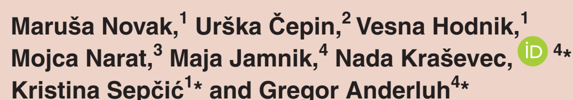
Molecular Microbiology (2019) 112(4), 1253–1269
doi:10.1111/mmi.14360
First published online 16 August 2019

Functional studies of aegerolysin and MACPF-like proteins in *Aspergillus niger*



Maruša Novak,[1] Urška Čepin,[2] Vesna Hodnik,[1] Mojca Narat,[3] Maja Jamnik,[4] Nada Kraševec, [4]* Kristina Sepčić[1]* and Gregor Anderluh[4]*

[1] *Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia.*
[2] *BioSistemika Ltd and National Institute of Biology, Ljubljana, Slovenia.*
[3] *Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia.*
[4] *Department of Molecular Biology and Nanobiotechnology, National Institute of Chemistry, Ljubljana, Slovenia.*

Summary

Proteins of the aegerolysin family have a high abundance in Fungi. Due to their specific binding to membrane lipids, and their membrane-permeabilization potential in concert with protein partner(s) belonging to a membrane-attack-complex/perforin (MACPF) superfamily, they were proposed as useful tools in different biotechnological and biomedical applications. In this work, we performed functional studies on expression of the genes encoding aegerolysin and MACPF-like proteins in *Aspergillus niger*. Our results suggest the sporulation process being crucial for strong induction of the expression of all these genes. However, deletion of either of the aegerolysin genes did not influence the growth, development, sporulation efficiency and phenotype of the mutants, indicating that aegerolysins are not key factors in the sporulation process. In all our expression studies we noticed a strong correlation in the expression of one aegerolysin and MACPF-like gene. Aegerolysins were confirmed to be secreted from the fungus. We also showed the specific interaction of a recombinant *A. niger* aegerolysin with an invertebrate-specific membrane sphingolipid. Moreover, using this protein labelled with mCherry we successfully stained insect cells membranes containing this particular sphingolipid. Our combined results suggest, that aegerolysins in this species, and probably also in other aspergilli, could be involved in defence against predators.

Accepted 28 July, 2019. *For correspondence. E-mail nada.krasevec@ki.si (N. K.); Tel. + 386 1 4760 262; Fax + 386 1 4760 300; E-mail kristina.sepcic@bf.uni-lj.si (K. S.); Tel. + 386 1 3203 419; Fax + 386 1 2573 390; E-mail gregor.anderluh@ki.si (G. A.); Tel. + 386 1 4760 210; Fax + 386 1 4760 300.

Introduction

The aegerolysin protein family (PF06355; InterPro IPR009413) is currently composed of more than 350 low-molecular-weight (15–20 kDa), acidic, β-structured proteins that have been found in several eukaryotic and bacterial taxa, and are particularly abundant in fungi (Berne *et al.*, 2009; Novak *et al.*, 2015; Butala *et al.*, 2017). The exact biological role of these proteins in fungi is still not clear. However, there is evidence that their production is developmentally regulated in the producing organism (reviewed in Butala *et al.* 2017 and Ota *et al.* 2014). For example, in several Basidiomycota, the expression and consequent production of aegerolysins have been shown to peak during the formation of primordia and young fruiting bodies (Fernandez Espinar and Labarère, 1997; Berne *et al.*, 2002; Lee *et al.*, 2002; Shim *et al.*, 2006; Joh *et al.*, 2007).

The family of proteins with membrane-attack-complex/perforin (MACPF) domain (PF01823) is a large protein family containing proteins from all kingdom of life, among which the animal-derived MACPF proteins are prevailing (Gilbert *et al.*, 2013). While roles of MACPF proteins in the producing organisms are generally well-defined, roles of fungal MACPF-like proteins that represent only a smaller group of approximately 200 proteins, remain to be elucidated (Anderluh *et al.*, 2014; Ota *et al.*, 2014). The only MACPF-like protein from genus *Aspergillus* described to date is SpoC1-C1C protein from *Aspergillus nidulans* that was highly expressed during later stages of sporulation and in spores (Stephens *et al.*, 1999).

The common feature of aegerolysins is their interactions with specific lipids in artificial and biological membranes (Sepčić *et al.*, 2004; Ota *et al.*, 2013; Skočaj *et al.*, 2014; Bhat *et al.*, 2015). Aegerolysins from the fungal genus *Pleurotus* are the most studied in this regard,

