurethane degradation by bacteria and fungi. For example, the lipases PueA and PueB from select Pseudomonads have been shown to contribute to the degradation of the model colloidal polyester polyurethane Impranil (12, 13). Impranil is a protected aliphatic polyester-polyurethane colloidal dispersion used for textile, leather, and aircraft fabric coatings (14). Additionally, it has been used as a model polymer for the study of the degradation of polyester polyurethanes (12, 14–16). For the last 30 years, organisms and their enzymes have been classified as being able to degrade polyurethanes using Impranil, with the first article describing this application published in 1984 (14, 17). For instance, an esterase produced by *Corynebacterium* was shown to hydrolyze polyester polyurethanes (18), and a polyester hydrolase from actinomycetes was able to degrade Impranil as well as Elastollan B85A-10 and C85A-10 (19). Also, many cutinase enzymes (EC 3.1.1.74) have been isolated and reported for their ability to degrade thermoplastics, such polyethylene terephthalate (PET) and other related polyesters (20, 21). Cutinases, originally discovered in fungi, are enzymes that hydrolyze the ester bonds of plant cutin (22). Previously, our group reported that *Papiliotrema laurentii* (synonym *Cryptococcus laurentii*), an environmental, non-motile, encapsulated saprotrophic fungus that is rarely associated with human infection (23–26), can hydrolyze polyester-based coatings in nutrient-deprived conditions (8, 27). However, the specific fungal factors involved in this process remained to be identified. Thus, the purpose of this work was to understand the responsible enzymatic factors involved in these degradative processes.

In this work, we demonstrate that *P. laurentii* 5307AH culture supernatants are capable of clearing Impranil. We isolated supernatant fractions with activity against Impranil, soluble esters, and identified the responsible putative hydrolytic enzyme as a true cutinase with high similarity to a cutinase-like enzyme from *Cryptococcus* sp. S-2 (28, 29). These findings lead to the hypothesis that cutinases and their respective biochemical activity represent a major degradative threat to surface coatings with polyester backbones. For instance, military performance topcoats for aerospace applications with polyester backbones are available commercially (Sherwin Williams, Aerospace Topcoats) and could be subjected to enzymatic degradation. Here, we provide key insights into the degradative mechanisms employed by *P. laurentii* to breakdown complex natural compounds and synthetic polyesters by directly linking the presence of the Plcut1 enzyme to hydrolytic activity.

## MATERIALS AND METHODS

### Culture conditions

*P. laurentii* strain 5307AH was isolated from an environmental consortium within an aircraft (8, 30), and its closest relatives are found within the Tremellales (Fig. S1). Cultures were maintained in glycerol stocks at −80°C, and overnight cultures were inoculated from tryptic soy agar (TSA) plates. Overnight cultures were grown from isolated colonies in sterile tryptic soy broth (TSB) at 27°C overnight. For strain requests, please contact Dr. Chia S. Hung (chia-suei.hung.2@us.af.mil).

### Screening culture supernatants for hydrolytic activity

Overnight *P. laurentii* 5307AH cultures were diluted 1:1,000 into 50 mL of TSB and were incubated for 20–24 h with shaking at 27°C. Clarified growth medium was obtained by centrifuging the cultures in 50-mL conical tubes at >7,200 × *g* for 10 min, and the supernatant was filter sterilized (0.22-µm filters). The clarified supernatant was concentrated 50-fold using 3,000 nominal molecular weight (MW) cutoff centrifugal concentrators (Pierce). Aliquots of 2–3 mL were injected onto a Sephacryl S-300 HR HiPrep size exclusion column by a Bio-Rad FPLC. Fractions were eluted in phosphate-buffered saline (PBS) (150 mM sodium chloride, 50 mM sodium phosphate, pH 7.4) at 0.5 mL/min. Fractions were collected in 5-mL aliquots and were screened for the ability to clear Impranil in TSA plates. For SDS-PAGE, the active fractions were reconcentrated (~40-fold) via 3,000 nominal molecular weight cutoff concentrators, and the samples were resolved in Novex Tris-Glycine gradient gels (4%–20%, Thermo Fisher).

Esterase activity in *P. laurentii* culture supernatants in TSB or TSB supplemented with glucose was monitored following a previously published protocol (12) with some modifications. Briefly, a single *P. laurentii* colony was inoculated in 10 mL of TSB followed by mixing to completely resuspend the inoculum. Next, the culture was split into two 5-mL cultures and glucose was added, except for control samples that contained no additional carbon source, to a final concentration of 2%. The cultures were incubated overnight with shaking at 27°C. The cultures were pelleted, and the supernatants were diluted 1:50 in fresh TSB. The diluted supernatant (495 µL) was mixed with 5 µL of a 25-mM 4-nitrophenyl hexanoate solution, and 150 µL of the reaction was immediately transferred to a 96-well plate. The $OD_{405nm}$ was monitored for 25 min using a Spectra Max M3 plate reader (Molecular Devices, CA, USA). Plcut1 levels were determined in parallel by Western blot (see section below).

### Cloning, protein purification, and activity assays with recombinant enzyme

To purify recombinant Plcut1, the predicted ORF was codon optimized and cloned into pET28a using standard cloning techniques, to generate pET29a::*plcut1* for the expression of His6-tagged Plcut1 in *Escherichia coli* BL21. Plcut1 was amplified by PCR using the primers and Plcut1 codon-optimized sequence listed in Table S1. Overnight cultures of the *E. coli* BL21 strain carrying pET29a::*plcut1* were diluted 1:100 in 1 L LB with kanamycin (50 µg/mL) and were grown to an $OD_{600nm}$ of 0.7 at 37°C. Protein expression was induced with 0.1 mM IPTG for 16–18 h at 18°C. Harvested cells were lysed by sonication, and the cleared supernatant was incubated with nickel beads (Qiagen) for 2 h at 4°C with rotation. The beads were washed with 50× bed volume with Tris buffer salt (TBS, 50 mM Tris-Cl, 150 mM NaCl, pH 7.4). His6-tagged recombinant Plcut1 was eluted with TBS containing 300-mm Imidazole and desalted using a desalting column (Bio-Rad). The protein was further purified by size exclusion chromatography (SEC) with an FPLC system using a Superdex 200 10/300 GL column (GE Healthcare). The TBS buffer was used as the mobile phase, and the flow rate was set at 0.4 mL/min.

