Copsin, a Novel Peptide-based Fungal Antibiotic Interfering with the Peptidoglycan Synthesis*

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Fungi and bacteria compete with an arsenal of secreted molecules for their ecological niche. This repertoire represents a rich and inexhaustible source for antibiotics and fungicides. Antimicrobial peptides are an emerging class of fungal defense molecules that are promising candidates for pharmaceutical applications. Based on a co-cultivation system, we studied the interaction of the coprophilous basidiomycete Coprinopsis cinerea with different bacterial species and identified a novel defense peptide, copsin. The polypeptide was recombinantly produced in Pichia pastoris, and the three-dimensional structure was solved by NMR. The cysteine stabilized α/β-fold with a unique disulfide connectivity, and an N-terminal pyroglutamate rendered copsin extremely stable against high temperatures and protease digestion. Copsin was bactericidal against a diversity of Gram-positive bacteria, including human pathogens such as Enterococcus faecium and Listeria monocytogenes. Characterization of the antibacterial activity revealed that copsin bound specifically to the peptidoglycan precursor lipid II and therefore interfered with the cell wall biosynthesis. In particular, and unlike lantibiotics and other defensins, the third position of the lipid II pentapeptide is essential for effective copsin binding. The unique structural properties of copsin make it a possible scaffold for new antibiotics.

Background: Secreted antibacterial substances of fungi provide a rich source for new antibiotics. 
Results: Copsin is a novel fungal antimicrobial peptide that binds in a unique manner to the cell wall precursor lipid II. 
Conclusion: As part of the defense strategy of a mushroom, copsin kills bacteria by inhibiting the cell wall synthesis. 
Significance: Copsin provides a novel highly stabilized scaffold for antibiotics.

Fungi and bacteria co-exist in a variety of environments, where fungi directly compete with heterotrophic bacteria. One of the best studied types of bacterial-fungal interactions is the antibiosis, where secreted substances play a key role in combating other microorganisms to defend a nutritional niche (1, 2). Secondary metabolites are the best characterized group of defense molecules, with the most prominent member being penicillin from the Penicillium chrysogenum mold (3). Various other metabolites discovered in studies of bacterial-fungal interactions have pharmaceutical applications, emphasizing the importance of gaining knowledge on molecular mechanisms of bacterial-fungal interactions.

Antimicrobial peptides (AMPs)2 are a class of defense molecules that participate in competing bacteria and other microbes as a part of an innate immune system. AMPs are a large and highly diverse group of low molecular mass proteins (<10 kDa), produced by both prokaryotes and eukaryotes. They are broadly classified according to their structure and amino acid sequence. Often, they are characterized by an amphipathic composition resulting in hydrophobic and cationic clusters (4). Plectasin was the first fungal defensin identified in the secretome of the ascomycete Pseudoplectania nigrella (5). It shows high similarity to plant and insect defensins with a core structural motif of a cysteine stabilized α/β-fold (CSαβ). Plectasin acts bactericidal by binding to the lipid II precursor and by inhibiting the peptidoglycan synthesis of predominantly Gram-positive bacteria (6). The cell wall biosynthetic pathways are a very effective target for antibacterial substances, as shown for a number of clinically applied antibiotics. The far biggest group of these drugs are the β-lactams, which inhibit the transpeptidation step of the peptidoglycan layer (7). In an era of increasing bacterial resistance against many commercially available antibiotics, AMPs are considered promising candidates for a new generation of antibiotics (8).

Here, we studied the interaction of the basidiomycete Coprinopsis cinerea with different bacterial species. To model the lifestyle of this coprophilous fungus, a system was developed, where the fungus was grown on medium-submerged glass beads. This method allowed for a co-cultivation with different bacterial species and made it possible to analyze the secretome of both fungus and bacteria.

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The atomic coordinates and structure factors (code 2MN5) have been deposited in the Protein Data Bank (http://wwpdb.org/).
The chemical shift assignments are available from the BioMagResBank under Number 1880.
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2 The abbreviations used are: AMP, antimicrobial peptide; CSαβ, cysteine stabilized α/β-fold; CCM, C. cinerea minimal medium; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; MHB, Mueller-Hinton broth; CF, carboxyfluorescein; MurNAc, N-acetyluramic acid.
The analysis of secreted proteins revealed that *C. cinerea* secreted AMPs that acted as a defense line against bacteria. One of these peptides, hereafter called copsin, was characterized further at a molecular and structural level, and its mode of action was determined both *in vitro* and *in vivo*.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Fungal Strains**—The *C. cinerea* strain Amut-Bmut (*A43mut B43mut pab1.2*) was used for all experiments involving the fungus. All chemicals, if not otherwise mentioned, were bought at the highest available purity from Sigma-Aldrich.