© 2019 John Wiley & Sons Ltd

and these aegerolysins have been shown to specifically target membrane domains enriched in sphingomyelin and cholesterol (Sepčić *et al.*, 2004; Tomita *et al.*, 2004; Bhat *et al.*, 2013; Ota *et al.*, 2013; Skočaj *et al.*, 2014). It was recently reported that some *Pleurotus* aegerolysins can also interact with ceramide phosphoethanolamine (CPE), the major membrane sphingolipid of invertebrates (and particularly of insects and molluscs), which is only found in trace amounts in higher taxa (Crone and Bridges, 1963; Itasaka *et al.*, 1973; Vacaru *et al.*, 2013). The interactions of these aegerolysins with CPE/cholesterol membrane systems is at least 1000-fold stronger than the interaction with sphingomyelin/cholesterol (Bhat *et al.*, 2015; Kishimoto *et al.*, 2016; Panevska *et al.*, 2019). Moreover, *Pleurotus* aegerolysins can effectively function as bi-component pore-forming complexes targeting artificial sphingomyelin/cholesterol vesicles and mammalian cell membranes when in combination with a 59-kDa, MACPF-like protein pleurotolysin B (Tomita *et al.*, 2004; Shibata *et al.*, 2010; Ota *et al.*, 2013; Lukoyanova *et al.*, 2015). Our recent data show that aegerolysins ostreolysin A6, pleurotolysin A2 and erylysin A, when combined with pleurotolysin B, effectively permeabilize artificial CPE/cholesterol membrane systems and insect cells. Moreover, these complexes show selective toxicity toward western corn rootworm larvae and adults and Colorado potato beetle larvae, indicating that their role in the fungi may be related to defence against invading organisms (Panevska *et al.*, 2019).

The fungal genus *Aspergillus* is of critical importance to humankind because these species are a source of many industrial applications, while also being important pathogens of humans, animals and crops, potent carcinogenic contaminants of food, and an important genetic model (de Vries *et al.*, 2017). To date, over 30 predicted aegerolysin proteins have been found in 18 *Aspergillus* species, but only a few have been described so far (Novak *et al.*, 2015). The first ever reported aegerolysin protein, Asphemolysin (AspHS), was isolated from the filamentous fungus *Aspergillus fumigatus*, the main causative agent of invasive aspergillosis in immunocompromised patients (Sakaguchi *et al.*, 1975). AspHS is a 15-kDa protein with a low isoelectric point (4.0). It was reported to be haemolytic and involved in the pathogenesis of *A. fumigatus*. AspHS was detected *in vivo* during experimental infection of mice by *A. fumigatus* spores, and anti-AspHS antibodies were shown to protect mice from infection by *A. fumigatus* spores (Ebina *et al.*, 1982). However, subsequent studies using wild-type and knock-out *aspHS* and *aspHS*-like mutant strains of *A. fumigatus* have shown that production of AspHS is not important for the progress of invasive alveolar aspergillosis in mice (Wartenberg *et al.*, 2011). Instead, AspHS has been suggested to have a role in potentiating other virulence mechanisms of the fungus (Rementeria *et al.*, 2005). Beside AspHS and AspHS-like aegerolysins from *A. fumigatus*, the examples from the literature include terrelysin from *A. terreus* (Nayak *et al.*, 2011), and AspHS homologues from *A. niger* (Braaksma *et al.*, 2010; Lu *et al.*, 2010). One of AspHS homologues from *A. niger*, terrelysin from *A. terreus* and AspHS from *A. fumigatus* were all reported to be secreted by these fungi, with the later one even being among the major proteins secreted (Braaksma *et al.*, 2010; Lu *et al.*, 2010; Wartenberg *et al.*, 2011; Nayak *et al.*, 2012).

Taking all of these aspects into an account, we investigated some characteristics and possible biological role(s) of aegerolysin and MACPF-like proteins in a saprophytic, ubiquitous fungus, *A. niger*. Here, a systematic study of the expression of genes encoding these proteins was carried out using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Aegerolysin gene deletion mutants were constructed and the impact of that deletion on growth and development of *A. niger* was determined. Secretion of aegerolysins was confirmed with Western blotting. In addition, we have analysed the lipid binding specificity of a recombinant *A. niger* aegerolysin by vesicle sedimentation assay and surface plasmon resonance (SPR) and shown that this binding is biologically relevant by staining membranes of insect cells with fluorescently labelled aegerolysin.

Results and discussion

The genome of A. niger *contains two aegerolysin and two MACPF-like genes*

Proteins encoded by genes derived from the two genomes of the fungus *A. niger* (CBS 513.88 [Pel *et al.*, 2007] and ATTC 1015 [Andersen *et al.*, 2011]) were searched with BLASTP algorithm using amino acid sequences of three fungal aegerolysins, OlyA from *P. ostreatus*, AspHS and AspHS-like from *A. fumigatus*, and of two fungal proteins with MACPF domain, PlyB from *P. ostreatus* and SpoC1-C1C of *A. nidulans*. Ostreolysin A, besides its highly homologous variant pleurotolysin A, till now remains the best functionally characterised protein from aegerolysin family (Sepčić *et al.*, 2004, Tomita *et al.*, 2004, Ota *et al.*, 2013, Bhat *et al.*, 2015, Lukoyanova *et al.*, 2015, Butala *et al.*, 2017, Panevska *et al.*, 2019). AspHS and AspHS-like proteins from *A. fumigatus*, belonging to *Aspergillus* genus, were chosen as other well-studied aegerolysins (Sakaguchi *et al.*, 1975; Ebina *et al.*, 1982; Wartenberg *et al.*, 2011; Rementeria *et al.*, 2005). In combination with hits from the Pfam database (PF06355 or PF01823), two nucleotide sequences encoding aegerolysin homologues, An19g00210 (*nigA1*) and An01g09980 (*nigA2*), and two nucleotide sequences encoding homologues of