For Impranil dispersion assays using recombinant Plcut1, Impranil (Lot: LP17M0022, Covestro) was diluted 1:20 in TBS. The resulting solution (10 µL) was mixed with 490 µL of TBS and 500 ng of Plcut1. Samples were briefly vortexed, and 150 µL was transferred to 96-well plates to monitor Impranil hydrolysis over time by measuring the $OD_{600nm}$

using a Spectra Max M3 plate reader. Impranil-coated glass slides were prepared by pipetting and gently spreading Impranil stock solutions directly onto the surface of the glass slides. The glass slides were incubated at room temperature for 3 d for the solvent to completely evaporate. Four microliters of Plcut1 (0.5 µg/µL) was drop casted onto the glass slide and incubated for 2 d at 95% RH, 27°C before imaging on a Keyence VK-X250 Microscope.

For determination of esterase activity using soluble esters, 5 µL of a 25-mM soluble ester substrate solution was mixed with 495 µL of TBS, followed by addition, or not, of 15 ng of recombinant Plcut1. One hundred fifty microliters was transferred to a 96-well plate, and the $OD_{405nm}$ was monitored over time using the same plate reader mentioned above. Impranil and ester hydrolysis assays were performed at room temperature.

### Western blots

Polyclonal antibodies against Plcut1 were generated by Pocono Rabbit Farm and Laboratory (Canadensis, PA, USA) following standard protocols. Protein samples resolved by SDS-PAGE were transferred onto PVDF membranes (Bio-Rad Turbo transfer system), followed by blocking with 4% milk in a PBS buffer containing 0.5% Tween-20 (PBS-T). Membranes were washed three times with PBS-T with gentle rocking for 5 min and were incubated with rabbit anti-Plcut1 serum diluted 1:2,000 in PBS-T overnight with gentle rocking at 4°C. Next day, the membranes were washed three times with PBS-T with gentle rocking. An Alexa Fluor 680-conjugated anti-rabbit secondary antibody (Invitrogen) was diluted 1:5,000 in PBS-T, and a ChemiDoc Imaging System (Bio-Rad) was used to detect the signals.

### Gas chromatography with mass spectroscopy (GC-MS) of hydrolyzed apple cutin

The ATR-IR (Cary 630 Diamond ATR-IR Accessory) of the isolated cutin sheets were identical to previously published IR spectra (31) for apple cutin with -OH stretches between 3,400–3,200 $cm^{-1}$ and the C=O stretch of the polyester at 1,731 $cm^{-1}$ (Fig. S2). The preparation of the hydrolyzed cutin for GC-MS was performed as previously published (32, 33). Briefly, the freeze-dried material in each vial from the enzymatic hydrolysis step was exposed to 0.2 mL of N-O-bis(trimethylsilyl)acetamide (Sigma) at 90°C for 25 min. The excess silylating agent was removed via evaporation using a stream of nitrogen gas. The sample was then dissolved in 1 mL of chloroform, filtered with a 0.2-µm PTFE syringe filter into the autosampler vials. A Trace 1310 Gas-Chromatography System with ISQ 7000 Single Quadrupole Mass Spectroscopy detector was used to analyze the hydrolysis products from the control and enzyme containing samples with electron impact (EI) ionization (70 eV, source temperature 200°C). Samples were injected directly onto a 30-m Rtx5-MS capillary column (Restek) using a splitless injection and a He carrier gas. The injector temperature was 250°C. The separations were performed using temperature profile starting at 80°C (holding for 2 min), followed by a 10°C/min temperature gradient to 150°C and holding for 5 min. Then, the temperature was increased to 300°C (5°C/min) and held for 2 additional minutes. The hydrolysis products were identified by comparing the mass spectra of each compound to the mass spectra contained in the five standard libraries present on the Chromeleon 7 software package (Thermo Fisher, 2019) and the NIST MS database within the program. No compounds were reported that had a matching probability, based on the fragmentation patterns, below 85%. We also focused on library matches that contained trimethylsilyl (TMS) groups as well.

## RESULTS

### *P. laurentii* supernatants clear Impranil dispersions

Recent findings showed that *P. laurentii* biofilms and planktonic cultures express factors capable of clearing Impranil and degrading polyester polyurethanes coatings (8, 27), but

it remained unclear if this occurred through a secreted factor. Thus, we hypothesized that *P. laurentii* secreted an active enzyme capable of hydrolyzing polyesters. To test this hypothesis, we used the dissolution of the Impranil dispersions by *P. laurentii* supernatants as a qualitative indication of hydrolytic activity (14). Filter-sterilized, concentrated supernatants from overnight cultures were drop casted onto Impranil-containing TSA agar plates. Incubation of the concentrated supernatants on TSA-Impranil plates leads to Impranil deterioration, as shown by clearing and creation of a "halo" in the area where the supernatant was spotted (Fig. 1A). The region exposed to the concentrated supernatant showed obvious signs of clearing compared to the control region where the colloid is unchanged.

## Identification of a hydrolytic protein in Impranil-clearing supernatant fractions

To identify the protein(s) responsible for Impranil clearing in the culture supernatants shown in Fig. 1A, we utilized SEC to isolate Impranil-degrading fractions. We isolated one fraction that induced Impranil clearing when compared to buffer controls (Fig. 1B). The hydrolytic and concentrated fractions were separated via SDS-PAGE, and a single prominent band was observed in the fraction with a molecular weight of ~22 kD (Fig. 1C).

