The Filament-specific Rep1-1 Repellent of the Phytopathogen *Ustilago maydis* Forms Functional Surface-active Amyloid-like Fibrils*

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### Abstract

Repellents of the maize pathogen *Ustilago maydis* are involved in formation of hydrophobic aerial hyphae and in cellular attachment. These peptides, called Rep1 to Rep1-11, are encoded by the rep1 gene and result from cleavage of the precursor protein Rep1 during passage of the secretion pathway. Using green fluorescent protein as a reporter, we here show that rep1 is expressed in filaments and not in the yeast form of *U. maydis*. *In situ* hybridization localized rep1 mRNA in the apex of the filament, which correlates with the expected site of secretion of the repellents into the cell wall. We also produced a synthetic peptide, Rep1-1. This peptide reduced the water surface tension to as low as 36 mJ m\(^{-2}\). In addition, it formed amyloid-like fibrils as was shown by negative staining, by thioflavin T fluorescence, and by x-ray diffraction. These fibrils were not soluble in SDS but could be dissociated with trifluoroacetic acid. The repellents in the hyphal cell wall had a similar solubility and also stained with thioflavin T, strongly indicating that they are present as amyloid fibrils. However, such fibrils could not be observed at the hyphal surface. This can be explained by the fact that the Rep1-1 filaments decrease in length at increasing concentrations. Taken together, we have identified the second class of fungal proteins that form functional amyloid-like filaments at the hyphal surface.

*Ustilago maydis* is the causal agent of smut in *Zea mays* (maize) and *Euchlaena mexicana* (Mexican teosinte). A filamentous pathogenic dikaryon is formed upon fusion of compatible yeast-like sporidia. Differentiation in the plant leads to the formation of diploid teliospores, which undergo meiosis ultimately resulting in haploid sporidia (see Refs. 1–3).

Fusion of haploid cells and development of an infectious dikaryon are controlled by the *a* and *b* mating type loci. The *a* locus regulates cell fusion through a pheromone-based recognition system (4), and the *b* locus controls post-fusion steps of pathogenic development, including hyphal growth. The latter locus encodes two unrelated homeodomain proteins, bE and bW, that form heterodimers when they are derived from different alleles (5, 6). This heterodimer regulates a number of genes, among which the rep1 gene (7–10). This gene is highly expressed resulting in 2.5% of the mRNA. It encodes a preprotein that, after processing at KEX2 recognition sites, results in 11 secreted peptides with a high sequence similarity. These peptides are localized in the cell wall of filaments, in an SDS-insoluble, but trifluoroacetic acid-extractable form (10). They are involved in formation of hydrophobic aerial hyphae (10) and in hyphal attachment to hydrophobic surfaces (11); as such they have functionally replaced hydrophobins in *U. maydis* (11).

Hydrophobins, which are not related to the repellents, fulfill a wide spectrum of functions in fungal development (12). They do so by forming an amphipathic protein film, which consists of amyloid-like fibrils (13–15). We here show that the repellent Rep1-1 also forms surface-active amyloid fibrils and that this peptide is specifically produced in filaments. Apparently, the amyloid structure is important for function in fungal development.

### Experimental Procedures

**Strains and Growth Conditions—**The *U. maydis* strains FB1 (*a1b1*, FB2 (*a2b2*) (16), and SG200 (*a1maf2 bE1/bW2*) (17) were used in this study. The non-isogenic strains FB1 and FB2 grow yeast-like in liquid medium. These strains are compatible and form a dikaryon upon mating that grows filamentous in contact with air. SG200 is a derivative of FB1 and harbors an active *b* mating type complex. As a consequence, this strain forms yeast-like cells in liquid medium and forms filaments at the water-air interface without the need for mating. *U. maydis* was grown at 25 °C using liquid or solidified (1.5% agarose) yeast extract, 0.4% peptone, 2% sucrose). For GFP expression analysis, 3 µl of cell suspension (2 × 10\(^7\) ml\(^{-1}\) cells) was seeded in a cut away within a 0.25-mm thin layer of solidified medium that had been sandwiched between a glass slide and a coverslip (Fig. 1A). A 0.17-mm layer of solidified medium was used for *in situ* hybridization.