protein with MACPF domain, An01g09970 (*nigB1*) and An09g03750 (*nigB2*), have been identified (Fig. S1). Following the naming of these proteins in *Pleurotus* sp., these predicted homologues have been named as nigerolysins (Nig), adding A and a number (1 or 2) for aegerolysins and B and a number (1 or 2) for MACPF-like proteins. From the positions of the genes in the *A. niger* genome it is evident that *nigA2* and *nigB1* genes form a bi-directional gene pair with 5′–5′ orientation (Fig. S1), as do *olyA* (*plyA*) and *plyB* genes in the *P. ostreatus* genome. Supplementary figure (Fig. S2) shows alignments and phylogenetical relations of NigA1 and NigA2 aegerolysin proteins to at least partly functionally studied fungal aegerolysins. MACPF-like proteins have low sequence identity (< 20%) and high numbers of introns coded in their genes (Anderluh *et al.*, 2014). Both proteins, NigB1 and NigB2, were confirmed by having MACPF-like signature (Y/F-G-X_2-F/Y-X_6-G-G) in protein models generated using MACPF/CDC proteins as templates.

Expression of genes that encode aegerolysin and MACPF-like proteins in A. niger *is linked to sporulation*

The aim of this study was to obtain the expression profiles of the genes of *A. niger* that encode aegerolysin and the MACPF-like proteins during growth and development, and to establish any links between strongly induced expression of these genes and particular developmental stages of *A. niger*. Therefore, the growth and development of the fungus were monitored macroscopically and microscopically at 8 or 16-h intervals (from 8 to 72 h), where the expression of *nigA1*, *nigA2*, *nigB1* and *nigB2* genes was analysed, along with the spores, using RT-qPCR (Fig. 1A and B). In concert with the fungal morphology, the expression levels of these four genes of interest also changed over time, following similar trends: an increase in gene expression to 40 h of growth, followed by a decrease to 48 and 56 h (Fig. 1B). The significant increases and the highest gene expression for all four genes of interest coincided with the development of the first conidiophores that contained few spores (at 32 h), followed by increased numbers of conidiophores that contained more spores (at 40 h). These significant increases and the highest gene expression (i.e., from 24 to 40 h) were statistically significant for all four genes of interest (Table S1). During the strong sporulation period (56–72 h), the expression of *nigA1*, *nigA2*, *nigB1* and *nigB2* genes continued to decrease. Similar correlations between aegerolysin gene expression and sporulation have been reported previously, for the *Alternaria gaisen* gene that encodes aegerolysin L152, the expression of which increased significantly after activation of sporulation following its exposure to light (Roberts *et al.*, 2011), and for the *A. oryzae* promoter of the *hlyA* gene, the expression of which was highest while *A. oryzae* was sporulating actively (i.e., 48–72 h after inoculation) (Bando *et al.*, 2011). These correlations are not only related to fungi, as the *B. thuringiensis* aegerolysin Cry34Ab1 was also synthesised during bacterial sporulation (Ellis *et al.*, 2002).

Wartenberg *et al.* (2011) observed similar expression profiles for aegerolysin genes *aspHS* and *aspHS-like*, although the expression of the genes was increased at an earlier developmental stage, i.e. during the exponential phase of the growth. Different manner of the growth of the fungi (with no sporulation in liquid media *vs*. plates) might explain the increased expression of the aegerolysin genes at different developmental stages. Also, correlation has been reported previously for the expression of the genes that encode aegerolysin and MACPF-like proteins (or for the production of these proteins) in primordia and fruiting bodies of *Moniliophthora perniciosa*, *Agrocybe aegerita* and *Agrocybe cylindracea*, although not for all of these homologues in each of these species (Shim *et al.*, 2006; Pires *et al.*, 2009; Wang *et al.*, 2013).

While aegerolysins have been detected in dormant *A. fumigatus* conidia (Teutschbein *et al.*, 2010; Wartenberg *et al.*, 2011; Suh *et al.*, 2012; Bayry *et al.*, 2014) and *P. ostreatus* basidiospores (Vidic *et al.*, 2005), in *A. niger* only the *nigA1* gene was barely detected. As because of the nature of the samples that contain many spores the RNA quantity and quality may be poor, this might impact lower aegerolysin gene detection using RT-qPCR than expected. This comparison is only partially valid as it compares asexual with sexual sporulation of fungi, although in both cases such spore formation and spores reflect a longer lasting life-form, during which Aspergilli or *P. ostreatus* mushrooms often encounter predators.

Due to the observed correlation between strongly induced expression of *nigA1*, *nigA2*, *nigB1* and *nigB2* genes and sporulation of *A. niger*, we investigated whether sporulation was indeed crucial for this strong induction of gene expression. Therefore, gene expression of these four genes was compared between sporulating (40 h – s, the same as 40 h in Fig. 1B) and non-sporulating fungal mycelia, using RT-qPCR (Fig. 1C). Sporulation was prevented either physically (growth between two porous cellophane membranes, 40 h – n) or genetically (non-sporulating mutants of *A. niger*: Δ*flbA*, Δ*brlA*; [Krijgsheld, Nitsche *et al.*, 2013]). Although conidiophores without spores have been reported to eventually develop when *A. niger* was grown between the two membranes (Krijgsheld, Bleichrodt *et al.*, 2013), this was not observed here from 24 to 72 h of growth (Fig. S3A). For the Δ*flbA* mutants, these do not form conidiophores; instead, they form many aerial hyphae (Lee and Adams, 1994; Wieser *et al.*, 1994). On the other hand, the Δ*brlA*