**C. cinerea in Competition with Bacteria on Glass Beads**—Preparation of the glass bead plates was adapted from a previously published protocol (9). In brief, 40 g of sterile borosilicate glass beads (5 mm) were poured into a Petri dish, and 15 ml of water were added. A bacterial suspension containing *B. subtilis* and *Pseudomonas aeruginosa* was used for all experiments (9). The bacteria were grown on YPD plates (1% (w/v) yeast extract, 2% (w/v) glucose, 2% (w/v) agar) for 4 days at 37 °C in the dark. Afterward, two mycelial plugs were cut from the margin of the mycelium and transferred to the center of a glass bead plate in a distance of 1 cm. After 60 h of letting the fungus grow on the beads (37 °C, dark), 4 ml of the medium underneath the fungus was replaced by 4 ml of a bacterial suspension (*Bacillus subtilis* 168, *Pseudomonas aeruginosa* 01, or *Escherichia coli* BL21) grown previously in CMCCM to an *A*<sub>660</sub> of 0.2. Both organisms were grown in competition for 48 h (37 °C, dark). For the fungal growth control, the medium was replaced by the same amount of CMCCM (4 ml). As control for the bacterial growth, the three bacterial strains were grown in the beads system independently for 48 h. Growth of the bacteria was monitored by measuring the *A*<sub>660</sub> of the medium.

**Purification of AMPs from Fungal Secretome**—*C. cinerea* was grown in a glass bead plate (19-cm diameter) with 200 g of glass beads and 80 ml of CMCCM for 5 days (at 37 °C in the dark). Subsequently, the medium was extracted and concentrated to 4 ml by lyophilization. The proteins were then precipitated with 2.2 M ammonium sulfate at 4 °C for 30 min. After centrifugation at 20,000 × g for 15 min, the pellet was dissolved in PBS, pH 7.4, and the proteins were digested with 100 mg/μl proteinase K (Roche) at 60 °C for 2 h. The solution was loaded in a 10-kDa molecular mass cutoff dialysis cassette (Thermo Scientific, Waltham, MA) and dialyzed against PBS, pH 7.4, at 4 °C for 24 h. The remaining proteins were applied to a Resource S cation exchange column (GE Healthcare) and eluted with a 100–600 mM NaCl gradient in 50 mM sodium phosphate buffer, pH 7.4. 0.5-ml fractions were collected, and the effluent monitored by absorbance at 280 nm on an AKTA purifier 10 system (GE Healthcare). The antibacterial activity of each fraction was tested in a disk diffusion assay. Therefore, an overnight *B. subtilis* culture grown in LB-Lennox medium was diluted in 8 ml of prewarmed (45 °C) water agar (1%) to an *A*<sub>660</sub> of 0.2 and poured on a LB agar plate. 40 μl of each fraction was loaded on a paper disk (Oxoid) placed on the solidified bacterial lawn. The diameter of the inhibition zone was determined after incubation at 37 °C for 24 h. The flow through and fractions showing activity were subjected to a reduction with 10 mM DTT at 37 °C for 45 min and alkylation with 40 mM iodoacetamide at 25 °C for 30 min in the dark. A proteolytic digest was performed with 0.25 μg/ml trypsin (Promega, Madison, WI) for 16 h at 37 °C, and the peptides were desalted on C18 ZipTip columns (Millipore, Billerica, MA). The LC-MS analysis was performed on a hybrid Velos LTQ Orbitrap mass spectrometer (Thermo Scientific) coupled to an Eksigent-nano-HPLC system (Eksigent Technologies, Framingham, MA). Separation of peptides was done on a self-made column (75 μm × 80 mm) packed with C18 AQ 3 μm resin (Bischoff GmbH, Essen, Germany). Peptides were eluted with a linear gradient from 2 to 31% acetonitrile in 53 min at a flow rate of 250 nl/min. MS and MS/MS spectra were acquired in the data-dependent mode with up to 20 collision induced dissociation spectra recorded in the ion trap using the most intense ions. All MS/MS spectra were searched against the p354_filteredMod_d *C. cinerea* database using the Mascot search algorithm v2.3 (Matrix Science, Boston, MA) with oxidation (methionine) as variable modification and carbamidomethyl (cysteine) as fixed modification. Further statistical validation was performed with Scaffold 4.0 (Proteome Software, Portland, OR) with a minimum protein probability of 90% and a minimum peptide probability of 50%. This program was also used for determining the total non-normalized spectral counts for a protein identified in the fractions active against *B. subtilis* and in the flow through.

**cDNA Synthesis and Cloning of the Copsin Precursor**—To extract RNA, 20 mg of lyophilized mycelium was lysed with 25 μl of 0.5-mm glass beads in three FastPrep steps, cooling the sample for 5 min on ice between each step. RNA was extracted with 1 ml of Qiazol (Qiagen) and 0.2 ml of chloroform. After a centrifugation at 12,000 × g at 4 °C for 15 min, RNA was recovered in the aqueous phase, washed on-column using the RNeasy Lipid Tissue Mini Kit (Qiagen), and eluted in RNase-free water. cDNA was synthesized from 2 μg of extracted RNA using the Transcripter Universal cDNA Master kit (Roche) following the manufacturer’s instructions.

The coding sequences of the copsin precursor gene were amplified from cDNA by PCR with the Phusion high fidelity DNA polymerase (Thermo Scientific) according to standard protocols (49): forward primer, 5′-CGGGATATCGAGAATTCCGTCGACTTAACAACGAGGGGACCTTTCACCTCTTTGCTCG-3′, and reverse primer, 5′-ACGCGTGCAGCTTAAACCAACGGGCAAGGG-3′. The PCR product was cloned into the pPICZA expression plasmid (Invitrogen) containing a Zeocin resistance gene using the EcoRI and Sall (Thermo Scientific) restriction sites. The resulting plasmid was linearized by the SacI restriction enzyme and transformed into *Pichia pastoris* (strain NRRLY11430) by electroproporation with 1.2 kV of charging voltage, 25 microfarad of capacitance, and 129 Ω of resistance (10). Positive clones were selected on YPD plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar) containing 100 μg/ml Zeocin (LabForce, Muttenz, Switzerland).