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* The abbreviations used are: GFP, green fluorescent protein; eGFP, enhanced GFP; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; ThT, thioflavin T.
Rep1 Reporter Construct and Rep1 Constitutive Expression—The promoter region of the rep1 gene was amplified from genomic DNA of FB1 using Phusion™ polymerase (Finnzymes) with oligonucleotide primers prRep-fw (CGCTAGCTGACCTGCGCTAAG) and prRep-rev (GTGCAGCGCTGATGGAAAG). The resulting 1844-bp PCR fragment was used as a template in a PCR reaction with nested PCR primers prRep-KpnI-fw (GGTACCGCAGCATCAGAG) and prRepNcoI-rev (TGGAAGCCATGGTTGATGTCGA), introducing KpnI and NcoI restriction sites, respectively. The resulting 1688-bp fragment was introduced in the SmaI site of pUC19 and was amplified in Escherichia coli DH5a. Subsequently, the KpnI/NcoI promoter fragment of Rep1 was introduced in the corresponding sites of pMF3c (19). As a result, egFP was placed under control of the Rep1 promoter, resulting in construct pMF3c-prRep.

The Otef promoter (19) was amplified by PCR using primers Otef-fw and Otef-rev (TACCATGACCCGTGATGTGGTGGT), where the asp1 coding sequence (10) was amplified using primers Rep-fw (TACCATGACCCGTGATGTGGTGGT) and Rep-rev (AGGCAGCCCGAGATGTGGTGGT), introducing Agal promoters in vector pMF3c (19), thereby removing the eGFP coding sequence. The resulting vector was named pMF3c-OR.

Transformation of U. maydis—pMF3c-OR and pMF3c-prRep were introduced in U. maydis after digestion with Agel thus linearizing the plasmid in the carboxin resistance cassette. U. maydis was transformed according to Brachmann et al. (19), and transformants were selected on carboxin-containing PDA plates (2 μg ml⁻¹). Targeting to the succinate dehydrogenase gene sdh2 (um00844) was confirmed by Southern analysis. To this end, U. maydis chromosomal DNA was isolated as described previously (20) and blotted on Hybond N⁺ membrane. Hybridization was performed at 60 °C in 0.5 M phosphate buffer, pH 7.2, 7% SDS, 10 mM EDTA, using a [α³²P]dCTP-labeled 1.9-kb NotI carboxin cassette as a probe.

Rep1 Expression Analysis Using in Situ Hybridization—In situ hybridization was performed according to Teertstra et al. (21). Cultures that had been grown in an agarose slab of 20 × 20 mm were fixed for 2 h at 20 °C with freshly prepared 4% paraformaldehyde in PBS, pH 7.4. After washing with buffer, the agarose slab cultures were placed overnight at −20 °C in excess 50% ethanol in PBS (v/v). The agarose slabs were placed on a coverslip (22 × 22 mm) and stepwise rehydrated (30% ethanol in PBS; 10% ethanol in PBS; and twice in PBS, each step for 10 min). The remaining fluid was removed with a piece of filter paper, and the coverslip with the agarose slab was placed in a hybridization chamber (Corning Inc.). 50 μl of hybridization buffer (100 mM NaCl, 0.5% SDS in 25 mM Tris·HCl, pH 9.0) was added, after which a Hybrid-blot (22 × 22 mm, Molecular Probes) was placed on top of the buffer. The hybridization buffer contained an EuUni PNA probe (120 nM) and a rep PNA probe (300 nM). The eukaryotic 18 S rRNA EuUni probe (ACCAGACTTGGCCCTTC) (22) and the rep mRNA probe (CATGCAGCTTTTGTTC) were (5') N-terminally labeled with fluorescein and Cy3, respectively. These probes had a Tₘ between 65 °C and 70 °C at the concentrations used (Applied Biosystems and Panagene). Hybridization was performed at 54°C in a water bath for 1 h. The agarose slabs were washed three times for 20 min with 10 mM Tris·HCl, pH 9.0, 1 mM EDTA (TE buffer) at 54 °C and mounted on microscope slides using Vectashield mounting medium (Vector Laboratories).