mutants form conidiophores with longer stalks than usual, but no swelling occurs and no vesicles are formed at the ends of the stalks (Adams *et al*., 1988). In the comparison of the expression levels of the *nigA1*, *nigA2*, *nigB1* and *nigB2* genes in non-sporulating fungal mycelia with sporulating mycelium, almost all of these were strongly reduced in the non-sporulating mycelia. The exception here was *nigB2* gene in the Δ*brlA* mutant (note also that for *nigB2* gene in the Δ*brlA* mutant, *t*-test for the sample 40 h – n could not be performed because the transcript copy number was too low

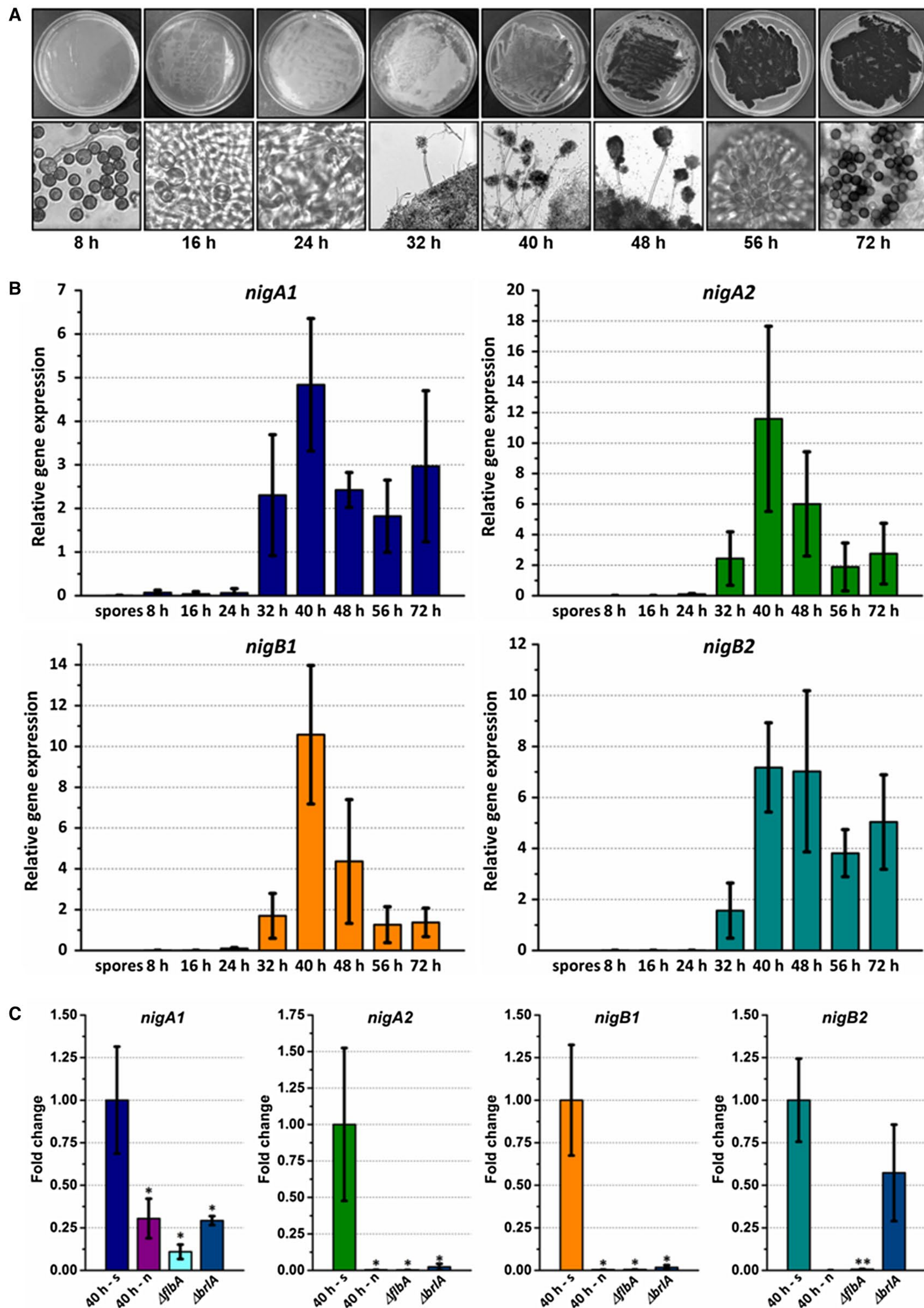


Fig. 1. Expression of genes that encode aegerolysin (*nigA1*, *nigA2*) and MACPF-like proteins (*nigB1*, *nigB2*) in *A. niger* is linked to sporulation.
A. Growth and development of *A. niger* at macroscopic (first row) and microscopic (second row) levels at 8-h or 16-h intervals (from 8 to 72 h).
B. Expression of *nigA1*, *nigA2*, *nigB1* and *nigB2* genes during growth of *A. niger*, as analysed using RT-qPCR. Relative gene expression per time point or in the sample of spores is given. Geometric mean of the relative transcript copy numbers of *actA* and *cox5* genes were used for normalisation. Data are means ± standard deviation, from three independent biological replicates.
C. Effect of physical or genetic prevention of sporulation on the expression of *nigA1*, *nigA2*, *nigB1* and *nigB2* genes, as analysed using RT-qPCR. Expression of these four genes was compared between sporulating (40 h – s = 40 h from B) and non-sporulating fungal mycelia (40 h – n, growth in between the two porous cellophane membranes; Δ*flbA* and Δ*brlA*, non-sporulating mutants of *A. niger* N402). Geometric mean of the relative transcript copy numbers of *actA* and *cox5* genes were used for normalisation. Relative gene expression is given compared to the control sample (fold change) in sporulating and non-sporulating strains of *A. niger*. Data are means ± standard deviation, from three independent biological replicates. *, $p < 0.05$; **, $p < 0,005$. *T*-test for sample 40 h – n for *nigB2* gene could not be performed because the quantity of the transcript was too low for reliable quantification with RT-qPCR. *nigA1*, gene encoding aegerolysin NigA1; *nigA2*, gene encoding aegerolysin NigA2; *nigB1*, gene encoding MACPF-like protein NigB1; *nigB2*, gene encoding MACPF-like protein NigB2; 40 h – s, sporulating *A. niger* N402 grown for 40 h; 40 h – n, non-sporulating *A. niger* N402 grown for 40 h between two porous cellophane membranes; Δ*flbA*, *A. niger* N402 mutant carrying a gene deletion of *flbA*; Δ*brlA*, *A. niger* N402 mutant with deleted gene for *brlA*. [Colour figure can be viewed at wileyonlinelibrary.com]

