INTRODUCTION

Aspergillus fumigatus, a saprophytic filamentous fungus, is known to cause fatal invasive aspergillosis (IA) among immunocompromised patients, and in recent years, the incidence of IA has soared up in patients who undergo immunosuppressive and cytotoxic chemotherapy (Latgé, 1999; Steinbach, Stevens, & Denning, 2003). Although three classes of antifungal drugs, including antifungal triazoles, amphotericin B, and echinocandin, have been currently used (Walsh et al., 2008), the mortality rate remains around 50% due to limited drug options, low efficiency, and drug resistance (Brown et al., 2012; Denning & Bromley, 2015). Therefore, new antifungal therapies are urgently needed.

The A. fumigatus cell wall is essential for survival, and its components are critical for fungal pathogenesis. Therefore, it is a unique target for drug development. Generally, a filamentous fungus initiates its life cycle from conidial germination, continues with hyphal growth, and terminates with conidiation, which involves a series of ordered morphological events, including the establishment of polarity (budding and germination), polar growth (mycelia elongation,
septation, and branching), and conidiation (Barhoum & Sharon, 2004; d’Enfert, 1997). These morphological events require a dynamic remodeling of the cell wall at the budding site, hyphal tip, and septation site. IA caused by A. fumigatus is featured with the penetration of growing hyphae into lung tissue and blood vessel. Therefore, the polar growth of A. fumigatus is vital for infection. However, it is still not well understood how A. fumigatus modulates the remodeling of cell wall during polar growth.

Chitin, an unbranched polymer of N-acetylglucosamine (GlcNAc), is a major component of the cells of most filamentous fungi (Bartnicki-Garcia, 1968) and contributes to the strength and integrity of the fungal cell wall and septum (Minke & Blackwell, 1978). It has been known that the cell wall chitin is predominantly deacetylated to chitosan, which is enzymatically generated by chitin deacetylase (CDA). CDAs have been identified in bacteria (Kadokura et al., 2007; Li, Wang, Wang, & Roseman, 2007), insects (Dixit et al., 2008), and fungi (reviewed in Zhao et al., 2010). According to the CAZY classification, CDAs (EC3.5.1.41) belong to the family 4 of carbohydrate esterases (ICE4) and share a conserved part of the primary amino acid structure named as the NodB homology domain or polysaccharide deacetylase domain (Caufrier, Martinou, Dupont, & Bouriotis, 2003; Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat, 2014). Putative members of the CE4 family are abundant in the genomes of chitin-containing fungi (www.cazy.org).

In fungi, CDAs participate in cell wall morphogenesis and integrity, spore formation, germling adhesion, and fungal autolysis (Baker, Specht, Donlin, & Lodge, 2007; Davis & Bartnicki-Garcia, 1984; Geoghegan & Gur, 2016; Matsuo, Tanaka, Matsuda, & Kawakurai, 2005; Zhao et al., 2010). Deacetylation of chitin in the cell wall affects virulence of Cryptococcus neoformans (Baker et al., 2007; El Gueddari, Rauchhaus, Moerschbacher, & Deising, 2002), Colletotrichum lindemuthianum (Blair et al., 2006), Magnaporthe oryzae (Kuroki et al., 2017), Pochonia chlamydosporia (Aranda-Martinez et al., 2018), and Pestalotiopsis sp. (Cord-Landwehr, Melcher, Kolkenbrock, & Moerschbacher, 2016). CDAs have been biochemically characterized with regard to substrate specificity in several fungi, such as Mucor rouxii (Kafetzopoulos, Martinou, & Bouriotis, 1993), Aspergillus nidulans (Alfonso, Nuer, Santamaria, & Reyes, 1995; Liu et al., 2017), C. lindemuthianum (Blair et al., 2006; Tsigos & Bouriotis, 1995), Puccinia graminis (Naqvi et al., 2016), Pestalotiopsis sp. (Cord-Landwehr et al., 2016), and Podospora anserina (Ho6bach et al., 2018). They preferentially deacetylate (GlcNAc)₃ and (GlcNAc)₂ with random, sequential, or processive mechanisms.

Chitin is one of the critical components of the A. fumigatus cell wall (Bernard & Latgé, 2001), and the genome of A. fumigatus contains seven putative CDA genes; however, their functions remain unknown. In this study, we cloned the A. fumigatus cod4 and cod7 genes and expressed them in E. coli with an appended short N-terminal His-tag. Biochemical characterization of the recombinant enzymes was carried out. We further deleted the cod4 and cod7 separately as well as both of them to construct the deletion mutants Δcod4, Δcod7, and Δcod4Δcod7. Our results showed that the Cod4 was an active CDA, whereas Cod7 was inactive; however, both of them contributed to polar growth and Cod4 was required for conidiation of A. fumigatus.

2 | EXPERIMENTAL PROCEDURES

2.1 | Strains and growth conditions

Aspergillus fumigatus strain YJ-407 (China General Microbiological Culture Collection Center, CGMCC0386) was maintained on potato glucose (2%) agar slant. A. fumigatus strain CEA17 (Weidner, d’Enfert, Koch, Mol, & Brakhage, 1998), a kind gift from C. d’Enfert, Institute of Pasteur, France, was propagated at 37°C on YGA (0.5% yeast extract, 2% glucose, and 1.5% Bacto agar), complete medium, or minimal medium with 0.5 mM sodium glutamate as a nitrogen source (Cove, 1966). 5 mM uridine and uracil were added when CEA17 or revertant strain was grown. Mycelium was grown in complete liquid medium at 37°C with constant shaking at 250 rpm. Mycelia were collected and washed with distilled water, and then frozen in liquid nitrogen and ground by pestles. The powder was then stored at -70°C for DNA, RNA, and protein extraction. Conidiospores were acquired by growing A. fumigatus strains on PDA plates with uridine and uracil for 36 hr at 37°C. The spores were collected first with distilled water, then washed twice with 0.05% Tween-20 in phosphate-buffered saline (PBS), and stored in 0.05% Tween-20 in PBS, and its concentration was confirmed via hemocytometer counting. Vectors and plasmids were propagated in Escherichia coli Trans-T1 (TransGen Biotech).

2.2 | Molecular cloning of the cod4 and cod7

Protein sequences were analyzed using Conserved Domain Search, SignalP3.0, and the TMHMM Server v.2.0. Protein sequence and AFUA number were retrieved from the CADRE Geno. Protein sequences were aligned using ClustalX 2.0.