**Production and Purification of Recombinant Copsin**—*P. pastoris* transformants were inoculated in BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 1.3% (w/v) YNB without
amino acids (BD Biosciences), 100 mM potassium phosphate buffer, pH 6, 1% (v/v) glycerol and cultured at 30 °C for 48 h. The cells were harvested by centrifugation at 3000 × g for 10 min; resuspended in P. pastoris minimal medium (1.3% (w/v) YNB without amino acids, 100 mM potassium phosphate buffer, pH 6, 0.4 µg/ml biotin, 0.5% (w/v) NH₄Cl, 1% (v/v) MeOH); and cultured at 30 °C for 72 h. Methanol was added to 1% (v/v) in a time interval of 12 h, and NH₄Cl was added to 0.5% (w/v) in a time interval of 24 h.

The culture broth was centrifuged at 3000 × g for 10 min, and the supernatant was concentrated in a 3.5-kDa Spectra/Por dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA) by a treatment with polyethylene glycol 6000 at 4 °C. The concentrated supernatant was dialyzed against 20 mM sodium phosphate, 50 mM NaCl buffer, pH 7 (buffer A), at 4 °C for 24 h. The protein solution was sterile-filtered and loaded on a self-made SP Sephadex cation exchange column equilibrated with buffer A. The column was washed with 180 mM NaCl, and bound proteins were eluted with 400 mM NaCl in 20 mM sodium phosphate buffer, pH 7. The eluent was subjected to a size exclusion chromatography for further polishing. The separation was performed on a Superdex75 column (HiLoad 16/60; GE Healthcare) equilibrated with 20 mM sodium phosphate, 50 mM NaCl buffer, pH 6. The effluent was monitored by absorbance at 210 nm. The fractions containing copsin were combined, and the molecular mass was determined by electrospray ionization-MS.

Production of 15N/13C-labeled Copsin in P. pastoris—The production of isotope-labeled copsin was adapted from a previously published protocol (11). In brief, P. pastoris transformants were inoculated in minimal medium supplemented with 0.5% (w/v) 15NH₄Cl (98 atom % 15N) and 13C₃-glycerol (99 atom % 13C), respectively, and cultivated in shake flasks at 30 °C to an A₅₉₀ of ∼35. After centrifugation at 3000 × g for 10 min, the cell pellet was dissolved in P. pastoris minimal medium containing 0.5% (w/v) 15NH₄Cl (98 atom % 15N) and [13C]methanol (99 atom % 13C). The culturing was performed at 30 °C for 3 days. [13C]methanol was added to 1% (v/v) in a time interval of 12 h, and 15NH₄Cl was added to 0.5% (w/v) in a time interval of 24 h. Purification of 15N/13C copsin was performed as described for the unlabeled product.

NMR Preparation of NMR Samples—Uniformly 15N/13C isotope-labeled copsin in 20 mM sodium phosphate, 50 mM NaCl buffer was prepared as described above.

NMR Spectroscopy—Sequence specific resonance assignments were obtained using a set of two-dimensional [15N,2H] and [13C,H] heteronuclear single quantum coherence, constant time [13C,1H] heteronuclear single quantum coherence, [13C,1H] total correlation spectroscopy (mixing time τm = 80 ms), exclusive correlation spectroscopy, [1H,1H] NOESY (τm = 40 ms), three-dimensional HN(CO)CA, and 13N/15N-resolved [1H,1H] total correlation spectroscopy (τm = 40 ms) spectra recorded on Bruker Avance III 500-, 600-, 700-, and 900-MHz NMR spectrometers at a temperature of 293 K. The data were processed with TopSpin 3.0 (Bruker, Billerica, MA) and analyzed with CARA 1.8.4. The protonation state of histidine residues was determined by recording a [15N,1H] heteronuclear multiple-quantum correlation spectroscopy spectrum with a long (22 ms) INEPT transfer period (12). Proline residues were assigned to cis and trans conformations based on chemical shifts of 13C and 15N nuclei (13).

Structure Calculation—Structure calculation was performed using two-dimensional [1H,1H] NOESY measured in H₂O and D₂O solutions and three-dimensional 13C,15N-resolved [1H,1H] NOESY spectra (τm = 40 and 60 ms), recorded on Bruker Avance 700- and 900-MHz spectrometers. Eight hydrogen bonds, identified based on correlations in long range HNCO spectra (14), were included as restraints. The NOEs were manually picked; the resulting peak list, the amino acid sequence, and the NOESY spectra were used as input for a structure calculation with the software CYANA 3.0 (15), using the simulated annealing protocol. In each of the seven cycles, 800 structures were calculated. The 40 structures with the lowest target function were then selected and used to calculate the final structure. At the outset of the structure calculation, no assumptions on disulfide bond topology were used, and the cysteines were defined as negative charged without a H⁺ atom. After the identification of the disulfide bonds (see next paragraph), the final structure calculation was performed, and the best 20 of 200 structures were subjected to constrained energy minimization using the software AMBER (16). The nonstandard N-terminal amino acid pyroglutamate was added to the library of AMBER. The determination of the secondary structure elements was based on the 20 final structures using the definitions of PyMOL. All 14 amide protons in the corresponding hydrogen bonds exchange slowly in D₂O (Δt > 10 min), and eight were contained in the long range HNCO experiment (see above). TALOS+ and PROCHECK determined a slightly higher content of secondary structure but contained the ones from PyMOL.