for the reliable quantification using RT-qPCR). These data indicated that sporulation process is indeed particularly important for the induction of expression of these four genes. However, while the formation of the spores appeared to be crucial for induction of the *nigA2* and *nigB1* genes, the formation of the conidiophores also appeared to be important to induce the expression of *nigB2* gene. Induction of *nigA1* expression appeared to be the least dependent on sporulation, as when sporulation was prevented physically, only the 40-h time point defined a statistically significant decrease in the expression of this gene compared to the sporulating mycelium (i.e., not at 24, 32, 48, 56, 72 h; Fig. S3B).

From these sets of RT-qPCR data, it is evident that the *nigA1* gene promoter is expressed at more instances than any of the other analysed promoters, as its expression was detected in considerable amounts at all of the times tested (with the exception of spores, Fig. 1B). Moreover, physical prevention of sporulation affected the *nigA1* gene expression dynamics the least (Fig. S3B). These observations support the findings of Bando *et al.* (2011), where they reported that the promoter that regulated expression of the *hlyA* gene in *A. oryzae* (i.e., a *nigA1* homologue) was more active than the promoter of the α-amylase gene, not only when *A. oryzae* was grown on plates at different temperatures and water activity, but also in liquid culture. To further support this, the *nigA1* gene was reported to be one of the 100 genes with the highest expression levels in the vegetative mycelium and aerial structures of *A. niger* (Bleichrodt *et al.*, 2013). Taking all of these into the account, aegerolysin NigA1 might have a more important role than the other three proteins, and/or NigA1 might have a different role than aegerolysin NigA2 in *A. niger*.

Expression of genes encoding aegerolysin and MACPF-like proteins in A. niger *is affected by different abiotic and biotic factors of the environment*

Expression of *nigA1*, *nigA2*, *nigB1* and *nigB2* genes might also be affected by different abiotic and/or biotic factors of the environment to which *A. niger* needs to adapt in its ecological niche. Therefore, *A. niger* N402 was exposed to different carbon sources (i.e., glucose, xylose, glycerol, arabinose, cellulose, starch, fructose, galactose, glucuronic acid, maltose, inulin, pectin, glucose with oleic acid, glucose with linolenic acid), different nitrogen sources (i.e., NH_4Cl, hydroxyproline), different stress factors (i.e., temperature stress, 15 or 42°C; anaerobic stress, atmosphere with no oxygen; osmotic stress, 1.5 M NaCl; oxidative stress, 15 mM H_2O_2; pH stress, pH 3 or 8; iron repletion, transfer from iron-depleted to iron-replete medium), and different organisms found in the soil (i.e., other *Aspergillus* species, bacteria of the genera *Streptomyces*, *Pseudomonas*, *Bacillus*). When exposed to the different abiotic factors, one was changed for a short period of time (i.e., 1 h) to define any immediate fungal response, while for the biotic factors, *A. niger* was co-cultivated with one of the organisms for 40 h, at one centimetre distance between the two.