The putative CDA gene was identified by searching the conserved domain of NodB that is homologous among the members of carbohydrate esterase family 4 in the genome database of A. fumigatus 293 using a Blastp program. A 1.040-bp and a 915-bp genomic DNA fragment were found to contain the entire ORF and named as cod4 and cod7, respectively.

Based on the nucleotide sequence, the primer pair cod4p1 (5′-ATGGGCAAGAAGCGCTTCT-3′)/cod4p2 (5′-TTATTCTTCTGAGATGGCC-3′) and cod7p1 (5′-ATGGGCAAGAAGCGCTTCT-3′)/cod7p2 (5′-CTCCTAAACCGGATTAACC-3′) were used to clone the cDNA of the cod4 and cod7 genes by PCR, respectively. The PCR products were subcloned into the Trans-T-Easy vector (TransGene) to obtain Teasy-cod4 and Teasy-cod7.

2.3 | Expression and purification of recombinant Cod4 and Cod7 in E. coli

The cDNA of the A. fumigatus cod4 or cod7 gene was amplified from Teasy-cod4 or Teasy-cod4 and subcloned into pET-21a (Novagen).
The recombinant E. coli BL21(DE3) strain (Novagen) harboring pET21-cod4 or pET21-cod7 was used to express the recombinant protein Cod4 or Cod7. One percent of transformant was inoculated into LB containing 100 µg/ml ampicillin and 50 µg/ml chloramphenicol, incubated at 37°C and 200 rpm for 2-3 hr to reach 0.6 at OD600, and then induced with a final concentration of 0.4 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at 28°C for 4 hr. The recombinant protein was purified by ÄKTA FPLC (GE Pharmacia) with a HisTrap HP column (Amersham Pharmacia Biotech). After purification, proteins were dialyzed, lyophilized, and stored at -70°C for later use. The purity of Cod4 or Cod7 was judged by SDS-PAGE, and the concentration was determined by the BCA method.

2.4 | Activity assay

The assay of Cod4 was carried out by using the acetic acid released from the substrates (Fukushima, Kitajima, & Sekiguchi, 2005). Standard enzyme assay was performed in a mixture containing 20 mM Tris-HCl (pH 7.4), 1 mM ZnCl₂, and 5 µl of pNP-(GlcNAc)₂ or (GlcNAc)₄ (1 mg/ml) in a total volume of 100 µl. The reaction was carried out at 37°C for 30 min and then stopped in boiled water for 10 min. Acetic acid released by the enzyme was quantified with the K-ACET kit (Megazyme). The amount of enzyme that releases 1 µmol of acetic acid from ethylene glycol chitin per minute is defined as one unit.

2.5 | Construction of the mutants and revertants

To delete the cod4, a knockout vector construction was designed to replace the entire coding region of the cod4 gene using primers 5′-GGACCCCAGCGACTGCAATG-3′ and 5′-TTTCGCAATTGAGCTCGT-3′.

To construct deletion mutant of the cod7, primers were designed to generated the upstream noncoding region (5′-GGGTTGTCGGCCCAATATCAAAAGGTGATACGACTT-3′ and 5′-GGTTCTAGAGATCCCCAGATTGACTGAG-3′; introduced NotI and Xhol sites are underlined) and downstream noncoding region (5′-GGTTCTCAGAGCTGCTGGAGTTTT-3′ and 5′-GGTTGTACCCTAGCCGGAAGCTGCTG-3′; introduced Xhol and KpnI sites are underlined) of the cod7. The mutant Δcod7 was obtained by knocking out the cod7 gene in the wild type with a similar protocol as described above. PCR confirmation of the Δcod7 mutant was carried out using primer pairs of 5′-CTAGAGTTAAGTATCGTAAAC-3′/5′-CTTCTTAATACCGCCTAGTC-3′ to amplify the pyrG and 5′-GGTTTCAGCTGCTGAGCTGAG-3′/5′-AGGAAGG CTTTGTTGTATGACT-3′ to amplify the neo. A 1-kg fragment was amplified from the upstream noncoding region of the cod7 gene using the primers 5′-GTGCGATGTCAGGGAAAGCAAG-3′ and 5′-ATCCCCAGATGACTGTA-3′ and used as a probe to confirm the Δcod7 mutant.

To generate the revertant strains or double mutant, the pyrG gene was first deleted in the Δcod4 or Δcod7 mutant using the method described by d’Enfert et al. (1996) to generate the Δcod4ΔpyrG or Δcod7ΔpyrG strain. Then, the wild-type copy of the cod4 or cod7 was transformed into the Δcod4ΔpyrG or Δcod7ΔpyrG strain to replace pyrG, respectively. The double mutant was constructed by deletion of the cod7 gene in the Δcod4ΔpyrG strain.

2.6 | Phenotypic analyses of the mutants

Growth kinetics of A. fumigatus strains was carried out by spotting 1 × 10⁵ conidiospores onto the center of a solid CM plate at 37°C or 50°C, respectively. The diameter of the colony was measured intermittently until the stationary phase, and the mean diameter was used to plot against the growth kinetics. This experiment was carried out in triplicate.

To test the sensitivity to antifungal reagents, the same amount of conidiospores freshly collected from the wild type, the mutants, and the revertant strains was spotted on CMU plates in the presence of 250 µg/ml calcofluor white or 250 µg/ml Congo red. After incubation at 37°C or 50°C for 24-48 hr, the plates were taken out and photographed.

To examine spore germination, 10 ml complete liquid medium was inoculated with 10⁷ freshly harvested conidia, poured into a petri dish containing a glass coverslip, and incubated at 37°C for the time indicated in each experiment. At the specified times, the coverslips with germinated spores were then washed with phosphate-buffered saline (PBS), incubated for 15 min with DAPI (1 µg/ml; Sigma), washed with PBS three times, and then incubated for 5 min with a 10 µg/ml solution of fluorescent
brightener 28 (Sigma), and washed again, and the germlings were photographed under microscope.

To count the number of germ tubes during spore germination, 10 ml of liquid CM was inoculated with 10⁶ spores in a petri plate containing sterilized glass coverslips and incubated at 37°C or 50°C. The coverslips with adhering germinated conidia were taken out, fixed in PFA solution (3.7% paraformaldehyde, 50 mM phosphate buffer, pH 7.0, and 0.1% Triton X-100), and observed and counted under differential interference contrast microscope.