Peptide Synthesis—The N-acetyl carboxymidy form of the Rep1-1 peptide HYEYKSYNAAGNVSVENKLVDASDLT-LGVDIL-NH₂ was assembled on an automatic ABI 433A Peptide Synthesizer using the ABI FastMoc 0.25-mmol protocols according to the instructions of the manufacturer. 9-Fluorenylmethoxycarbonyl (Fmoc)-amino acid derivatives, activated in situ using O-(benzotriazol-1-yl)-N,N,N'N'-tetramethyl-uronium hexafluorophosphate/N-hydroxybenzotriazole and N,N' disopropylethylamine in N-methylpyrrolidone were used in coupling steps of 45 min. The peptide was deprotected and cleaved from the resin by treatment with 10 ml of trifluoroacetic acid, 0.25 ml of H₂O₂, and 0.25 ml of triisopropylsilane for 2 h at room temperature. Finally, the peptide was precipitated in methyl-tert-butylenether/n-hexane (1/1, v/v), dissolved in 60 ml of tert-ButOH/water (1/1, v/v), and lyophilized. Preparative HPLC was performed using a semiautomatic HPLC system (Applied Biosystems) with a preparative reversed-phase column Phenomenex Luna C8 (100 Å, 10 μm, 250 × 21.2 mm, Phenomenex), a UV detector operating at 214 nm, and a flow rate of 12 ml min⁻¹. Analytical HPLC was performed using an automatic HPLC system (Shimadzu) with an analytical reversed-phase column Phenomenex Luna C8 (100 Å, 5 μm, 250 × 4.6 mm), a UV detector operating at 214 nm, and a flow rate of 1 ml min⁻¹. The buffers used were 0.1% trifluoroacetic acid in water/acetonitrile (95/5, buffer A) and 0.1% trifluoroacetic acid in acetonitrile/water (60/40, buffer B). The peptide was eluted with a linear gradient from 100% A to 100% B over 48 min (analytical) or with a linear gradient from 25% A to 77% B over 81 min (preparative). The peptide was characterized using matrix-assisted laser desorption/ionization time-of-flight (Kratos AXIMA CFR) with bovine insulin-oxidized B chain as the external reference (monoisotopic [M+H]+ = 3494.6513) and α-cyano-4-hydroxycinnamic acid as matrix. The calculated and detected monoisotopic masses [M+H]+ for Rep1-1 (C₁₇₀H₂₆₃N₄₄O₅₇) were determined as 3832.9034 and 3832.424, respectively.

SDS-PAGE—Proteins were separated on 12% SDS-PAGE gels and stained with colloidal Coomassie R-250 (23).

Surface Activity—Surface activity was measured with the Crüss Drop Shape Analysis system DSA10 Mk2 (Krüss) using 18-μl droplets of Rep1-1 solution.

Thioflavin T Fluorescence—Strains were grown on square siliconized coverslips of 22 × 22 mm (Hampton research) to induce filamentous growth. To this end, a 30-μl droplet of water containing 10⁶ cells from a culture in logarithmic phase was placed in the middle of the coverslip. Cells were grown under humid conditions for 40 h (11). The water was removed with a pipette, and the coverslips with attached hyphae were submerged in 7.5 μM ThT in PBS for 30 min in the dark. For yeast-like growth, strains were grown in YEPS at 28 °C. After washing with H₂O once, cells were resuspended in 7.5 μM ThT in PBS and incubated for 30 min in the dark. 5 μl of the suspensi-
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RESULTS