Using RT-qPCR, we found the expression of these genes that encode aegerolysin and MACPF-like proteins in *A. niger* significantly affected by various abiotic factors (Table 1, Table S2). These abiotic factors could not be attributed to one particular group (e.g., temperature stress), but were numerous and occurred for all of the groups tested (with the exception of the osmotic stress). These data show that when *A. niger* was exposed to different carbon sources, the expression of the *nigA1* gene (with the exception of pectin) was strongly reduced, while the expression of the *nigA2* and *nigB1* genes, and also of the *nigB2* gene (although not in all the cases, and not as strongly as for *nigA2* and *nigB1*) was strongly induced. On the other hand, these significant effects were similar for all four genes when *A. niger* was exposed to the abiotic factors across other groups; i.e., the expression was strongly reduced. Among these factors, the greatest effects were seen for high temperature (i.e., 42°C). McDonagh *et al.* (2008) compared *A. fumigatus* transcriptomes obtained

Table 1. Significant changes in the expression of genes encoding aegerolysin (*nigA1*, *nigA2*) and MACPF-like proteins (*nigB1*, *nigB2*) in *A. niger* after exposure to different abiotic factors.

	Abiotic factor	*nigA1*	*nigA2*	*nigB1*	*nigB2*
Different carbon sources	Glucose	–	++	++	
	Xylose	–––			
	Glycerol	––	+	+	
	Arabinose	–––			
	Cellulose			+	
	Starch		+++	++	
	Fructose	–––	++	+	
	Galactose	–––	+++	++	
	Glucuronic acid		++	++	+
	Maltose	–––	+		
	Inulin		+++	+	+
	Pectin	++	+		+
	Glucose + oleic acid				––
	Glucose + linolenic acid				––
Different nitrogen sources	NH_4Cl				
	Hydroxyproline		––	–––	
Temperature stress	15°C	–			
	42°C	–	–	–	
Anaerobic stress	No oxygen		–	–	
Osmotic stress	NaCl				
Oxidative stress	H_2O_2	––		–	
pH stress	pH 3			––	–
	pH 8		–	–	
Iron repletion	+ Fe				–

The significance of increase (+: $p < 0.05$, ++: $p < 0.005$, +++: $p < 0.001$) or decrease (–: $p < 0.05$, ––: $p < 0.005$, –––: $p < 0.001$) in the expression of genes. An empty field denotes no significance. For details on relative gene expressions and fold changes (relative to corresponding control) with standard deviations of listed genes after exposure to different abiotic factors see Table S2.

during its *in vivo* infection and growth and *in vitro* growth when exposed to various abiotic factors, to determine which factors *A. fumigatus* needs to adapt to after the onset of an infection. Among others, they showed that the expression of *aspHS* aegerolysin gene was strongly reduced at 12 to 14 h post-infection (germinating hyphae), which was primarily due to iron depletion, while acid shift and oxidative and anaerobic stress did not affect *aspHS* expression. Although our data are only in agreement in terms of responses to oxidative stress and low pH, and not iron depletion and anaerobic stress, these differences might be attributed to monitoring the expression of the *aspHS* and *nigA2* genes at different developmental stages of the fungi (germinating hyphae *vs*. sporulating fungus, respectively).

The expression of the genes that encode aegerolysin and MACPF-like proteins appeared also to be affected by various biotic factors (Table 2, Table S3). Among these, *nigA1* gene turned out to be the most responsive, with its expression significantly up-regulated in the presence of bacteria of the genera *Bacillus* and *Pseudomonas*. On the contrary, the expression of the other aegerolysin gene, *nigA2*, was not significantly affected in any of these cases. As seen for *nigA1* gene, co-cultivation with *P. aeruginosa* strongly induced the expression of *nigB2* gene, whereas co-cultivation with *A. brasiliensis* strongly reduced the expression. The expression of *nigB1* gene, as in case of *nigA2* gene, was not significantly affected by any of the biotic factors. It should be noted that only a small number of organisms representing a few of the groups of soil organisms were chosen for co-cultivation with *A. niger* here. Nevertheless, the data obtained do indicate a possible role for the aegerolysin NigA1 in the interactions of *A. niger* with soil bacteria, such as those of the genera *Pseudomonas* and *Bacillus*; however, its precise role remains to be determined.

Table 2. Significant changes in the expression of genes encoding aegerolysin (*nigA1*, *nigA2*) and MACPF-like proteins (*nigB1*, *nigB2*) in *A. niger* after exposure to different biotic factors.

Biotic factor	*nigA1*	*nigA2*	*nigB1*	*nigB2*
Aspergillus brasiliensis				–
Aspergillus terreus				
Streptomyces anulatus				
Streptomyces netropsis				
Pseudomonas aeruginosa	+			+
Pseudomonas fluorescens	+			
Bacillus cereus	+			
Bacillus subtilis	+			

The significance of increase (+: $p < 0.05$) or decrease (–: $p < 0.05$) in the expression of genes. An empty field denotes no significance. For details on relative gene expressions and fold changes (relative to corresponding control) with standard deviations of listed genes after exposure to different biotic factors see Table S3.

In summary, the data further support our hypothesis that the role of the aegerolysin NigA1 in the fungus *A. niger* is different from the role of aegerolysin NigA2. Additionally, for all of the data obtained with RT-qPCR, there was high correlation of expression of the gene that encodes aegerolysin NigA2 and the gene that encodes MACPF-like protein NigB1. As these two genes form a bi-directional gene pair with 5′–5′ orientation, the observed correlation might not be surprising and might indicate that these two proteins function together, as has already been shown for the bi-directional gene pair *olyA* (*plyA*) and *plyB* in the fungus *P. ostreatus* (Tomita *et al.*, 2004; Ota *et al.*, 2013).