Conidia were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 4 hr or overnight at 4°C. Cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate, washed three times in 0.1 M phosphate, postfixed in 1% osmium tetroxide, for 2–4 hr in 0.1 M phosphate and then for 15–20 min in methanol 30%, 50%, 70%, 85%, 95%, and 100%, respectively, and postfixed in 2% uranyl acetate–methanol 30%. Cells were rinsed, dehydrated, and embedded in Epon 812 for the floating sheet method. The section was examined with H-600 electron microscope (Hitachi).

### TABLE 1
Primers used in polarity and virulence analyses by real-time PCR

| Forward primer | Reverse primer |
|---------------|---------------|
| **TBP**       | 5′-CCACCTTGAAACACATTGTT-3′ |
| **cdc42**     | 5′-GGAGCTTGGTCTGTTAAATTACGC-3′ |
| **rsr1**      | 5′-GGCCGACAATACGCTCATC-3′ |
| **rho3**      | 5′-TGGTTGGAATGAGAAATAAG-3′ |
| **rho1**      | 5′-GGCTTTACGTCAATTTCCCACG-3′ |
| **sur2**      | 5′-GGGATCTTGACTCTTCAATTC-3′ |
| **sepa**      | 5′-GCCGCGGTGACACGTTG-3′ |
| **kipA**      | 5′-CTTCTTACAAGCGGTGACG-3′ |
| **lag1**      | 5′-GGATGCGATTGGGGCCTGATG-3′ |
| **swoC**      | 5′-AACTATGCAAGCCTGAGG-3′ |
| **amb**       | 5′-GGGATCTTGACTCTTCAATTC-3′ |
| **pksp**      | 5′-GCCGCGGTGACACGTTG-3′ |
| **fos1**      | 5′-CTTCTTACAAGCGGTGACG-3′ |
| **rhbA**      | 5′-GGCTTTACGTCAATTTCCCACG-3′ |
| **pabA**      | 5′-GGATGCGATTGGGGCCTGATG-3′ |
| **lysF**      | 5′-AACTATGCAAGCCTGAGG-3′ |

**FIGURE 1**
Expression and purification of Cod4 and Cod7. E. coli harboring pET21-cod4 or pET21-cod7 was induced with 0.6 mM IPTG at 28°C for 10 hr. Recombinant proteins were purified by ÄKTA FPLC system (GE Pharmacia) with a HisTrap HP column (Amersham Pharmacia Biotech). The purity of Cod4 or Cod7 was judged by 10% of SDS-PAGE. Ctrl, E. coli harboring pET-21a; M, marker.
2.7 | Analysis of the cell wall

Cell wall components were isolated and determined as described by Yan et al. (2013). Cell wall chitin was isolated as described by White, Farina, and Fulton (1979). The purification procedures included an alkaline treatment, which followed an acidic environment. Deacetylation degree of chitin was determined by the IR spectrophotometry method (Muzzarelli, Tanfani, Scarpini, & Laterza, 1980).

| Substrate          | Activity (U/mg) | Substrate          | Activity (U/mg) |
|--------------------|----------------|--------------------|----------------|
| pNP-GlcNAc         | 0.75           | GlcNAc             | 1.47           |
| pNP-(GlcNAc)₂      | 1.34           | GlcNAc₂            | 3.57           |
| pNP-(GlcNAc)₃      | 2.58           | GlcNAc₃            | 13.45          |
| pNP-(GlcNAc)₄      | 2.37           | GlcNAc₄            | 18.98          |
| pNP-(GlcNAc)₅      | Nd             | GlcNAc₅            | 4.56           |
| pNP-(GlcNAc)₆      | Nd             | GlcNAc₆            | 1.34           |

Note: Standard enzyme assays were performed in a mixture containing 20 mM Tris-HCl (pH 7.4), 1 mM ZnCl₂, and 5 μl of 1 mg/ml pNP-(GlcNAc)₁₋₆ or (GlcNAc)₁₋₆. Reaction mixture was incubated at 37°C for 30 min and then boiled at 100°C for 10 min to stop the reaction. Acetic acid released by the enzyme was quantified with the K-ACET kit (Megazyme). The amount of enzyme that releases 1 μmol of acetate from ethylene glycol chitin per minute is defined as one unit.

2.8 | Real-time PCR

Examination of the expression level of genes by relative real-time RT-PCR analysis was performed as described previously (Yan et al., 2013). The primers used for specific genes in this study are shown in Table 1. First, total RNAs were extracted using TRizol (Invitrogen). The cDNA synthesis was carried out from total RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Then, the PCR was performed by using SYBR® Premix Ex Taq™ (Takara). A triplicate of samples was tested in each assay, and each experiment was repeated 3 times. To testify the contamination of fungal genomic DNA, negative controls were set up for each gene.

3 | RESULTS AND DISCUSSION

3.1 | Expression and biochemical characterization of recombinant CDAs

Based on the Blast results, seven genes were found to encode putative CDAs, named as cod1-7. Analysis of the complete cDNA sequences with SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP/) predicted that the cod4 (AFUA_5G09130) and cod7 (AFUA_6G05030) were soluble CDAs without signal peptide or transmembrane domain, suggesting these two CDAs are soluble.

![Graph of substrate specificity of Cod4](image1.png)

**TABLE 2** Substrate specificity of Cod4

| Substrate Activity (U/mg) | Substrate Activity (U/mg) |
|--------------------------|--------------------------|
| pNP-GlcNAc 0.75          | GlcNAc 1.47              |
| pNP-(GlcNAc)₂ 1.34       | GlcNAc₂ 3.57             |
| pNP-(GlcNAc)₃ 2.58       | GlcNAc₃ 13.45            |
| pNP-(GlcNAc)₄ 2.37       | GlcNAc₄ 18.98            |
| pNP-(GlcNAc)₅ Nd         | GlcNAc₅ 4.56             |
| pNP-(GlcNAc)₆ Nd         | GlcNAc₆ 1.34             |

Note: Standard enzyme assays were performed in a mixture containing 20 mM Tris-HCl (pH 7.4), 1 mM ZnCl₂, and 5 μl of 1 mg/ml pNP-(GlcNAc)₁₋₆ or (GlcNAc)₁₋₆. Reaction mixture was incubated at 37°C for 30 min and then boiled at 100°C for 10 min to stop the reaction. Acetic acid released by the enzyme was quantified with the K-ACET kit (Megazyme). The amount of enzyme that releases 1 μmol of acetate per minute is defined as one unit.