To determine the identity of the protein in the active fractions, peptide fragments from a tryptic digest were sequenced using liquid chromatography tandem mass spectrometry (LC-MS/MS). A total of seven peptide sequences were identified and are listed in Table S2. Top BLAST hits for peptides (1) and (2) are listed in Table S3. These peptides encoded similarities to a range of putative cutinases and esterases from a broad range of organisms.

The peptides detected by MS were used to search the *P. laurentii* 5307AH genome (accession #PRJNA500119) and were found to match segments of a predicted ORF with a putative cutinase domain (Papla1 543643, herein named "Plcut1"). Similarly, BLAST

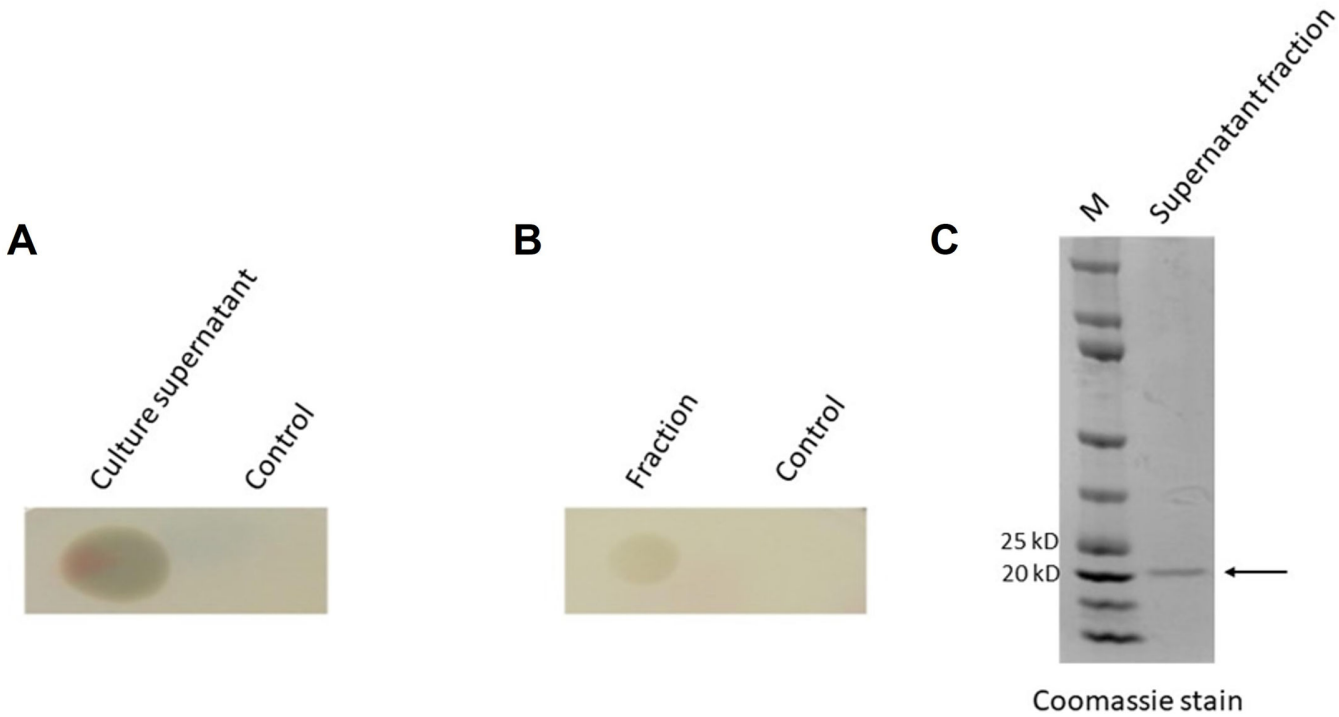


**FIG 1** *P. laurentii* supernatants hydrolyze Impranil. (A) 50× concentrated supernatant disperse Impranil suspended in a TSA plate. (B) Isolation of an Impranil-clearing fraction through size exclusion chromatography. (C) SDS-PAGE analysis of the fraction described in (B), showing a single prominent band with a size of ~22 kD (arrow).

searches using the full Plcut1 sequence returned hits against proteins from the family of esterases and cutinases (Table S4). Cutinases are present across the fungal tree of life and their distribution across the Dikarya is shown (Fig. 2; Table S5). The Plcut1 ORF (237 residues long) was found to encode a 20 amino acid N-terminal signal peptide cleavage site using SignalP (34), which would result in a 217 amino acid mature protein with a predicted MW of 22,413 Da. The predicted MW of Plcut1 is closely aligned with the experimental MW (Fig. 1C). The *in silico* structure of Plcut1 is shown and it does not possess a lid over the enzyme site (Fig. S3). Multiple sequence alignments showed that Plcut1 harbors substantial sequence similarities with other cutinases (22, 28, 35–37), including the classical α/β hydrolase catalytic triad (Fig. 3). Phylogenetic analysis clusters Plcut1 with the cutinase-like enzymes from *Moesziomyces antarcticus* and *Cryptococcus* sp. S-2, and with the putative cutinases from *Alternaria alternata* and *Aureobasidium pullulans* EXF-150 (Fig. 4).