Aegerolysin gene deletion mutants of A. niger *show no obvious phenotypic changes*

To better understand the importance of aegerolysins for *A. niger*, two aegerolysin gene deletion mutants were prepared that were derived from the parental strain N402, Δ*nigA1* and Δ*nigA2*. When comparing their growth and development, sporulation efficiency, and growth dynamics and phenotype while exposed to different abiotic factors to these for the wild-type strain, no differences between the strains were observed (Fig. S4). This indicated that aegerolysins are not vital for the survival of the fungus *A. niger* when exposed to the abiotic factors tested, and are most likely not the key factors for growth and developmental processes in this organism; e.g., the sporulation process, as has been suggested for some aegerolysins in fungi (Fernandez Espinar & Labarère, 1997; Lee *et al.*, 2002; Vidic *et al.*, 2005; Berne *et al.*, 2009; Pires *et al.*, 2009; Kurahashi *et al.*, 2013; Nayak *et al.*, 2013; Ota *et al.*, 2014). Our observations are similar to those reported by Wartenberg *et al.* (2011) for aegerolysin single-gene and double-gene deletion mutants in *A. fumigatus*, where deletion of *aspHS* and/or *aspHS-like* aegerolysin genes did not affect the growth and development of *A. fumigatus* on different media, nor its haemolytic and cytolytic activity and virulence. This thus defines these two proteins as dispensable for all of these processes. It is therefore possible that the lack of any obvious phenotypic changes for the aegerolysin gene deletion mutants in the present study means that these aegerolysins are involved in processes that have not been investigated here, and/or other proteins have taken on the role of aegerolysins. If the latter is true, this role would most likely be taken by some other proteins and not aegerolysins, as Wartenberg *et al.* (2011) observed no difference between aegerolysin single-gene and double-gene deletion mutants, and as we observed strong decrease in the expression of the *nigA2* gene in the deletion mutant Δ*nigA1* (Table S4), which is the exact opposite of what might be expected in the case of role acquisition.

Aegerolysins from A. niger *are secreted into the medium*

According to the expression profiles obtained for the *nigA1* and *nigA2* genes during growth and development of *A. niger*, secretion of aegerolysins was followed by mouse polyclonal antibodies against recombinant NigA2, while *A. niger* was grown on plates over 8 to 120 h. In agreement with the expression profiles for aegerolysin genes *nigA1* and *nigA2* during growth and development of *A. niger*, at least one aegerolysin was detected in the fungal secretome at all of the times tested (Fig. 2). Also, secretion of aegerolysins was detected beneath the whole surface of the mycelium during the first 24 h of growth, and this moved towards the periphery of the colony when the conidiophores with conidia started to form, which was in agreement with previous reports on protein secretion in *A. niger* by Levin *et al.* (2007) and Wösten *et al.* (1991).

Our results thus confirmed the previous findings that aegerolysin NigA2 is being secreted from the fungus *A.*

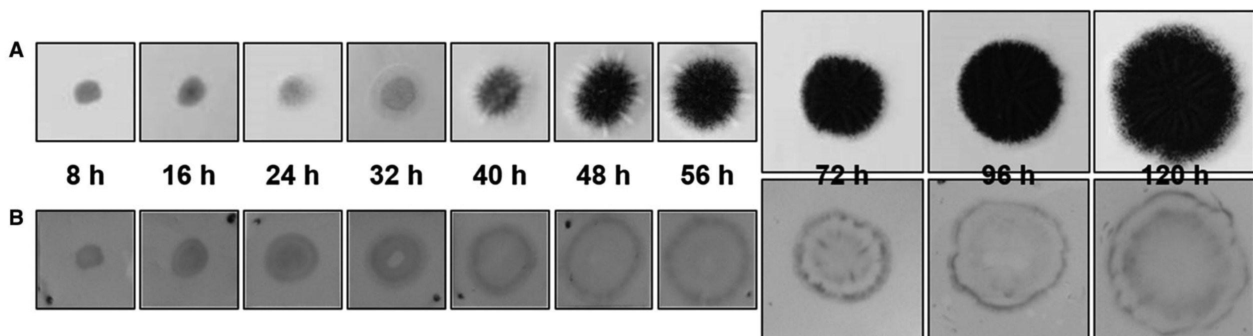


Fig. 2. Detection of aegerolysins during growth and development of *A. niger*. Spores of wild-type strain were point inoculated and incubated in the dark for 8 to 120 h in 8-h, 16-h or 24-h intervals (A). Aegerolysins in the fungal secretome were detected using Western blotting, with mouse polyclonal antibodies against recombinant NigA2 as primary antibodies (B).

niger even though the amino-acid sequence does not contain any signal peptides (Braaksma *et al.*, 2010; Lu *et al.*, 2010; Nitsche *et al.*, 2012). Also, other aegerolysins have been previously reported to be secreted and these include AspHS from the fungus *A. fumigatus* (Wartenberg *et al.*, 2011), terrelysin from the fungus *A. terreus* (Nayak *et al.*, 2012), and RahU from the bacterium *P. aeruginosa* (Rao *et al.*, 2008). Altogether, these data firmly indicate that physiological role for aegerolysin proteins in fungi is in the extracellular environment, possibly involving interactions with other (micro)organisms.