NMR, Identification of Disulfide Bonds—In addition to the visual analysis of structures calculated without disulfide bonds, three established methods for the determination of disulfide bonds based on NMR data were used: (a) ambiguous intersulfur restraints (17), (b) probability of a certain disulfide bond ensemble (18), and (c) the averaged target function (19). (a) The cysteines were forced to form disulfide bonds by the CYANA algorithm using ambiguous distance restraints, for every cysteine, between one specific cysteine and all other cysteines (15). Clustering of at least two sulfur atoms together satisfies the restraint. Standard distances between the sulfur and carbon atoms S-S’ (upper/lower limit, 2.1/2.0 Å), S-C’ (3.1/3.0 Å), and C-S’ (3.1/3.0 Å) were used. The topology of the disulfide bond of the best 40 structures selected on the basis of their energy was analyzed. (b) An initial set of 800 conformers was calculated with no explicit constraint for disulfide bonds and the sulfur H⁺ atoms removed. Subsequently the interatomic distances between all cysteine C’ atoms were extracted and averaged over the 40 final lowest energy structures. Based on the initial structure ensemble, all conceivable disulfide patterns were formed (10–40; 18–48; 22–50; 35–54; 3–32; 25–57/10–40; 18–48; 22–35; 50–54; 3–32; 25–57/10–40; 18–48; 22–50; 35–50; 3–32; 25–57/10–48; 18–40; 22–50; 35–54; 3–32; 25–57/10–48; 18–40; 22–50; 35–50; 3–32; 25–57/10–18; 48–40; 22–35; 50–54; 3–32;
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Table 1

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<th>Disulfide bond pattern</th>
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<th>Violations</th>
<th>Weight factor</th>
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*In target function and violation analysis, for every topology a structure calculation was performed using CYANA (15). The disulfides were given as fixed constraints determined by the standard three upper and lower distance limits (see text). Target function and violations are shown averaged over the 40 selected best energy structures.

1. Distance violations.
2. van der Waals (VdW) violations.
3. Characterization of disulfide bond patterns by cysteine Cα–Cα distance analysis as previously described (18). Averaged distances were extracted from the structure ensemble generated without any disulfide constraints. Subsequently weights were assigned to every disulfide pattern. The weight is directly proportional to the likelihood of certain disulfide bond patterns to be realized in the final structure.

*Disulfide bonding connectivity of the final structure as described under "Results." Variations of single disulfide bonds from this assignment are shown in bold in the first column.

25–57/10–18; 48–40; 22–54; 35–50; 3–32; 25–57/10–40; 18–22; 48–50; 35–54; 3–32; 25–57/10–40; 18–50; 48–22; 35–54; 3–32; 25–57/10–40; 18–48; 22–50; 35–54; 3–32; 25–57/10–40; and 32–25, and a weighting factor representing the probability of a specific disulfide bond topology was then calculated for all these possibilities (Table 1). (c) The averaged target function of the 40 best CYANA structures was compared for conceivable fixed disulfide bond connectivities, and the number of violations was analyzed (Table 1).

Effect of Temperature, pH, and Proteases on Copsin Activity—The pH stability of copsin was determined after incubation for 1 h at room temperature in a pH gradient of buffered solutions, including 250 mM KCl/HCl buffer (pH 2), 100 mM sodium acetate buffer (pH 4 and 5), 250 mM sodium phosphate buffer (pH 6), and 250 mM HEPES buffer (pH 8). As a control, copsin form stock solution in 20 mM sodium phosphate, 50 mM NaCl buffer, pH 6, was loaded on one disk. Thermal stability was tested in PBS buffer, pH 7.4, at 4, 25, 50, 70, and 90 °C for 1 h of incubation.

The effect of proteases such as pepsin, trypsin, and proteinase K on the activity of copsin was investigated by incubation with respective proteases at 37 °C for 5 h. The cfu/ml were determined in intervals of 30 min by plating serial dilutions on LB agar plates. The MIC was defined as the lowest concentration of copsin, where no visible growth was observed. The MBC is defined as the concentration of polypeptide that killed 99.9% of bacteria after 20–24 h of incubation. All determinations were performed at least in duplicate.

Killing Kinetics—B. subtilis was grown in MHB to an A600 of 0.6 and diluted to an A600 of 0.1 in MHB supplemented with 50 mM sodium phosphate, pH 6. The cfu/ml was determined by plating serial dilutions on LB agar plates. The MIC is defined as the lowest concentration of copsin, where no visible growth was observed. The MBC is defined as the concentration of polypeptide that killed 99.9% of bacteria after 20–24 h of incubation. All determinations were performed at least in duplicate.

Light Microscopy—For TAMRA labeling, copsin was dissolved in 1 M NaHCO3 buffer, pH 8.0, to 1.6 mg/ml. TAMRA (Invitrogen) was added to a final concentration of 1 mg/ml and incubated at room temperature for 1 h. The excess dye was removed by passing the solution through a prepacked Sephadex G-25 M PD-10 desalting column (GE Healthcare) in 20 mM sodium phosphate, 50 mM NaCl buffer, pH 6.0.