## Plcut1 shows esterase and Impranil-degrading activity

Because Plcut1 shows homology to proteins in the esterase family, we tested whether the purified recombinant enzyme (Fig. 5A) displays soluble esterase activity *in vitro* (36). Our results showed that recombinant Plcut1 exhibits esterase activity and hydrolyzes *p*-nitrophenyl soluble esters with chain lengths smaller than eight carbons (Fig. 5B). Also, we determined that Plcut1 esterase activity is temperature and pH dependent because the enzyme lost activity at 37°C and below pH 7.4 against 4-nitrophenyl hexanoate (Fig. S4). Moreover, we found that Plcut1 can clear Impranil in agar plates, as indicated by the formation of the halo where the enzyme was spotted (Fig. 5C). In addition, liquid Impranil reactions containing Plcut1 displayed a drastic decrease in $OD_{600nm}$ in contrast to buffer-only controls (Fig. 5D). These data suggest that Plcut1 is the responsible factor

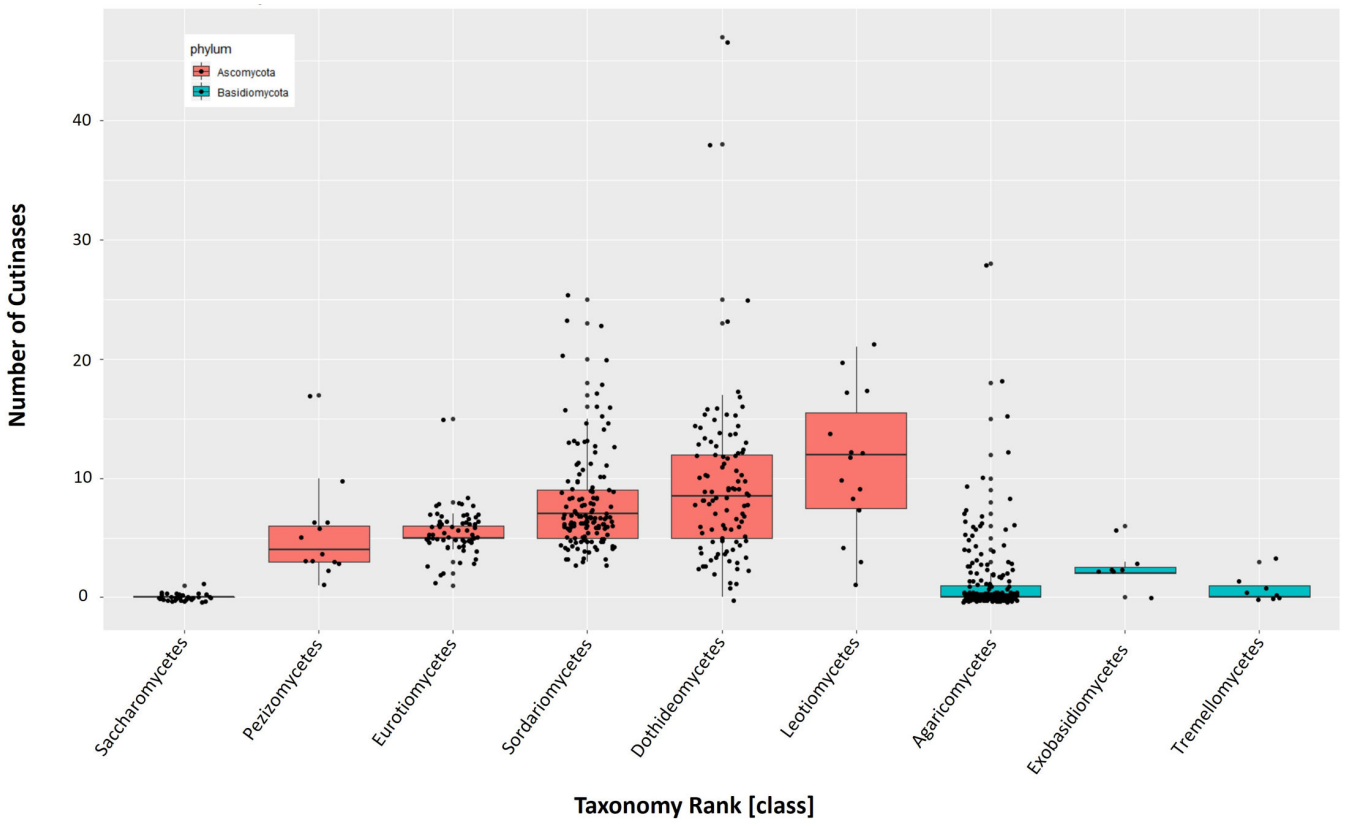


**FIG 2** Cutinase distribution in the Dikarya. We identified cutinases in 595 previously published genomes across the Dikarya based on their functional annotation available on MycoCosm at https://mycocosm.jgi.doe.gov/dikarya.