![Effect of pH, temperature, and metal ions on activity of Cod4](image2.png)

**FIGURE 2** Effect of pH, temperature, and metal ions on activity of Cod4. Standard activity assay was carried out by adding 10 μg of purified Cod4 to a mixture containing 20 mM Tris-HCl (pH 7.4), 1 mM ZnCl₂, and 5 μl of 1 mg/ml pNP-(GlcNAc)₁₋₆ in a total volume of 100 μl, and the reaction mixture was incubated at 37°C for 30 min. Reactions were terminated by boiling the mixture at 100°C for 10 min. Acetic acid released by the enzyme was quantified with the K-ACET kit (Megazyme). The amount of enzyme that releases 1 μmol of acetate per minute is defined as one unit. To determine the temperature optimum for activity, reactions were performed at 20–60°C under otherwise standard conditions. To determine the pH optimum for activity, reactions were performed at pH 5.0–9.0 under otherwise standard conditions. The effect of metal ions was tested by adding 5 mM of EDTA or various metal ions to the reaction mixtures. Control, Zn²⁺.
proteins and different from other ones that have been reported. To understand the functions of these two soluble CDAs, both cod4 and cod7 genes were chosen for further study. The cod4 gene is 1,040 bp in length and contains two introns and three exons. A 927-bp region of the cod4 cDNA encodes a protein of 308 aa (XP_753725.1). The cod7 gene is 1,111 bp in length and contains three introns and four exons. A 918-bp region of the cod7 cDNA encodes a protein of 306 aa (XM_742482.1). Cod4 and Cod7 share a similarity of 80%.
The cDNA of the cod4 or cod7 gene was cloned and expressed in E. coli BL21(DE3). The recombinant Cod4 and Cod7, containing a short N-terminal His-tag, were purified to homogeneity by Ni²⁺ affinity chromatography. A 35-kDa of recombinant Cod4 and Cod7 protein were purified to homogeneity, respectively (Figure 1).

Under standard assay conditions, a variety of substrates were tested. As summarized in Table 2, Cod4 showed the highest CDA activity toward pNP-β-(GlcNAc)₃ and (GlcNAc)₄, deacetylated pNP-β-GlcNAc, (GlcNAc)₂ and (GlcNAc)₃ at a lower rate, and no activity toward pNP-β-(GlcNAc)₅, GlcNAc, and (GlcNAc)₆. In addition, Cod4 was inactive on 4-nitrophenyl butyrate, 4-nitrophenyl palmitate, 4-nitrophenyl, and acetylxylan. These results clearly demonstrate that Cod4 is an active CDA in A. fumigatus. Studies of the effect of temperature and pH on CDA activity of Cod4 toward pNP-β-(GlcNAc)₃ showed optima of approximately 37°C and pH 7.1, respectively (Figure 2).

The activity of CE4 deacetylases depends on bivalent metals (Blair, Schuttelkopf, MacRae, & Aalten, 2005; Taylor et al., 2006). In our study, the CDA activity of Cod4 was completely inhibited by the addition of 5 mM EDTA. Of several bivalent metal ions tested, Zn²⁺, Ni²⁺, and Ca²⁺ were beneficial for activity, while Mn²⁺, Fe²⁺, Co²⁺, Cu²⁺, and Mg²⁺ inhibited the CDA activity (Figure 2). These results clearly demonstrate that Cod4 is an active CDA in A. fumigatus. Studies of the effect of temperature and pH on CDA activity of Cod4 toward pNP-β-(GlcNAc)₃ showed optima of approximately 37°C and pH 7.1, respectively (Figure 2).

The activity of CE4 deacetylases depends on bivalent metals (Blair, Schuttelkopf, MacRae, & Aalten, 2005; Taylor et al., 2006). In our study, the CDA activity of Cod4 was completely inhibited by the addition of 5 mM EDTA. Of several bivalent metal ions tested, Zn²⁺, Ni²⁺, and Ca²⁺ were beneficial for activity, while Mn²⁺, Fe²⁺, Co²⁺, Cu²⁺, and Mg²⁺ inhibited the CDA activity (Figure 2). These results clearly demonstrate that Cod4 is an active CDA in A. fumigatus. Studies of the effect of temperature and pH on CDA activity of Cod4 toward pNP-β-(GlcNAc)₃ showed optima of approximately 37°C and pH 7.1, respectively (Figure 2).

In this study by using pNP-(GlcNAc)₁₋₆ as substrates, which are chitooligosaccharides labeled by p-nitrophenol at their reducing end, we observed a low CDA activity toward pNP-GlcNAc. Almost no activity toward GlcNAc was detected. These results demonstrate that Cod4 is inactive on the reducing end of chitooligosaccharides and similar to the CDAs from other fungi. The other hand, Cod4 showed the highest activity toward pNP-(GlcNAc)₃ and (GlcNAc)₄. As p-nitrophenol ring can be treated as a sugar ring analog at the reducing end, it is reasonable to conclude that Cod4 prefers (GlcNAc)₄ and is less active on chitooligosaccharides with DP > 5.

It is proposed that CDAs have four subsites (−2, −1, 0, and +1). The enzyme strongly recognizes a sequence of four GlcNAc residues of the substrate, and the N-acetyl group in GlcNAc residue at subsite 0 is exclusively deacetylated (Zhao et al., 2010). The reducing-end residue of chitooligosaccharides is undetectable (Zhao et al., 2010). For example, CDA from C. lindemuthianum can fully deacetylate (GlcNAc)₂ and (GlcNAc)₃, whereas the reducing-end residue of (GlcNAc)₂ could not be deacetylated (Tokuyasu, Ono, Ohnishi-Kameyama, Hayashi, & Mori, 1997). It seems justified to conclude that the four fungal CDAs analyzed in detail so far all prefer to deacetylate the position next to the reducing end when chitin tetramer is used as a substrate, but they differ in the further conversions of the substrate (Hoßbach et al., 2018).