Bacteria were grown in LB medium to an A600 of 0.3 and incubated with TAMRA-copsin at 0.5× MIC (0.5 μg/ml) for B. subtilis and 2× MIC (16 μg/ml) for S. carnosus for 10 min. The cells were washed twice with PBS and then immobilized on...
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a poly-l-lysine-coated coverslip. After washing away the unbound cells with sterile water, BODIPY-vancomycin (Invitrogen) was used at 1 μg/ml concentration for staining on the coverslip. The cells were observed under a 100× oil immersion objective (Zeiss, Oberkochen, Germany) on a spinning disk confocal microscope (Visitron, Puchheim, Germany) and imaged with an Evolve 512 EMCCD camera (Photometrics, Tucson, AZ) using standard filter sets for GFP (excitation/emission, 488/525 nm) and rhodamine (540/575 nm). Images were processed using Image J v1.46.

Binding of Copsin to Cell Wall Precursors—2 nmol of each purified C55P, lipid I, lipid II (21), or lipid III (22) were incubated in the presence of increasing molar copsin concentrations from 1:0.5–1:4 (lipid-copsin precursor) in a total volume of 30 μl. After incubation for 20 min at 25 °C, the mixture was extracted with 2:1 (v/v) n-butanol/pyridine acetate (pH 4.2), analyzed by TLC as described earlier (23, 24). Analysis was carried out by phorbol phosphomolybdic acid staining.

Synthesis of UDP-MurNAc Peptides by Staphylococcus aureus MurA-F Enzymes—UDP-MurNAc-pp was synthesized as described (6) with modifications. UDP-GlcNAc (100 nmol) was incubated with 15 μg of each of the recombinant histidine-tagged muropeptide synthetases MurA to MurF in 50 mM Bis-Tris propane, pH 8, 25 mM (NH₄)₂SO₄, 5 mM MgCl₂, 5 mM KCl, 0.5 mM DTT, 2 mM ATP, 2 mM phosphoenolpyruvate, 2 mM NADPH, 1 mM of each amino acid (l-Ala, d-Glu, l-Lys, and d-Ala), 10% Me₂SO in a total volume of 100 μl for 120 min at 30 °C. 33 μl of the reaction mixture, corresponding to 25 nmol of UDP-MurNAc-pp, were used in a MraY synthesis assay without further purification (23). UDP-MurNAc-peptide variants with shortened stem peptide, i.e., UDP-MurNAc-di-peptide and -tripeptide, were synthesized in the presence of the corresponding subset of muropeptide synthetases and were used for the synthesis of lipid I (dipeptide) and lipid I (tripeptide), respectively. After synthesis, lipid intermediates were incubated at room temperature with increasing molar ratios of copsin for 20 min. TLC analysis was performed as described above.

Carboxyfluorescein (CF) Efflux from Lipid II Containing Liposomes—Large unilamellar vesicles were prepared by the extrusion technique, essentially as previously described (25). Vesicles were made of 1,2-dioleoyl-sn-glycerol-3-phosphocholine supplemented with 0.5 mol % lipid II (referring to the total amount of phospholipid). CF-loaded vesicles were prepared with 50 mM CF and then diluted in 1.5 ml of buffer (50 mM MES-KOH, 100 mM K₂SO₄, pH 6.0) at a final concentration of 25 μM phospholipid on a phosphorous base. After addition of 1 μM peptide (nisin or copsin), the increase of fluorescence intensity was measured at 520 nm (excitation at 492 nm) on an RF-5301 spectrophotometer (Shimadzu, Kyoto, Japan) at room temperature. Leakage was documented relative to the total amount of marker release after solubilization of the vesicles by addition of 10 μl of 20% Triton X-100.

RESULTS

Interaction of C. cinerea and Bacteria—Dung of herbivores, the natural substrate of C. cinerea, is a rather complex environment. The fungus grows on a solid substrate in high humidity and is confronted with a complex diversity of bacteria and other microbes (26). To mimic this substrate and to study the interactions of C. cinerea with bacteria, an artificial system was applied in which the fungus was grown on glass beads submerged in liquid medium (9). Because the fungal mycelium was kept in place by the beads layer, the medium supporting fungal growth could be replaced and also allowed for the co-cultivation with either B. subtilis (strain 168), P. aeruginosa (strain PA01), or E. coli (strain BL21). In addition, the growth of both organisms in competition could be monitored easily (Fig. 1).

The Gram-negative bacteria P. aeruginosa and E. coli had an inhibitory effect on the growth of C. cinerea (Fig. 1A). B. subtilis, the Gram-positive species tested, did not affect the expansion of the fungal mycelium, but C. cinerea had an inhibitory impact on B. subtilis (Fig. 1B). P. aeruginosa was not affected at the beginning of the co-cultivation, but the culture reached the stationary phase much earlier than the fungal-free control. The growth of E. coli did not display any dependence on C. cinerea. Our results showed that C. cinerea was indeed interacting in a species-specific way with bacteria. In particular, we noticed an antagonistic behavior in competition with B. subtilis and P. aeruginosa.