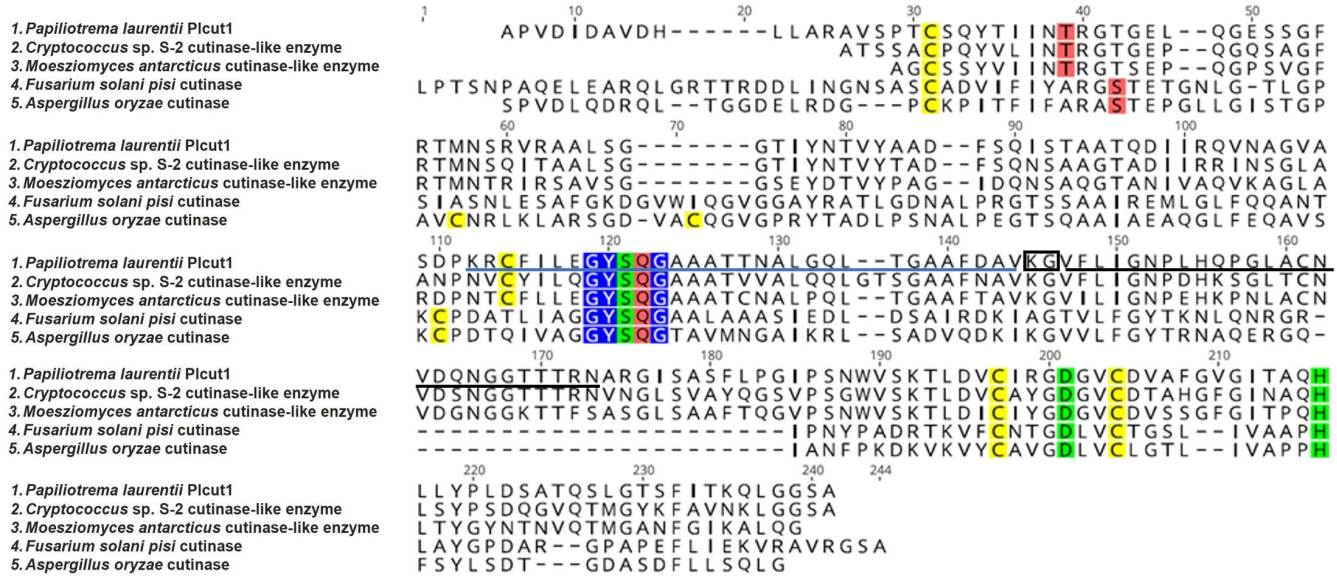


**FIG 3** Cutinase and cutinase-like amino acid alignment. Papla1 543643 ("Plcut1") sequence was aligned using MUSCLE, as described in the Methods. The invariant cysteines are highlighted in yellow, the lipase box (Gly-X1-Ser-X2-Gly) is highlighted in blue, the catalytic triad residues are highlighted in green, and the oxyanion hole residues are highlighted in red. Blue and black underlines highlight the peptides shown in Table S3. Boxed residues emphasize overlapping amino acids between the underlined peptides.

for the Impranil-clearing activity originally observed with the culture supernatants (Fig. 1A).

Furthermore, NMR spectroscopy was performed to quantitatively determine hydrolysis rates based on monitoring the hydrolysis products from Impranil incubated with Plcut1. The spectra are shown in Fig. S5 and indicate that the addition of the enzyme increased the rate of Impranil hydrolysis by 17-fold (1.0 ± 0.1 mM/day). This rate was roughly twice as fast as the rate of hydrolysis observed with an identical density of a commercial lipase with high Impranil hydrolysis rate (0.55 ± 0.08 mM/day) (14).

Plcut1 degradation of Impranil was not limited to liquid solutions as Plcut1 was also observed to degrade solid Impranil coatings (Fig. S6). In these images, the cracking of the coating was not observed if the enzyme was not present. Interestingly, *P. laurentii* cultures grown in minimal media with Impranil as the sole carbon source displayed higher survivability when compared to control cultures after 48 h, (Fig. S7) indicating hydrolytic activity and that the products released from Impranil deterioration can support the growth of *P. laurentii*.

To investigate the role of Plcut1 in the esterase activity present in culture supernatants, we inoculated *P. laurentii* cultures in media containing an excess of glucose. Glucose supplementation has been shown to repress hydrolase expression (12, 41). Western blot analysis of *P. laurentii* culture supernatants grown in TSB showed substantial levels of Plcut1 (Fig. 6A). In contrast, Plcut1 was undetectable in culture supernatants of *P. laurentii* grown in TSB supplemented with 2% glucose. Importantly, we found esterase activity against 4-nitrophenyl hexanoate in supernatants of *P. laurentii* TSB cultures, but not in supernatants from TSB supplemented with glucose (Fig. 6B), even though these cultures contained similar protein concentrations.

## *P. laurentii* Plcut1 is a bona fide cutinase

Cutinases are hydrolases secreted by many microorganisms to break the ester bonds of cutin, the waxy polyester component of the protective cuticle at plant surfaces and may also hydrolyze these bonds in xenobiotic polyester polymers (42, 43). As shown in Fig. 3, Plcut1 displays similarities to putative cutinases from other microorganisms. Thus, we