It is proposed that CDAs have four subsites (−2, −1, 0, and +1). The enzyme strongly recognizes a sequence of four GlcNAc residues of the substrate, and the N-acetyl group in GlcNAc residue at subsite 0 is exclusively deacetylated. More recently, Liu et al. (2017) described the crystal structure and substrate-binding modes of AnCDA (XP_682649.1) from A. nidulans. AnCDA is active toward (GlcNAc)₂₋₆ and inactive toward the GlcNAc and mono-deacetylation of (GlcNAc)₂. It has been confirmed that deacetylation catalyzed by AnCDA occurs at random positions, except for the reducing end. AnCDA adopts (β/α)₈ barrel topology. Similar to CiCDA (Blair et al., 2006; Sarkar, Gupta, Chakraborty, Senapatii, & Gachhui, 2017), the active site of AnCDA contains the His-His-Asp metal-binding triad (H97, H101, D48), a catalytic acid (His196, aiding sugar departure), and a catalytic base (Asp47, activating the nucleophilic water). The −1 sugar can make several interactions with Asp47 and His101, while the sugar bound in subsite +1 interacts with Leu139

FIGURE 4 Southern blotting of the mutants and revertants. The mutants and revertant strains were constructed as described under Section 2. After PCR confirmation of the mutants and revertants, the positive strains were further confirmed by Southern blot using a 1-kb fragment amplified from the upstream noncoding region of the cod4 or cod7 gene as a probe.
and Leu194 that form a hydrophobic pocket, which is important for effective catalysis and preferable deacetylated site. Cod4 contains the His-His-Asp metal-binding triad (H87, H91, D14), a catalytic acid His248 and a catalytic base Asp12 (Figure 4b). As compared with AnCDA, it seems that Asp12 and His91 are responsible for interaction with the −1 sugar, while at subsite +1 the counterparts of Leu139 and Leu194 were not found; indeed, they are replaced by hydrophobic amino acids Gly123 and Ile247, respectively (Figure 3b).

On the other hand, in contrast to Cod4, Cod7 did not show any CDA activity toward the substrates tested in this study though Cod7 shares a similarity of 80% with Cod4. We further compared these two proteins with other reported members of the CE4 family (Andrés et al., 2014; Blair & van Aalten, 2004; Blair et al., 2005; Fadouloglou et al., 2017; Shaik, Cendron, Percudani, & Zanotti, 2011), including alignment, secondary structure, and three-dimensional structure. Blast search revealed that Cod4 and Cod7 shared 67% of similarity with a putative peptidoglycan deacetylase from Helicobacter pylori (HpPgdA; Shaik et al., 2011). As shown in Figure 4a, Cod4 and Cod7 share a similar folding pattern with HpPgdA (PDB ID: 3QBU). Therefore, three-dimensional structures of Cod4 and Cod7 were simulated with SWISS-MODEL (Figure 3c). As indicated in Figure 4b,c, the amino acid residues required for catalysis and Zn2+ binding are conserved in Cod4, Cod7, and HpPgdA except the Motif 1, in which the conserved TF(⁄Y)DD are AYDD in both Cod7 and HpPgdA (Figure 3b,c). Like other CDAs, in Cod4 a catalytic triad, T8-D12-H248, is identified to be required for deacetylation and H87-H91-D14 is for Zn2+ binding. In HpPgdA, the corresponding residues of the catalytic triad Thr-Asp-His are A7, D11, and H247. Previously,
it has been shown that HpPgdA is inactive on peptidoglycans and polyamines. Similarly, Cod7 was inactive on N-acetyl-oligosaccharides. Therefore, it is reasonable to conclude that, as in HpPgdA, Cod7 is unable to catalyze deacetylation due to the substitution of T8 by A8.

3.2 | Functional analyses of the cod4 and cod7 gene

To evaluate the physiological function of the cod4 and cod7 in A. fumigatus, the deletion mutants were constructed by replacing the cod4 or cod7 with pyrG as described under Section 2, respectively. As a result, the Δ cod4 and Δ cod7 were obtained and confirmed by PCR and Southern blotting analysis of XbaI-digested genomic DNA, in which the wild-type 7.9-kb XbaI fragment was converted into a 5.4-kb XbaI fragment (Figure 5). The double mutant Δ cod4 Δ cod7 and revertant strains of the Δ cod4 and Δ cod7 were constructed and confirmed by Southern blot (Figure 4).

The growth rate was determined on solid complete medium at 37°C and 50°C. As shown in Figure 5, the growth rate of the Δ cod4 mutant was higher than that of the wild type or the revertant strain, while the growth rate of the Δ cod7 and Δ cod4 Δ cod7 was similar to that of the wild type.

Considering that CDAs are the enzymes that deacetylate fungal cell wall chitin, we further analyzed the cell wall of the mutants. When the Δ cod4, Δ cod7, and Δ cod4 Δ cod7 mutants were grown on solid complete medium containing calcofluor white or Congo red, all three mutants were similar to the wild type at both 37°C and 50°C (Figure 6). These observations demonstrate that deletion of the cod4, cod7, or both does not affect the cell wall integrity of A. fumigatus.

We further analyzed the cell wall contents of the mutants. As summarized in Table 3, as compared with the wild type, the Δ cod4 mutant showed decreases in glycoprotein (by 5%) and β-glucan (by 10%) and increases in α-glucan (by 8%) and chitin (by 21%). Meanwhile, glycoprotein, α-glucan, and chitin in the Δ cod7 decreased by 6%-11%, whereas β-glucan increased by 6%. The Δ cod4 Δ cod7 showed a similar pattern with the Δ cod7 mutant but displayed more severe decreases in glycoprotein, α-glucan, and chitin. Although the cell wall integrity was not affected in the mutants, the cell wall contents were changed in all three mutants. Also Cod4 and Cod7 showed different effects on cell wall contents. Deletion of the cod4 led to a significant increase in cell wall chitin, while deletion of the cod4 caused a slight increase in β-glucan, which suggests their functions might be different.

### TABLE 3 Cell wall components of the Δ cod4, Δ cod7, and Δ cod4 Δ cod7 mutants

| Strains         | Alkali-soluble | Alkali-insoluble |
|-----------------|----------------|------------------|
|                 | Glycoprotein (μg) | α-Glucan (μg) | Chitin (μg) | β-Glucan (μg) |
| Wild type       | 132 ± 3 (100%)   | 501 ± 27 (100%) | 286 ± 21 (100%) | 1,182 ± 31 (100%) |
| Δ cod4          | 125 ± 2 (95%)    | 543 ± 4 (108%)  | 345 ± 6 (121%)  | 1,063 ± 17 (90%)  |
| Δ cod7          | 127 ± 3 (96%)    | 473 ± 23 (94%)  | 256 ± 37 (89%)  | 1,254 ± 25 (106%) |
| Δ cod4 Δ cod7   | 115 ± 2 (87%)    | 443 ± 43 (88%)  | 235 ± 17 (82%)  | 1,263 ± 32 (107%) |

Note: Three aliquots of 10 mg lyophilized mycelia were used as independent samples for cell wall analysis. The experiments were repeated three times from different biological samples. The values are expressed as micrograms of cell wall component per 10 mg dry mycelia (± SD).
Furthermore, both Δcod4 and Δcod4Δcod7 mutants showed an increased acetylation degree of cell wall chitin, while the Δcod7 was similar to the wild type (Table 4). This result is consistent with the observation that Cod7 was inactive on chitin, indicating that Cod4 is an active CDA responsible for the deacetylation of cell wall chitin whereas Cod7 does not act as a CDA enzyme in A. fumigatus.