Identification and Recombinant Production of Secreted AMPs—The co-cultivation experiments suggested a secreted fungal substance with a negative effect on B. subtilis growth (Fig. 1B). To examine whether the fungus in axenic culture secretes an antibacterial substance, unchallenged C. cinerea was grown in the glass beads system in minimal medium. After 5 days, the medium was collected and concentrated, and an antibacterial activity was shown in a disk diffusion assay on B. subtilis. To identify putative AMPs involved in this inhibition, proteins were precipitated with ammonium sulfate and digested with proteinase K at 60 °C. The remaining proteins were separated on a cation exchange column and evaluated according to their activity against B. subtilis in a disk diffusion assay (Fig. 2, A and B). Fractions that displayed an inhibition zone against B. subtilis were treated with reducing agent followed by a tryptic digest and subjected to a MS measurement. Five C. cinerea-derived proteins were identified in the active fractions. These proteins were quantified by spectral counting, and the relative amount of each protein was correlated to the activity in the disk diffusion assay (Fig. 2C). The relative concentrations of the predicted protein CC1G_13813 were congruent to the activity profile.

The transcript of the CC1G_13813 locus was analyzed by reverse transcriptase-initiated PCR. The cDNA sequence revealed an open reading frame encoding a precursor protein with in total 184 amino acids. In silico analysis suggested a signal peptide (position 1–23), a pro-peptide (position 24–127), and a C-terminal domain of 57 amino acids corresponding to the mature AMP, which we termed copsin.

To obtain pure copsin, the cDNA encoding the native signal sequence was cloned in a pPICZa expression vector and transformed in P. pastoris. The secreted recombinant product was purified by cation exchange chromatography and yielded a mature polypeptide with a monoisotopic mass of 6059.5 Da, determined by electrospray ionization-MS (theoretical monoisotopic mass, 6059.5 Da).
Sequencing of the N terminus by electrospray ionization-MS/MS of recombinant copsin and of the native polypeptide from *C. cinerea* revealed the same peptide, both with an N-terminal glutamine converted to a pyroglutamate (data not shown). These findings showed that the recombinant preprotein was processed in *P. pastoris* in the same way as in the host fungus *C. cinerea*. Mature copsin contained 12 Cys residues, all involved in a disulfide bond as displayed by a mass shift of 12 Da after a reduction with DTT. This was further confirmed by characteristic $^{13}$C NMR chemical shifts, which differ from reduced Cys residues. $[^{1}H,^{15}N]$ heteronuclear multiple bond correlation spectroscopy NMR spectra recorded at pH 6.8 and 7.4 exhibited a stable positive charge on the His$_{26}$ side chain and a delta protonated, uncharged His$_{21}$. Thus, a net positive charge of +7 was assigned to the polypeptide. A sequence comparison on the AMP database and by the Blastp algorithm exhibited maximum identities of 20–27% of copsin with defensins from invertebrates, fungi, and plants, such as plectasin from *P. nigrella* or eurocin from *Eurotium amstelodami*, both assigned to the fungal phylum of ascomycota (Fig. 3A) (5, 27, 28).

**Structural Features of Copsin—**The three-dimensional structure was determined using NMR spectroscopy. An almost complete assignment of resonances was obtained (99.1% of backbone and 85.7% of side chain resonances assigned). The input data for the structure calculation and the characterization of the NMR structure are summarized in Table 2. The structure of copsin contains one $\alpha$-helix (residues 15–23) followed by two $\beta$-strands (residues 36–40 and 46–50) and is stabilized by six disulfide bonds (Fig. 3B). The two $\beta$-strands form a small antiparallel sheet structure. These secondary structural elements exhibited a high sequence identity to known CS$\alpha$$\beta$ defensins, whereas the loop regions and the termini were very unique in their length and composition. Standard identification of disulfide bonds following proteolytic digest could not be
applied, because of a lack of single disulfide bond cleavage products. A comparison of a bundle of NMR structures calculated without disulfide constraints with the three-dimensional structures of other defensins strongly suggests three conserved disulfide bonds: these bonds connect the \(\alpha\)-helix to the second \(\beta\)-strand (Cys18–Cys48 and Cys22–Cys50) and the N-terminal region to the first \(\beta\)-strand (Cys10–Cys40) (Fig. 3B). Three additional disulfides stabilize copsin, linking the termini to the loop between the \(\alpha\)-helix and the \(\beta\)-sheet. Given that Cys22 is linked to Cys50, it follows that the two cysteines Cys35 and Cys54 are connected. For the remaining two disulfide bonds between the cysteines Cys5, Cys25, Cys32, and Cys57, no direct lead could be obtained from the structural data. To corroborate the four disulfide bonds obtained based on the structure and to find the most probable connectivities for the two remaining ones the most likely combinations are Cys3–Cys32 and Cys25–Cys57 (Table 1). Thus, the four structural based disulfide bonds were confirmed, and for the two remaining ones the most likely combinations are Cys3–Cys32 and Cys25–Cys57.

The compactness of the structure with its six disulfide bonds and the modifications at the termini are mainly responsible for the very high stability of copsin. The activity was completely retained after heat treatment or incubation at different pH values shown in a disk diffusion assay (Fig. 4, A and B). Copsin exhibited a strong resistance toward different proteases, such as proteinase K, trypsin, or pepsin (Fig. 4C). However, antibacterial activity was lost when treated with a reducing agent, supporting the pivotal role of disulfide bonds for the structural integrity of copsin.