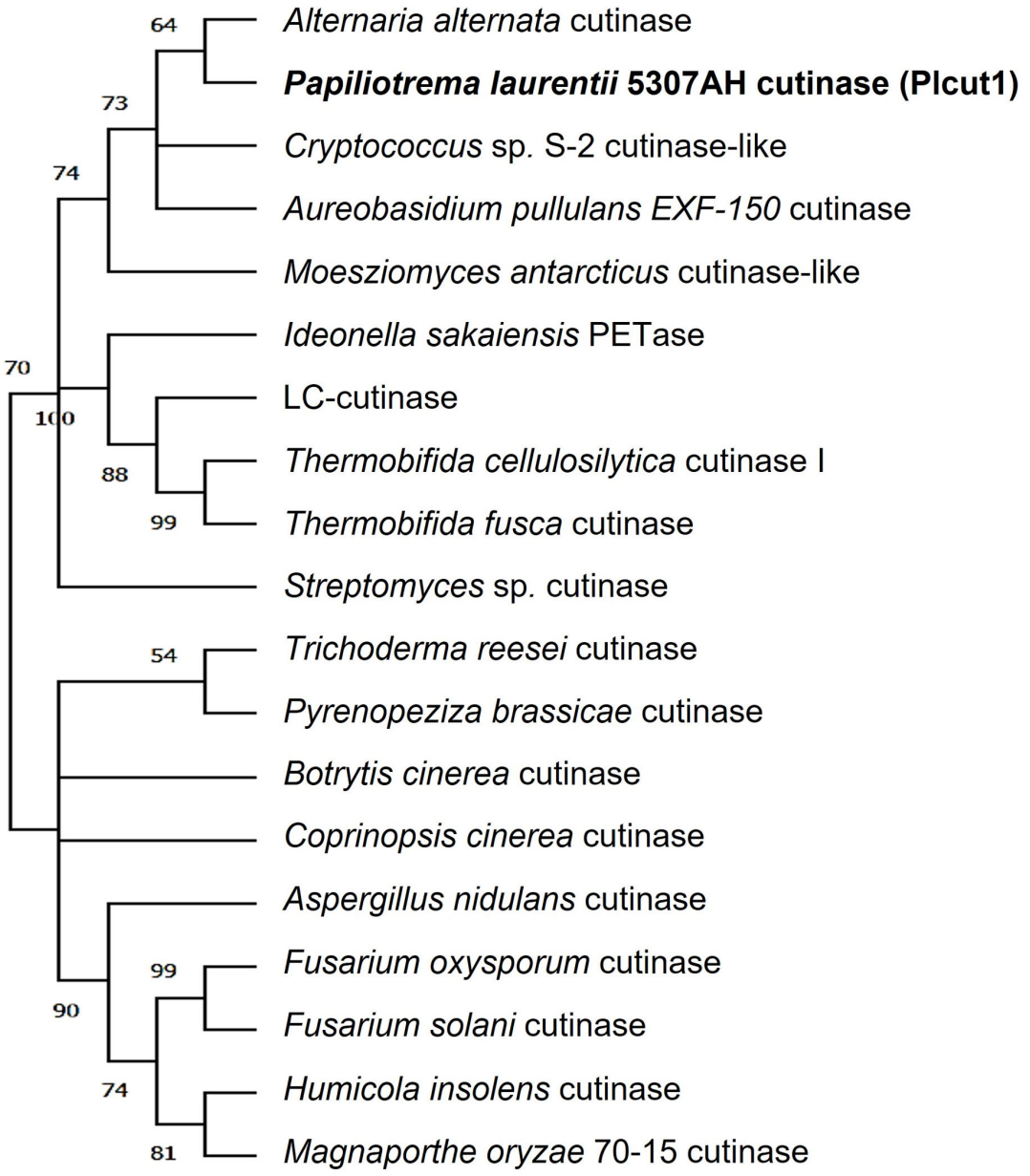


**FIG 4** Phylogenetic relationship of Plcut1. The evolutionary history was inferred by using the maximum likelihood method and the JTT matrix-based model (38). The bootstrap consensus tree inferred from 200 replicates (39) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test 200 replicates are shown next to the branches (39). Initial tree(s) for the heuristic search was obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with superior log likelihood value. There was a total of 327 positions in the final data set. Evolutionary analyses were conducted in MEGA11 (40). Accession numbers for the sequences used here are provided in Table S7.

tested the ability of the recombinant Plcut1 to hydrolyze cutin. Apple cutin is a well-established substrate for cutinases (44–46). Plcut1-dependent deterioration of the cutin sheet was observed as indicated by an increase in turbidity in the reactions (Fig. 7A). Comparison of the gas chromatograms of the negative control (gray trace) and the reactions containing Plcut1 (black trace) showed unique peaks that were only detectable in the reactions containing Plcut1 (Fig. 7B). Table S6 provides a list of the compounds identified through GC-MS after cutin hydrolysis. The compounds identified were a mixture of long alkyl chain alcohols and carboxylic acids with some of the most