### 3.3 | Morphogenesis of the mutants

The biological functions of fungal CDAs have been extensively studied in plant pathogenic fungi and yeasts (Cord-Landwehr et al., 2016; El Gueddari et al., 2002; Zhao et al., 2010). Cbp1, a CDA from the rice blast fungus *M. oryzae*, is confirmed to be critical for appressorium formation, which involves in tip growth and requires accumulation of chitosan at the tips of germ tubes (Kuroki et al., 2017). In *S. cerevisiae*, conversion of chitin to chitosan by either Cda1 or Cda2 is required for formation of the second layer of the spore cell wall, which is important for spores to retain its structural rigidity and resistance to various stresses (Christodoulidou, Briza, Ellinger, & Bouriotis, 1999). CDA from *S. pombe* is also required for proper spore formation (Matsuo et al., 2005), whereas in *C. neoformans*, Cda1, Cda2, and Cda3 are confirmed as virulence factors and responsible for providing cell wall integrity during vegetative growth (Baker et al., 2007; Baker, Specht, & Lodge, 2011; Upadhya et al., 2016).

The developmental process of filamentous fungi is featured with the establishment and maintenance of polarity. The nucleus, meanwhile, undergoes several mitotic divisions, and new nuclei move out into the germ tube. Septation takes place after the third nuclear division by placement of a cross-wall at the basal end of the germ tube (Harris, Hamer, Sharpless, & Hamer, 1997; Harris, Hofmann, Tedford, & Lee, 1999; Momany & Taylor, 2000). When the *A. fumigatus* wild-type conidiospores were cultivated in liquid CM media at 37°C, at the early stage of germination, the wild type

| Strains       | Acetylation (%) |
|---------------|-----------------|
| Wild type     | 58.5 ± 2.3      |
| Δcod4         | 68.8 ± 2.3      |
| Δcod7         | 54.3 ± 4.8      |
| Δcod4Δcod7    | 76.3 ± 3.6      |

Note: Cell wall chitin was isolated as described under Section 2. Degree of deacetylation of chitosan was determined by the IR spectrophotometry.

![FIGURE 7](image_url)  
Fluorescent observation of nuclear and cell wall of Δcod4, Δcod7, and Δcod4Δcod7 mutants. Ten milliliters of complete liquid medium was inoculated with 10⁷ freshly harvested conidia, poured into a petri dish containing a glass coverslip, and incubated at 37°C for the time indicated in each experiment. At the specified times, the coverslides with adhering germlings were removed and fixed in a fixative solution (4% formaldehyde, 50 mM phosphate buffer, pH 7.0, and 0.2% Triton X-100) for 30 min. The coverslips with germinated spores were then washed with phosphate-buffered saline (PBS), incubated for 15 min with DAPI (1 μg/ml; Sigma), washed with PBS three times, then incubated for 5 min with a 10 μg/ml solution of fluorescent brightener 28 (Sigma), and washed again, and the germlings were photographed using a microscope.
elongated mostly toward one direction, the second germ tube and the first germ tube showed typically bipolar pattern at an angle of 180 degrees, and the second germ tube and the first seption occurred after four rounds of mitosis (7 hr). The septum formed at the basal area of the first germ tube (Figure 7). In comparison with the wild type, the second germ tube formed earlier in the Δcod4, Δcod7, and double mutant. The second germ tube of all three mutants occurred at a 120° angle only after the second mitosis (5–6 hr), and the third germ tube was found after the third or the fourth nuclear division (6–7 hr). All spores of three mutants germinated at 7 hr and 14%–24% of them had the third germ tube, while all wild-type spores germinated at 8 hr and only 2% of had the third germ tube (Table 5). These observations suggest that all three mutants germinate earlier than the wild type and display abnormal polarized growth.

Besides abnormal polar growth, conidiation was also affected in the mutants. At either 37°C or 50°C, the conidiospores produced by the Δcod4 and Δcod4Δcod7 were dramatically increased, while the conidiospores produced by the Δcod7 were similar to those of the wild type (Table 6). Under electron microscope, the conidia of the Δcod4 mutant displayed a thickened cell wall (Figure 8). These results indicate that Cod4 is vital to conidiation in A. fumigatus. It is likely that the significantly increased conidiospores, as well as thickened conidial cell wall, is a strategy to survive under the stress condition led by deletion of the cod4.

Although Cod7 was inactive on chitin, our results indicate that Cod7 involves in polarity of A. fumigatus. A similar result has been observed in H. pylori. Although HpPgdA is inactive on peptidoglycan, expression of the HpPgdA encoding gene HP0310 is induced when H. pylori is held in contact with macrophages. The HP0310 deletion mutant presents larger acetylation as compared with its wild type and displays an increased susceptibility to lysozyme degradation (Shaik et al., 2011).

As the mutants were associated with polarity, we further checked expression of the genes that are involved in the polarized growth of A. fumigatus by RT-PCR. Among the genes tested, amb, rsr1/bud1, cdc42, rho1, rho3, and sepA/bni1 involve in actin rearrangement and are thus required for polarity of fungi, kipA involves in regulation of microtublin, and lag1, sur2, and swoC involve in morphology of hyphae. As shown in Figure 9, in the Δcod4 mutant, expression levels of all genes except swoA were higher than those in the wild type; in the Δcod7 mutant, expression levels of all genes except sepA were higher than those of the wild type; and in the double mutant, all genes except amb and sepA were highly expressed. These results confirm that both cod4 and cod7 are required for normal polarized growth of A. fumigatus.
It should be pointed out that although both genes contribute to polarity, our results demonstrate that functions of Cod4 and Cod7 are different. Apparently, Cod7 does not act as an enzyme that directly deacetylates cell wall chitin. It is still unknown how Cod7 contributes to polar growth of *A. fumigatus*. We also tested the virulence of both \(\Delta\)cod4 and \(\Delta\)cod7 mutants with immunocompromised mouse model; however, we were unable to find any significant change in virulence.