**Antibacterial Profile of Copsin**—The antibacterial activity of recombinant copsin was tested in disk diffusion assays against a variety of Gram-positive and Gram-negative bacteria. For selected species, the MICs and MBCs were determined by the microdilution broth method in MHB at pH 7.3 (Table 3). Copsin exhibited MIC values in the low microgram per milliliter range for Gram-positive bacteria, such as *B. subtilis, Listeria*...
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### TABLE 2

Structural statistics of the 20 best NMR structures of copsin

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<th>A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>B&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>Target function</td>
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<sup>a</sup> No constraint for disulfide linkage was used.
<sup>b</sup> Each disulfide bond was explicitly defined by upper and lower distance limit restraints between the sulfur and carbon atoms of the two linked cysteines: Cys<sup>3</sup>–Cys<sup>32</sup>, Cys<sup>10</sup>–Cys<sup>18</sup>, Cys<sup>22</sup>–Cys<sup>48</sup>, Cys<sup>25</sup>–Cys<sup>50</sup>, and Cys<sup>35</sup>–Cys<sup>54</sup>.
<sup>c</sup> 20 structures of column B with the lowest target function were energy minimized using Amber (16).
<sup>d</sup> H-bond constraints were identified from HNCO (14) experiments and slow exchanging amide protons in D<sub>2</sub>O.
<sup>e</sup> Statistics computed for the best 20 structures (see column C).
<sup>f</sup> Based on structured residue range, residues 3–57.
<sup>g</sup> Ramachandran plot, as defined by the program Procheck (48).

Spp., and Enterococcus spp., including a vanA type vancomycin resistant Enterococcus faecium strain. Most potent activity was detected against Listeria monocytogenes with MIC values of 0.25–0.5 μg/mL. A change in pH to 6 in MHB did not affect the activity of copsin, determined against B. subtilis and S. carnosus. Gram-negative species such as E. coli were not affected in viability when exposed to copsin.

MICs and MBCs did not differ more than 2-fold in their value indicating a bactericidal effect of copsin on Gram-positive bacteria. To verify this finding, a kill curve assay using B. subtilis was performed in MHB at pH 6 and 7.3 (Fig. 5). Independent of the pH, a clear reduction of the viable count was observed after 30 min of incubation, supporting a bactericidal activity of copsin.

**Molecular Target of Copsin in Bacteria**—The antibacterial activity and specificity of copsin was comparable with cell wall biosynthesis interfering antibiotics such as plecacin or vancomycin (6). We therefore determined the cellular localization of copsin on B. subtilis and S. carnosus cells during exponential growth phase, using TAMRA-labeled copsin and BODIPY-FL-labeled vancomycin as a control. Fig. 6 shows a dual staining of BODIPY-vancomycin- and TAMRA-labeled copsin. Vancomycin is known to localize specifically to sites of active cell wall synthesis (29). Copsin exhibited binding at the cell surface, preferably in curved regions of the rod-shaped bacterium B. subtilis and similarly distributed on the spherical-shaped S. carnosus cells. A co-localization with vancomycin was found at the cell septa.

To gain further insights on the molecular targets, we studied the binding affinity of copsin to essential precursors of bacterial cell wall synthesis in vitro. Lipid I was synthesized as N-acetyl-muramiduramic acid-pentapeptide (MurNAC-pentapeptide) linked to the bactoprenol-phosphophate lipid carrier (bactoprenol-phosphophosphate, C<sub>55</sub>–PP). The pentapeptide was composed of L-alanyl-γ-D-glutamyl-L-lysyl-D-alanyl-D-alanine, a composition found in Staphylococcus spp. For lipid II, GlicNAC was attached to the MurNAC moiety of lipid I (Fig. 7A). Binding studies included bactoprenol-phosphate (C<sub>55</sub>–P) and lipid II, a wall teichoic acid synthesis precursor consisting of GlicNAC linked to C<sub>55</sub>–PP (30). Copsin was incubated with the synthesized...
compounds in a defined molar ratio for 20 min, and the free substrate was analyzed by TLC (Fig. 7B). It was found that copsin bound in a 1:1 molar ratio to lipid I and II but had no affinity for C55-P and lipid III.

These findings suggested specific interaction of copsin with lipids I and II and indicated that MurNAc-pentapeptide might be pivotal for a stable complex. To examine whether the peptide chain was necessary for binding to copsin, truncated versions of the pentapeptide were synthesized, and the binding affinity to copsin was analyzed (Fig. 7C). Copsin showed a strongly reduced affinity for the lipid I-dipeptide (L-Ala–D-Glu) in comparison to the lipid I-tripeptide (L-Ala–D-Glu–L-Lys). This result demonstrated that the third position of the pentapeptide is essential for stable binding of copsin to the peptidoglycan precursor.