Repellents Are Specifically Expressed in Filaments of U. maydis—Strains FB1 and SG200 were transformed with pMF3c-prRep, which contains the eGFP gene behind the 1688-bp rep1 promoter. Southern analysis showed that the plasmid had been targeted into the sdh2 gene in transformants WR1 and WR2, which have an FB1 and a SG200 background, respectively (data not shown; see “Experimental Procedures” for details). These strains were grown on slices of nitrate minimal medium in between an object glass and coverslip (Fig. 1A). Cells of the FB1 derivative WR1 grew mainly yeast-like on the agar layer (Fig. 1B). Some filaments were formed that grew into the air (Fig. 1D). These filaments, but not the yeast-like cells, showed GFP fluorescence (Fig. 1, C and E). Cells of the SG200 derivative WR2, which encompasses active a- and b-mating type complexes, grew yeast-like on the agarose medium (Fig. 1F). From this layer, hyphae grew into the air (Fig. 1H) as well as into the medium (Fig. 1J). At a depth of 0.5 mm the filaments adopted a yeast-like morphology again (Fig. 1F). Both the aerial and substrate hyphae expressed eGFP, whereas expression was not observed in the yeasts (Fig. 1, G and I).

GFP fluorescence correlated with in situ hybridization using an rep1 PNA probe that binds to 5 of the 12 repeats within this gene. The PNA probe was found to hybridize in filaments of SG200, whereas no hybridization was observed in the SG200Δrep1 strain (Fig. 2, C and D). The 18 S PNA probe was detected both in yeasts and filaments of these strains in a co-hybridization experiment (Fig. 2, A and B). Interestingly, the rep1 probe was mainly observed in the apical part of hyphae of the SG200 strain.

The Repellent Peptide 1-1 Forms Amyloid Fibrils in Vitro—Peptide Rep1-1 was synthesized chemically (see “Experimental Procedures”). Similar to the peptide residing in the cell wall (10) synthetic Rep1-1 was only soluble in water or 2% SDS after treatment with trifluoroacetic acid. The peptide of 3.7 kDa ran at an apparent molecular mass of 21 kDa (data not shown), indicating that Rep1-1 forms oligomers.

The water surface activity of the peptide at a concentration of 100 μg ml⁻¹ was hard to measure. The shape of the drop hanging at the tip of a syringe changed readily (Fig. 3B) and often detached after a few minutes. In those cases that the droplet remained attached, surface activity was reduced from 72 to 36 mN m⁻¹ in a 1-h period (Fig. 3A). A similar decrease was observed at 30 μg ml⁻¹, but in this case it took 90 min to reach this surface tension. Reduction in the water surface tension was accompanied with the formation of a rigid protein film, as was shown by sucking back the water droplet into the syringe (Fig. 3C). Taken together, the Rep1-1 peptide self-assembles into a rigid highly surface-active protein membrane at the water-air interface.

Rep1-1 did not only assemble at the water-air interface, it also aggregated in the aqueous solution, as was shown by the reduction in the amount of soluble peptide as monitored by SDS-PAGE (Fig. 4A). The aggregates that had already formed within a 30-min period could be pelleted upon centrifugation and were soluble again in water after trifluoroacetic acid treatment (data not shown). Aggregate formation could be acceler-
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**FIGURE 1.** Localization of rep1 expression. A, cells of strain FB1 (B–E) and SG200 (F–I) were seeded in an air slot on top of a thin layer of solidified medium sandwiched between a glass slide and a coverslip, allowing the cells to grow into the medium as well as into the air. Cells were monitored by phase contrast (B, D, F, and H) and by fluorescence microscopy (C, E, G, and I). Cells of B, C, F, and G were growing in the agar medium (position indicated by a thin arrow in A), and cells of D, E, H, and I were growing in the air (position indicated by a thick arrow in A). Bar indicates 100 μm. Asterisks in B and F indicate clumps of yeast cells.

**FIGURE 2.** In situ co-hybridization of 18 S rRNA (A and B) and rep1 mRNA (C and D) in SG200 (A, C, and E) and in SG200Δrep1 (B, D, and F). E and F represent overlays of A and C, and B and D, respectively. Bar represents 20 μm.

**FIGURE 3.** Rep1-1 (100 μg ml⁻¹) forms a highly surface-active protein film at the water-air interface. A, temporal reduction of the water surface tension of the aqueous Rep1-1 solution. B, reduction of the water surface tension is accompanied by a change of the shape of the hanging droplet. C, a protein film remains attached to the tip of a syringe after sucking back the aqueous solution into the pipette.