In conclusion, our results suggest that Cod4 is a soluble CDA and involved in polarity and conidiation in *A. fumigatus*. On the other hand, although Cod7 is unable to deacetylate the N-acetylated polysaccharides recognized by the typical CDAs, it also contributes to polarity of *A. fumigatus*; however, its mechanism needs further investigation.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31320103901) and partially supported by Bagui Scholar Program Fund (2016A24) of Guangxi Zhuang Autonomus Region to CJ.

CONFLICT OF INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

CJ conceived, administered, and supervised the project; validated the data with equal contributions from MX and XZ; carried out visualization experiments; and acquired funding. YL developed the methodology, curated the data, and implemented the software; MX and XZ carried out investigation and analyzed the data with support from YL, and wrote the original draft. All authors reviewed and edited the manuscript, and gave the final approval for publication.

ETHICAL APPROVAL

None required.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

ORCID

Cheng Jin  
https://orcid.org/0000-0002-1514-6374

REFERENCES

Alfonso, C., Nuero, O. M., Santamaría, F., & Reyes, F. (1995). Purification of a heat-stable chitin deacetylase from *Aspergillus nidulans* and its role in cell wall degradation. *Current Microbiology*, 30(1), 49–54. https://doi.org/10.1007/BF00294524

Andrés, E., Albesa-Jové, D., Biarnés, X., Moerschbacher, B. M., Guerin, M. E., & Planas, A. (2014). Structural basis of chitin oligosaccharide...
deacetylation. Angewandte Chemie International Edition, 53, 6882–6887. https://doi.org/10.1002/anie.201400220
Aranda-Martinez, A., Grijolli-Romero, L., Aragunde, H., Sancho-Vello, E., Blänsé, X., Lopez-Lorca, L. V., & Planas, A. (2018). Expression and specificity of a chitin deacetylase from the nematophagous fungus Pochonia chlamydosporia potentially involved in pathogenicity. Scientific Reports, 8, 2170. https://doi.org/10.1038/s41598-018-19902-0
Baker, L. G., Specht, C. A., Donlin, M. J., & Lodge, J. K. (2007). Chitosan, the deacetylated form of chitin, is necessary for cell wall integrity in Cryptococcus neoformans. Eukaryotic Cell, 6, 855–867.
Baker, L. G., Specht, C. A., & Lodge. J. K. (2011). Cell wall chitosan is necessary for virulence in the opportunistic pathogen Cryptococcus neoformans. Eukaryotic Cell, 10, 1264–1268. https://doi.org/10.1128/EC.05138-11
Barhoorn, S., & Sharon, A. (2004). cAMP regulation of pathogenic and saprophytic fungal spore germination. Fungal Genetics and Biology, 41, 317–326. https://doi.org/10.1016/j.fgb.2003.11.011
Bartnicki-Garcia, S. (1968). Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annual Review of Microbiology, 22(1), 87–108. https://doi.org/10.1146/annurev.mi.22.100168.000511
Bernard, M., & Latgé, J. (2001). Chitinase-mediated chitosan gene expression in Aspergillus nidulans: a role in sexual development. EMBO Journal, 20, 1769–1775. https://doi.org/10.1093/EMBOJ/20.7.1769
Blair, D. E., Schuttelkopf, A. W., MacRae, J. I., & van Aalten, D. M. F. (2004). Structures of Bacillus subtilis PdaA, a family 4 carbohydrate esterase, and a complex with N-acetyl-glucosamine. FEBS Letters, 570, 13–19.
Brown, G. D., Denning, D. W., Gow, N. A., Levitz, S. M., Netea, M. G., & White, T. C. (2012). Hidden killers: Human fungal infections. Science Translational Medicine, 4, 165rv113. https://doi.org/10.1126/scitranslmed.3004404
Caufrier, F., Martinou, A., Dupont, C., & Bourriotis, V. (2003). Carbohydrate esterase family 4 enzymes: Substrate specificity. Carbohydrate Research, 338, 687–692. https://doi.org/10.1006/CSRE0008-6215(03)00028-7
Christodoulou, A., Briza, P., Ellinger, A., & Bourriotis, V. (1999). Yeast ascospor wall assembly requires two chitin deacetylase isozymes. FEBS Letters, 460, 275–279. https://doi.org/10.1016/S0014-5793(99)01334-4
Cord-Landwehr, S., Melcher, R. L. J., Kolkenbrock, S., & Moerschbacher, B. M. A. (2016). Chitin deacetylase from the endophytic fungus Pestalotiopsis sp. efficiently inactivates the elicitor activity of chitin oligomers in rice cells. Science Reports, 6(1), 38018. https://doi.org/10.1038/srep38018
Cove, D. J. (1966). The induction and repression of nitrate reductase in the fungus Aspergillus nidulans. Biochimica Et Biophysica Acta (BBA) - Enzymology and Biological Oxidation, 113(1), 51–56. https://doi.org/10.1016/S0006-2659(66)80120-0
Davis, L. L., & Bartnicki-Garcia, S. (1984). The co-ordination of chitosan and chitin synthesis in Mucor rouxii. Journal of General Microbiology, 130, 2095–2102. https://doi.org/10.1099/00221287-130-8-2095
D'Enfert, C. (1997). Fungal spore germination: Insights from the molecular genetics of Aspergillus nidulans and Neurospora crassa. Fungal Genetics and Biology, 21, 163–172. https://doi.org/10.1006/fgbi.1997.0975
D'Enfert, C., Diaquin, M., Delit, A., Wuscher, N., Debeaupuis, J. P., Huere, M., & Latgé, J. P. (1996). Attenuated virulence of uridine-uracil auxotrophs of Aspergillus fumigatus. Infection and Immunity, 64(10), 4401–4405.
Denning, D. W., & Bromley, M. J. (2015). How to bolster the antifungal pipeline: Few drugs are coming to market, but opportunities for drug development exist. Science, 347(6229), 1414–1416.
Dixit, R., Arakane, Y., Specht, C. A., Richard, C., Kramer, K. J., Beeman, R. W., & Muthukrishnan, S. (2008). Domain organization and phylogenetic analysis of proteins from the chitin deacetylase gene family of Tribolium castaneum and three other species of insects. Insect Biochemistry and Molecular Biology, 38(4), 440–451. https://doi.org/10.1016/j.ibmb.2007.12.002
El Gueddari, N. E., Rauchhaus, U., Moerschbacher, B. M., & Deising, H. B. (2002). Developmentally regulated conversion of surface-exposed chitin to chitosan in cell walls of plant pathogenic fungi. New Phytologist, 156, 103–112. https://doi.org/10.1046/j.1469-8137.2002.00487.x
Fadoulouglo, V. E., Balomenou, S., Aivaliotis, M., Kotsifaki, D., Arnaouteli, S., Tomatsidou, A., ... Kokkinidis, M. (2017). An unusual α-carbon hydroxylation of proline promotes active-site maturation. Journal of the American Chemical Society, 139(15), 5330–5337. https://doi.org/10.1021/jacs.6b12209
Fukushima, T., Kitajima, T., & Sekiguchi, J. A. (2005). Polysaccharide deacetylase homologue, PdA, in Bacillus subtilis acts as an N-acetylglumatic acid deacetylase in vitro. Journal of Bacteriology, 187(4), 1287–1292.
Geoghegan, I. A., & Gurr, S. J. (2016). Chitosan mediates germing adhesion in Magnaporthe oryzae and is required for surface sensing and germing morphogenesis. PLoS Path, 12, e1005703. https://doi.org/10.1371/journal.ppat.1005703
Harris, S. D., Hamer, L., Sharpless, K. E., & Hamer, J. E. (1997). The Aspergillus nidulans sepA gene encodes an FH 1/2 protein involved in cytokinesis and the maintenance of cellular polarity. EMBO Journal, 16, 3474–3483. https://doi.org/10.1093/EMBOJ/16.12.3474
Harris, S. D., Hofmann, A. F., Tedford, H. W., & Lee, M. P. (1999). Identification and characterization of genes required for hyphal morphogenesis in the filamentous fungus Aspergillus nidulans. Genetics, 151, 1015–1025.
Hofbäch, J., Bußwinkel, F., Kranz, A., Wattjes, J., Cord-Landwehr, S., & Moerschbacher, B. M. (2018). A chitin deacetylase of Podospora anserina has two functional chitin binding domains and a unique mode of action. Carbohydrate Polymers, 183, 1–10. https://doi.org/10.1016/j.carbpol.2017.11.015
Javed, S., Ahmad, M., Ahmad, M. M., Hamid, R., Khan, M. A., & Musarrat, J. (2013). Chitinases: An update. Journal of Pharmacy and Bioallied Sciences, 5, 21–29. https://doi.org/10.4103/0975-7406.106559
Kadourka, K., Rokutani, A., Yamamoto, M., Ikegami, T., Sugita, H., Itou, S., ...Nishio, T. (2007). purification and characterization of Vibrio parahaemolyticus extracellular chitinase and chitin oligosaccharide deacetylase involved in the production of heterodisaccharide from chitin. applied microbiology and biotechnology, 75(2), 357–365. https://doi.org/10.1007/s00253-006-0831-6
Kafetzopoulos, D., Martinou, A., & Bourriotis, V. (1993). Bioconversion of chitin to chitosan: Purification and characterization of chitin deacetylase from Mucor rouxii. Proceedings of the National Academy of Sciences of the United States of America, 90, 2564–2568. https://doi.org/10.1073/pnas.90.7.2564
Kuroki, M., Okauchi, K., Yoshida, S., Ohno, Y., Murata, S., Nakajima, Y., ... & Kamakura, T. (2017). Chitin-deacetylase activity induces appressorium differentiation in the rice blast fungus Magnaporthe oryzae. Scientific Reports, 7, 9697. https://doi.org/10.1038/s41598-017-10322-0
Latgé, J.-P. (1999). Aspergillus fumigatus and Aspergillosis. Clinical Microbiology Reviews, 12(2), 310–350.
Li, X., Wang, L.-X., Wang, X., & Roseman, S. (2007). The chitin catabolic cascade in the marine bacterium Vibrio cholerae: Characterization of a unique chitin oligosaccharide deacetylase. Glycobiology, 17(12), 1377-1387. https://doi.org/10.1093/glycob/cwm096