Based on the hypothesis that copsin bound to lipid II, we wanted to know whether copsin was able to permeabilize the bacterial membrane, similar to the mode of action of nisin, a pore-forming lantibiotic (25, 31). Two assays were performed: the first relied on CF efflux from lipid II containing liposomes (Fig. 8A), and the second relied on the potassium efflux of B. subtilis cells (data not shown). In both assays, no pore formation was detected. Importantly, preincubation of the lipid II containing liposomes with copsin inhibited the action of nisin, which was used as a positive control (Fig. 8B). These results showed that copsin interacted specifically with lipid II at the extracellular side of the bacterial membrane and prevented the binding of nisin but did not lead to pore formation.
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FIGURE 7. Binding of copsin to cell wall precursors. A, schematic of the peptidoglycan precursor lipid II and the wall teichoic acid precursor lipid III. M, MurNAc; G, GlcNAc; P, phosphate. B, purified cell wall precursors were incubated with increasing molar ratios of copsin. After extraction with n-butanol/pyridine acetate (pH 4.2), the samples were analyzed by TLC, which displayed a binding of copsin to lipids I and II. C, truncated versions of lipid I (lipid I-diopptide and lipid I-tripptide) as well as lipid I-pentapeptide were synthesized, using purified enzymes from S. aureus. Copsin was added in increasing molar ratios to the lipid I versions. After incubation for 20 min at room temperature, samples were extracted as described and analyzed by TLC. The result showed that copsin binds to the third position of the pentapeptide.

DISCUSSION

Co-cultivation studies of C. cinerea with bacteria led to the identification of the peptide-based antibiotic copsin, to our knowledge the first defensin identified and characterized in the fungal phylum of basidiomycota. Growing fungi on the surface of inert glass beads submerged in liquid medium provides a reproducible and easy to handle model system for studying the interaction with bacteria. In comparison to a confrontation assay on an agar plate or in a pure liquid culture, this setup has the big advantage of combining a solid surface and a humid environment, two pivotal factors for growth of bacteria and fungi in nature. Furthermore, it allows for a repeatable extraction of secreted fungal and bacterial substances to perform an appropriate analysis at the metabolomic and proteomic level. For our assays, B. subtilis, E. coli, and P. aeruginosa were selected as competitors, which are well characterized and are known to interact with fungi (1, 32, 33). Under the conditions tested, we observed an antagonistic behavior of the fungus with B. subtilis and P. aeruginosa and a more commensal association with E. coli. These differentiated interactions suggest an interdependent community of C. cinerea and bacteria on herbivorous dung to preserve their nutritional niche. Different studies demonstrated that AMPs act as regulators of microbial diversity, for example, α-defensins in mice or arminin peptides in Hydra species (34, 35). Based on the inhibition found against B. subtilis, we developed a novel workflow for the purification and identification of highly stable and active antibacterial peptides and proteins. This analytical method involved a quantitative MS measurement, where we finally selected copsin for further investigations.

P. pastoris is a highly efficient system for the heterologous production of secreted proteins at high yields in shake flasks and bioreactors (36). It ensured also a correct processing of copsin, because P. pastoris expresses a Kex2 protease that cleaves the pro-region at the lysine-arginine site, frequently identified in defensins (37). The three-dimensional structure of recombinant copsin was solved by NMR and revealed a CSαβ fold with a net charge of +7 at neutral pH. The CSαβ structural motif is a major characteristic of defensins of plants, invertebrates, and fungi and is differentiating them from vertebrate defensins with a common motif of a triple-stranded β-sheet structure (38). Copsin is stabilized by a unique connectivity of six cysteine bonds in contrast to most other CSαβ defensins, which are linked by three or four disulfide bonds. The structural compactness combined with an N-terminal pyroglutamate and a C-terminal cysteine involved in a disulfide bond render copsin extremely stable in a wide pH and temperature range and insensitive to proteases.

Analysis of predicted fungal proteomes revealed different classes of CSαβ defensins primarily in the phylum of ascomycota (37, 39). Based on the structural similarity, an orthologous relation of copsin to these fungal defensins seems to be likely and is further supported by the presence of CSαβ-like bacterio-
cins as a possible common ancestor (38, 39). However, because the similarity is limited to the secondary structural elements, we cannot exclude a structural convergence toward a particular function such as a highly stabilized fold. More comprehensive studies are needed to conclusively define the relationship between different classes of fungal defensins.

Microscopy studies and binding assays with cell wall precursors revealed lipid II as the molecular target of copsin. Lipid II is a highly conserved molecule throughout the bacterial kingdom and is accessible at the extracellular side of the bacterial membrane. Binding to this essential building block is an effective way to interfere with a proper cell wall synthesis and consequently to kill a bacterium (40). In addition, it is unlikely that toxic effects on, for example, mammalian cells would occur because of the unique structural composition of this peptidoglycan precursor. Antibiotics found to interact with lipid II exert their actions over defined binding sites, such as vancomycin, which interacts with the d-Ala residues of the pentapeptide (29). In depth studies of plectasin exhibited that the N-terminal GFGC motif is found in many other defensins (6). This motif is found in many other defensins such as MGD-1 or eurycin but is absent in copsin (28, 41). Binding studies of copsin with truncated versions of lipid I revealed that, instead of d-Ala and D-Glu, the third amino acid in the pentapeptide side chain is crucial for binding to copsin, independently of whether L-Lys or diaminopimelic acid is located at this position. This finding is consistent with the strong activity of copsin against a vancomycin-resistant E. faecium strain, where d-Lac is located at the C-terminal site of lipid II instead of d-Ala (42).

Copsin exhibited a distinct antibacterial profile differentiating it from other fungal defensins such as plectasin or micasin (5, 37). Besides Enterococcus spp., copsin exerted its most potent activity against L. monocytogenes, a food-borne pathogen causing severe forms of listeriosis in animals and humans (43, 44). The exceptional stability of copsin and the potent activity against bacteria are important features for further applications in clinics or food industry.

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REFERENCES

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