Repellent Rep1-1 forms functional amyloid-like fibrils when aggregated by shaking, probably explained by creating a large water-air interface (Fig. 4A). Fluorescence of ThT was measured to address whether Rep1-1 forms amyloid-like fibrils upon aggregation. 30 μg ml⁻¹ of Rep1-1 in water was incubated with ThT 30 min after solubilization with or without additional vortexing for 3 min. In both samples an increase in fluorescence at A₄₈₅ was measured (Fig. 4B). Amyloid fibril formation was also indicated by negative staining. Long fibrils were observed when 3 μl of a solution of 50 μg ml⁻¹ Rep1-1 was dried down on a grid (Fig. 4D). In contrast, a more amorphous structure was observed at 100 μg ml⁻¹ (Fig. 4E). Finally, an amyloid-like nature was indicated by X-ray diffraction (Fig. 4C). Precipitated Rep1-1 showed a diffraction at 4.7 and 10 Å. These diffractions represent the inter-β-strand distance, resulting from the main-chain hydrogen bond, and the intersheet spacing between β-sheets (25, 26), respectively. The diffraction at 3.7 Å is also often found in diffraction patterns of amyloids (27, 28).
Repellents Form Amyloid Fibrils in Vivo—Scanning electron microscopy did not show amyloid fibrils at the surface of dikaryotic hyphae that grew out of a water droplet (Fig. 5A). In contrast, hydrophobin amyloids could be clearly visualized at the surface of A. niger spores (Fig. 5B). This result could be explained by the absence of amyloid fibrils in vivo or by the high concentration of the repellents in the cell wall (see Fig. 4E). To address this, cells of SG200 were stained with ThT. Yeast cells that had grown in the liquid medium did not stain with ThT (Fig. 6F). In contrast, hyphae that had grown out of the water droplet did stain (Fig. 6F). However, this was also observed with a SG200Δrep1 strain (Fig. 6D). This suggests that other cell wall proteins also form amyloids when exposed to air. Indeed, yeast cells of SG200 and SG200Δrep1 stained with ThT when they were air-dried (data not shown). Because these experiments did not indicate that repellents form amyloid in vivo, rep1 was placed under regulation of the strong constitutive Otef promoter in vector pMF3c-OR. Formation of hydrophobic aerial hyphae was restored when this vector was introduced in SG200Δrep1, showing that the gene was functionally expressed. In contrast to yeast cells of SG200 (Fig. 6F) or SG200Δrep1 (data not shown) that had been grown in liquid, yeast cells harboring pMF3-OR did stain with ThT (Fig. 6H). Staining correlated with the presence of rep1 mRNA as detected by Northern hybridization (data not shown). Taken together, it is concluded that repellents form amyloid in vivo but that also other cell wall proteins do so when in contact with air.

DISCUSSION

Class I hydrophobins fulfill a wide spectrum of functions in filamentous fungi. These proteins function by forming a rigid, surface-active film consisting of amyloid-like fibrils (12, 13). Recently, it was shown that repellents of U. maydis have replaced class I hydrophobins in forming hydrophobic aerial hyphae and in hyphal attachment to hydrophobic surfaces (11). We here showed that repellents, like class I hydrophobins, are filament-specific and that they form a rigid, surface active amyloid-like layer.

Previously, it was shown that rep1 is 50-fold up-regulated in a compatible cross between FB1 and FB2 when compared with the parental strains (10). However, expression in the monokaryons was still considerable (±0.05% of the mRNA). There-
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FIGURE 6. Visualization of amyloid fibrils by ThT fluorescence at the surface of yeast cells and hyphae of U. maydis. Bright field microscopy (A, C, E, and G) and ThT staining (B, D, F, and H) of SG200 (A, B, E, and F), SG200Δrep1 (C and D), and SG200Δrep1OR (G and H). Amyloid fibrils are detected at the surface of aerial hyphae of strain SG-200 (B) and its derivative SG200Δrep1 (D). In contrast, amyloid fibrils are not observed at the surface of yeast cells of strain SG200 grown in liquid culture (F). However, they are detected at yeast cells of SG200Δrep1OR that express rep1 from the constitutive Otef promoter (H). Bar indicates 20 μm.