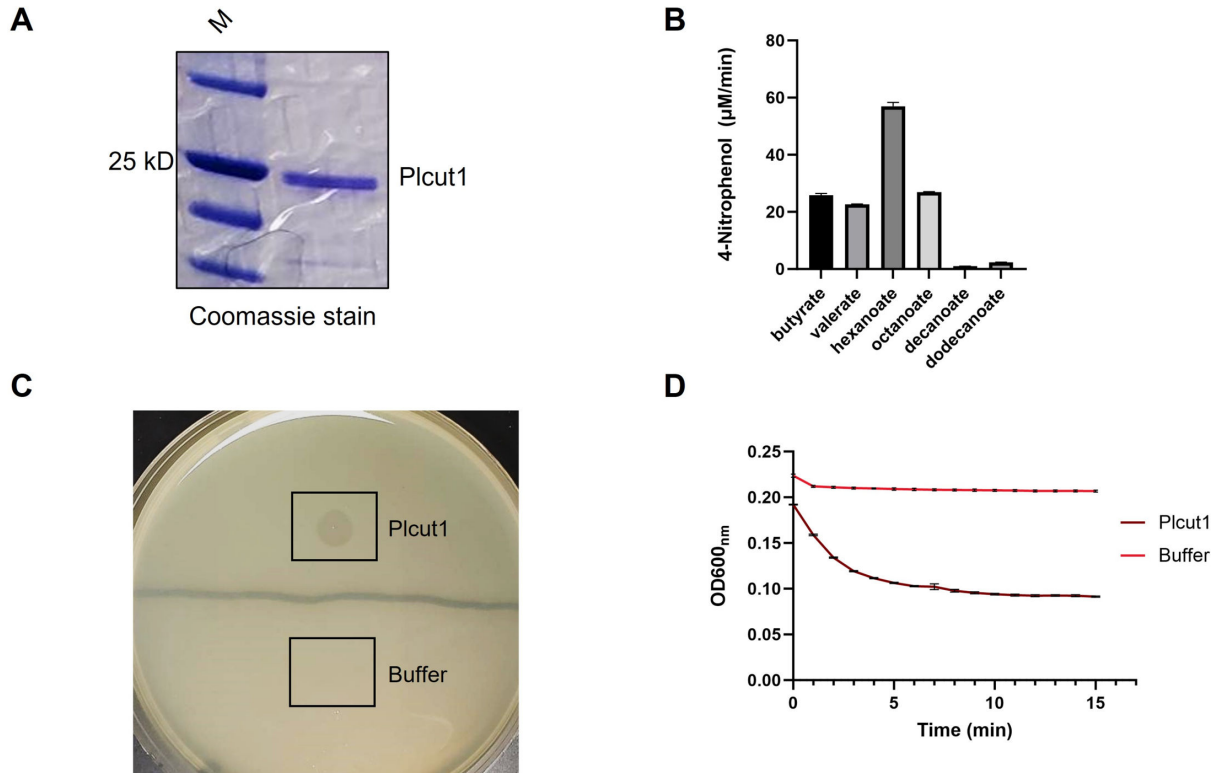


**FIG 5** Hydrolysis of select soluble esters and Impranil-clearing activity of Plcut1. Plcut1 gene was codon optimized, and the predicted signal peptide (amino acids 1–20) was removed before cloning into pET28a. (A) SDS-PAGE followed by Coomassie staining of Plcut1 preparations used for esterase and Impranil-degrading activity assays. (B) Esterase activity assay. Colorimetric assay based on the release of nitrophenol after hydrolysis of select 4-nitrophenyl soluble esterase substrates by Plcut1. (C) TSA plates supplemented with Impranil were exposed to a 10-µL (2-µg) drop of Plcut1, and Impranil clearing was monitored. (D) Impranil-clearing liquid assays. Impranil hydrolysis was monitored by tracking the $OD_{600nm}$ after addition of recombinant cutinase to Impranil solutions.

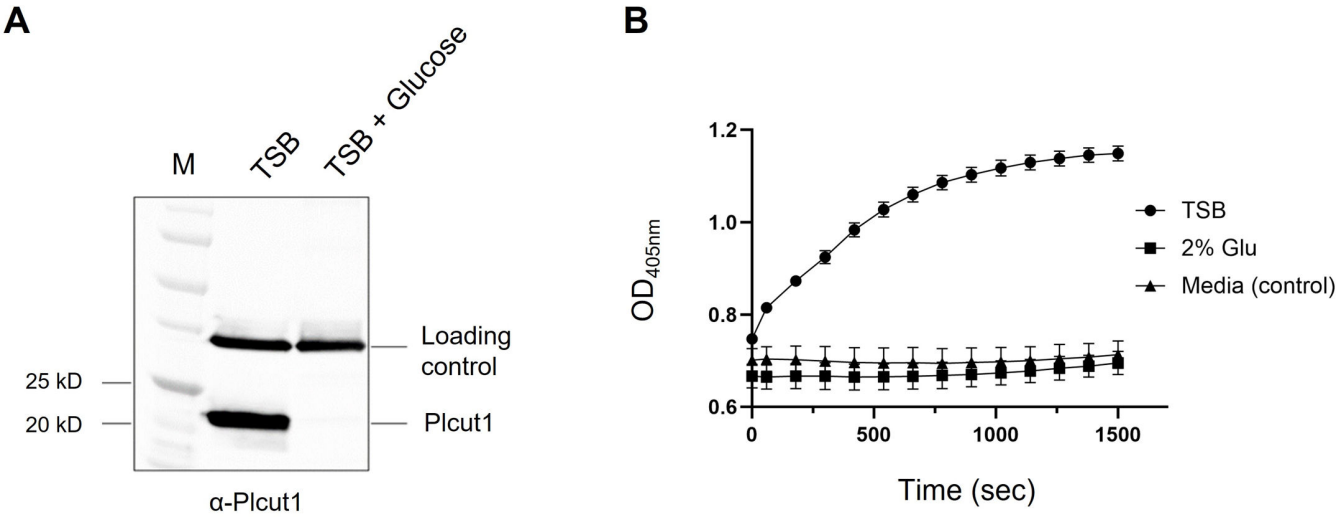


**FIG 6** Plcut1 expression and esterase activity in cell-free supernatants of *P. laurentii* grown in TSB or TSB supplemented with glucose. (A) Western blot of cell-free supernatants from *P. laurentii* cultures grown in TSB or TSB supplemented with 2% glucose. (B) Esterase activity of cell-free supernatants from indicated media. 4-Nitrophenyl hexanoate was used as a substrate, and its hydrolysis was monitored by measuring the $OD_{405nm}$.

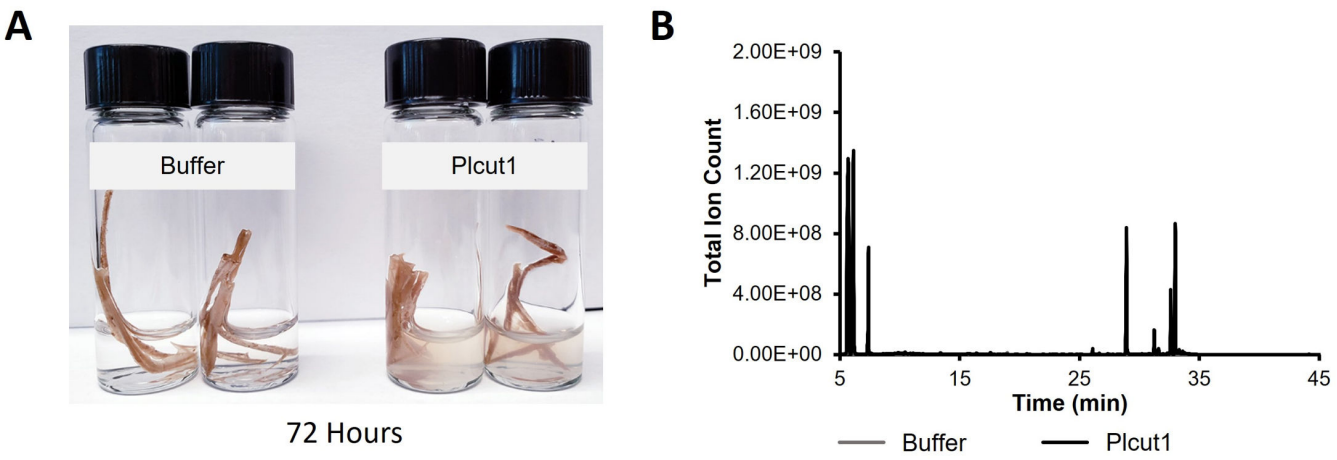


**FIG 7** Plcut1 hydrolyzes apple cutin. (A) Images of vials containing apple cutin incubated with recombinant Plcut1 or buffer controls (no Plcut1) in PBS after 72 h at 27°C. (B) Representative overlaid EI-GC-MS total ion count chromatograms from the silylated cutin hydrolysis products with Plcut1 and without Plcut1 (buffer).