Liu, Z., Gay, L. M., Tuveng, T. R., Agger, J. W., Westereng, B., Mathiesen, G., ...Ejsing, V. G. H. (2017). Structure and function of a broad-specificity chitin deacetylase from Aspergillus nidulans FGSC A4. Scientific Reports, 7, 1746. https://doi.org/10.1038/s41598-017-02043-1

Lombard, V., Golconda Ramulu, H., Drula, E., Coutinho, P. M., & Shaik, M. M., Cendron, L., Percudani, R., & Zanotti, G. (2011). The structure ofAspergillus fumigatusstrain RY1. Journal of Biological Chemistry, 286, 10182-10193. https://doi.org/10.1074/jbc.M110.184262

Muzzarelli, R. A. A., Tanfani, F., Scarpini, G., & Laterza, G. (1980). The coding chitin deacetylase is required for proper spore formation in Aspergillus nidulans FGSC A4. Carbohydrate Research, 82, 299–306. https://doi.org/10.1016/0008-6215(80)90054-8

Nasq, S., Cord-Landwehr, S., Singh, R., Bernard, F., Kolkenbrock, S., El Gueddari, N. E., & Moerschbacher, B. M. (2016). A recombinant fungal chitin deacetylase produces fully defined chitosan oligomers with novel patterns of acetylation. Applied and Environment Microbiology, 82(22), 6645–6655. https://doi.org/10.1128/AEM.01961-16

Sarkar, S., Gupta, S., Chakraborty, W., Senapati, S., & Gachhui, R. (2017). Homology modeling, molecular docking and molecular dynamics studies of the catalytic domain of chitin deacetylase from Cryptococcus laurentii strain RY1. International Journal of Biological Macromolecules, 104, 1682–1691. https://doi.org/10.1016/j.ijbiomac.2017.03.057

Shaik, M. M., Cendron, L., Percudani, R., & Zanotti, G. (2011). The structure ofHelicobacter pyloriHP0310 reveals an atypical peptidoglycan deacetylase. PLoSOne, 6(4), e19207. https://doi.org/10.1371/journal.pone.0019207

Steinbach, W. J., Stevens, D. A., & Denning, D. W. (2003). Combination and sequential antifungal therapy for invasive aspergillosis: Review of published in vitro and in vivo interactions and 6281 clinical cases from 1966 to 2001. Clinical Infectious Diseases, 37(Suppl. 3), S188–S244. https://doi.org/10.1086/376524

Taylor, E. J., Gloster, T. M., Turkenburg, J. P., Vincent, F., Brzozowski, A. M., Dupont, C., ...Davies, G. J. (2006). Structure and activity of two metal ion-dependent acetylxylan esterases involved in plant cell wall degradation reveals a close similarity to peptidoglycan deacetylases.