Therefore, we addressed whether yeast cells do express rep1. Using GFP as a reporter, it was shown that rep1 was only expressed in the few filaments formed by the FB1 strain. Expression was observed from the moment yeasts switched to filamentous growth. Hyphae in the FB1 strain were formed at the water-air interface, and most of them grew out into the air. Formation of filaments was much more pronounced in the co-isogenic SG200 strain that contains a compatible combination of α- and β-mating-type genes. In this case, hyphae did not only grow into the air but also into the agar medium. Both the aerial and vegetative hyphae were shown to express GFP from the rep1 promoter, and the fluorescence intensity was similar to that of hyphae of FB1. Taken together, it is concluded that rep1 is filament-specific rather than mating-type-regulated as was concluded previously (10).

In situ hybridization correlated with GFP fluorescence. Fluorescence was observed in filaments and not in yeast cells. Interestingly, in contrast to GFP, the rep1 probe was mainly observed in the apical part of the hypha. This difference can be explained by the stability of the GFP protein and/or its diffusion in the cytoplasm. Our results indicate that the rep1 mRNA is synthesized in the apex or is transported to this site. Trafficking of mRNA to the apex has been proposed to occur in U. maydis (29). In the case of the rep1 mRNA this would make sense, because the encoding protein is expected to leave the hyphae at the apex (30–32).

Repellents were originally identified by adopting the procedure to purify class I hydrophobins from cell walls (10). Like the hydrophobins, repellents could only be extracted from the cell wall with trifluoroacetic acid, after which these peptides were soluble in water or aqueous detergent (10, 33, 34). Synthetically produced Rep1-1 showed similar solubility as repellents and hydrophobins in the cell wall. It was only soluble in water or SDS after trifluoroacetic acid treatment. Like the class I hydrophobin, the Rep1-1 peptide assembled into a surface-active rigid membrane at the water-air interface. The decrease in water surface tension to 36 mJ m⁻² is similar to that of hydrophobins and other microbial surface-active peptides involved in aerial growth and attachment (35–39). The water-soluble form of the peptide, which seems to be an oligomer based on its behavior in SDS-PAGE, also aggregated readily in a SDS-insoluble form in the aqueous solution. This contrasts the class I hydrophobins (34), which have four di-sulfide bridges that prevent premature assembly in the aqueous environment (40). How the repellent prevents self-assembly in the cell is not yet known. The unprocessed pro-protein Rep1 may have a function in this.

The SDS-insoluble form of the synthetic repellent was shown to be of an amyloid-like nature. It stained with ThT, and it showed the typical x-ray diffraction pattern of amyloids. Scanning electron microscopy did not reveal amyloid fibrils at the surface of filaments. This suggested that repellents do not form amyloid in vivo. However, Rep1-1 was shown not to aggregate in detectable long fibrils at a high concentration, as is assumed to be the case in the cell wall (10). The absence of detectable fibrils at a high protein concentration can be explained by a high number of nucleation points, as was reported previously for hydrophobins (32). ThT staining was performed to strengthen the hypothesis that repellents do form amyloid in vivo. Yeast cells that do not express rep1 in liquid medium did not stain with ThT. In contrast, these cells did stain when the gene was expressed from the constitutive O2tef promoter. Taken together, we conclude that at least Rep1-1 forms amyloid-like fibrils in vitro as well as in vivo. The similarity between the repellents (10) suggests that other peptides of the family partic-
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Repellent Rep1-1 forms functional amyloid-like fibrils. In liquid medium, Rep1-1 forms fibrils that are stable in the absence of air. Contact with air induces amyloid formation, similar to other cell wall proteins. This suggests that other cell wall proteins can also form amyloid-like structures. The repellent Rep1-1 has been shown to form fibrils that are similar to those formed by other cell wall proteins, indicating that this is a common property of cell wall proteins.

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