significant products in the $C_{16}$-$C_{18}$ lengths. These products are consistent with cutin hydrolysis (47). Thus, these data confirmed that the Plcut1 is a true cutinase and is capable of hydrolyzing apple cutin.

## DISCUSSION

Microbial biodegradation of polyester polyurethane-based materials has been observed for a variety of fungi and bacteria (13, 48, 49), including one member of the related fungal genus, *Cryptococcus* (48, 50, 51). In this study, we described the isolation and biochemical characterization of the *P. laurentii* cutinase, Plcut1. We showed that recombinant Plcut1 hydrolyzes soluble esters with preference toward the shorter-chain substrates 4-nitrophenyl butyrate, -valerate, hexanoate, and octanoate. These findings align with previous work showing that esterases preferentially break shorter-chain fatty acids, whereas lipases have a larger substrate range (52). Furthermore, Plcut1 was found to disperse solutions and degrade solid coatings composed of Impranil, likely through ester hydrolysis (27). Impranil has been widely used to screen hydrolytic activity of bacterial and fungal enzymes, including cutinases (53–57). The degradation of Impranil solid coatings and apple cutin by Plcut1 suggests that this enzyme has hydrolytic activity at phase interfaces involving solid materials. These findings provide support to the idea that Plcut1 could play a major role in biodegradation of solid coatings in the built environment, such as the surface coatings of an aircraft, where this fungus was originally isolated, by exporting an enzyme capable of degrading solid ester-based polymers. Degradation of these surface coatings could compromise and negatively impact the material or instrumentation directly underneath. Unwanted and uncontrolled growth of microbes in the built environment, perhaps supported by the hydrolysis of surface coatings such as Impranil, could also pose a threat to the health of the warfighter and other staff.

The hydrolytic activity of this enzyme is likely encoded by a classic serine esterase catalytic triad in Plcut1, such as the one encoded by other related cutinases (58, 59), and further experimentation is underway to confirm its involvement in hydrolysis. We demonstrated that Plcut1 is a bona fide cutinase, which is known for optimal enzymatic activity toward a broad range of substrates, including plastics, at temperatures of 20–70°C and pH 6–9 without the need of cofactors (60–65). Unexpectedly, Plcut1 displayed hydrolytic activity toward soluble esters at 4°C while maintaining optimal activity up to 37°C, where a sharp decline in activity was observed. These data contrast the optimal activity of the *Fusarium oxysporum* cutinase, which displays maximum activity at 40°C (66). Even though *P. laurentii* and *F. oxysporum* are mesophilic fungi, we show that *P. laurentii* is armed with a cutinase capable of hydrolyzing ester-based polymers in

colder temperatures, potentially aiding its survival in a broad range of temperatures and environments, such as those experienced by aircraft during their lifetimes.

Remarkably, *P. laurentii* biofilms grow on polymer-coated surfaces with no additional nutrients and still display hydrolytic activity (8, 27). In such a scenario, the smaller monomeric products resulting from hydrolysis could potentially serve as a viable carbon source for *P. laurentii*. Indeed, we found that *P. laurentii* utilized Impranil as the sole carbon source to sustain survivability, which supports this hypothesis. Thus, under the low nutrient conditions encountered by *P. laurentii* in the environment, for example when colonizing vehicle coatings or other polymer-based surfaces, we speculate that cutinase expression could be induced to degrade these polymers and acquire nutrients. Natural plant cutin and many commercially important polymers are made of polyester backbones (67, 68) and thus equally susceptible to cutinases, which are found all over the Dikarya.

Previous work has shown that *P. laurentii* 5307AH degrades polyester coatings, but the causative factors remained to be identified. The work presented here provides an empirical view on the enzymatic machinery utilized by *P. laurentii* to hydrolyze ester-based materials. In support of our hypothesis, we have identified an active cutinase (Plcut1) in culture supernatants with hydrolytic activity toward polyester-based compounds in both liquid and solid formats. Further experimentation with Plcut1 mutants will provide insights into the biological importance of Plcut1 for polymer biodegradation in nutrient-deprived environments and to gain a more complete understanding of the factors that are important for polymer biodegradation by *P. laurentii*. This work directly links Plcut1 levels to hydrolytic activity, which strongly suggests that Plcut1 is playing a key role in biodegradation and potentially represents a mechanism by which this yeast acquires nutrients in carbon-depleted environments, but further *in situ* experimentation is needed to assess the presence of this enzyme in the environment at or around *P. laurentii*-contaminated sites. Finally, the findings presented here suggest that there could be other fungal species involved in cutinase-dependent biodegradation of human-made materials because cutinases are found across the Dikarya and highlights the necessity to further understand the promiscuous nature of these enzymes to develop functional inhibitors that could prevent or mitigate cutinase-driven biodeterioration.

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## DATA AVAILABILITY

The authors confirm that the data supporting the findings in this work are available within the article or its supplementary materials.

## ADDITIONAL FILES

The following material is available online.

### Supplemental Material

**Supplemental material (AEM01694-23-s0001.docx).** Supplemental methods, Table S1 to S6, and Figure S1 to S7.

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