NigA2 specifically binds to an invertebrate sphingolipid in combination with cholesterol

Aegerolysins from the fungal genus *Pleurotus*: ostreolysin A, ostreolysin A6, pleurotolysin A, and pleurotolysin A2, were shown in previous studies to specifically interact with sphingomyelin/ cholesterol domains of artificial and biological membranes (Sepčić *et al.*, 2004; Tomita *et al.*, 2004; Bhat *et al.*, 2013; 2015; Ota *et al.*, 2013). These have also been proposed as non-toxic biomarkers of membrane rafts (Bhat *et al.*, 2013; Skočaj *et al.*, 2014; Kishimoto *et al.*, 2016). Recently, ostreolysin A and pleurotolysin A2, along with erylysin A from *P. eryngii*, were reported to interact even more specifically (K_d, 1.2–12 nM) with binary equimolar lipid vesicles composed of CPE and cholesterol, and pleurotolysin A2 was able to bind also CPE-containing vesicles devoid of cholesterol (Bhat *et al.*, 2015). This interaction allowed the use of these aegerolysins as useful molecular markers of the CPE distribution in insect cells and tissues, and for detection of the bloodstream form of *Trypanosoma brucei* (Bhat *et al.*, 2015; Yamaji-Hasegawa *et al.*, 2016, Panevska *et al.*, 2019). Moreover, when combined with their MACPF protein partner, these *Pleurotus* aegerolysins can effectively permeabilize lipid vesicles containing physiologically relevant CPE concentrations and insect cells, and can act as potent bioinsecticides against selected coleopteran pests (Panevska *et al.*, 2019).

Because of NigA2 higher similarity to aegerolysins from the fungal genus *Pleurotus* compared to NigA1, binding of NigA2 to different multilamellar lipid vesicles and large unilamellar vesicles (LUVs) was verified using the sedimentation assay and SPR, respectively. In both, we confirmed the specific interactions of NigA2 with equimolar CPE: cholesterol vesicles, while no interactions were seen with CPE: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), sphingomyelin: cholesterol, or POPC: cholesterol vesicles (Fig. 3A and B). NigA2 binding to equimolar CPE: cholesterol LUVs was concentration dependent and irreversible, as seen from the SPR sensorgrams during the dissociation phase (Fig. 3B). Although the exact dissociation constant could not be determined here, it was indicative that NigA2 can associate with CPE: cholesterol membranes in the low nanomolar range, which suggests strong affinity of NigA2 for these membranes and coincides with the reports of Bhat *et al.* (2015) and Panevska *et al.* (2019) for CPE: cholesterol membrane affinities of ostreolysin A, ostreolysin A6, pleurotolysin A2 and erylysin A from *Pleurotus* spp.

To determine whether the interaction of NigA2 with CPE is biologically relevant as was shown for aegerolysins from the fungal genus *Pleurotus* (Bhat *et al.*, 2015, Panevska *et al.*, 2019), fluorescently labelled NigA2, NigA2-mCherry, was obtained. Its binding to insect Sf9 cells (pupal epithelial ovarian cells of moth *Spodoptera frugiperda*, containing several CPE species [Panevska *et al.*, 2019]) or mammalian Caco-2 cells (human epithelial colorectal adenocarcinoma cells; containing only trace amounts of CPE [Vacaru *et al.*, 2009]) was verified. In parallel, fluorescently labelled ostreolysin A from *P. ostreatus*, ostreolysin A-mCherry (Skočaj *et al.*, 2014), was used. Both aegerolysins, NigA2-mCherry and ostreolysin A-mCherry, bound to the membranes of Sf9 cells (Fig. 3C), however, approximately 10-times higher concentration of NigA2-mCherry compared to ostreolysin A-mCherry was needed to stain those membranes. Lower affinity of the NigA2 for Sf9 cell membranes could at least in part be explained by its different binding specificity. Sf9 cells contain CPE and sphingomyelin (Gerbal *et al.*, 2000) and small amounts of cholesterol (Marheineke *et al.*, 1998) in their membranes; and while NigA2 binds to CPE: cholesterol only, ostreolysin A is known to bind also sphingomyelin: cholesterol (Sepčić *et al.*, 2004; Tomita *et al.*, 2004; Bhat *et al.*, 2013; 2015; Ota *et al.*, 2013) and CPE: cholesterol (Bhat *et al.*, 2015). Also in accordance with reported ostreolysin A binding specificity and our sedimentation assay and SPR results, we were able to stain membranes of Caco-2 cells, which predominately contain sphingomyelin, only with ostreolysin A-mCherry, but not NigA2-mCherry (Fig. 3C). The binding to CPE, or to CPE: cholesterol, could be the common characteristic of aegerolysin proteins suggesting their role in defence against invertebrate predators that have a combination of these molecular targets in their membranes.

Many fungi defend themselves against microbial competitors and animal predators using chemical defence strategy, including toxic proteins (Künzler, 2018). The hypothesized role of these aegerolysin and MACPF-like proteins in fungal defence is based on upregulation by biotic stress using bacteria, which could further be corroborated more convincingly by showing upregulation of these genes in response to animal predation. The next step to the observed lipid specificity and confirmed binding to insect Sf9 cells would be to show the toxicity of these

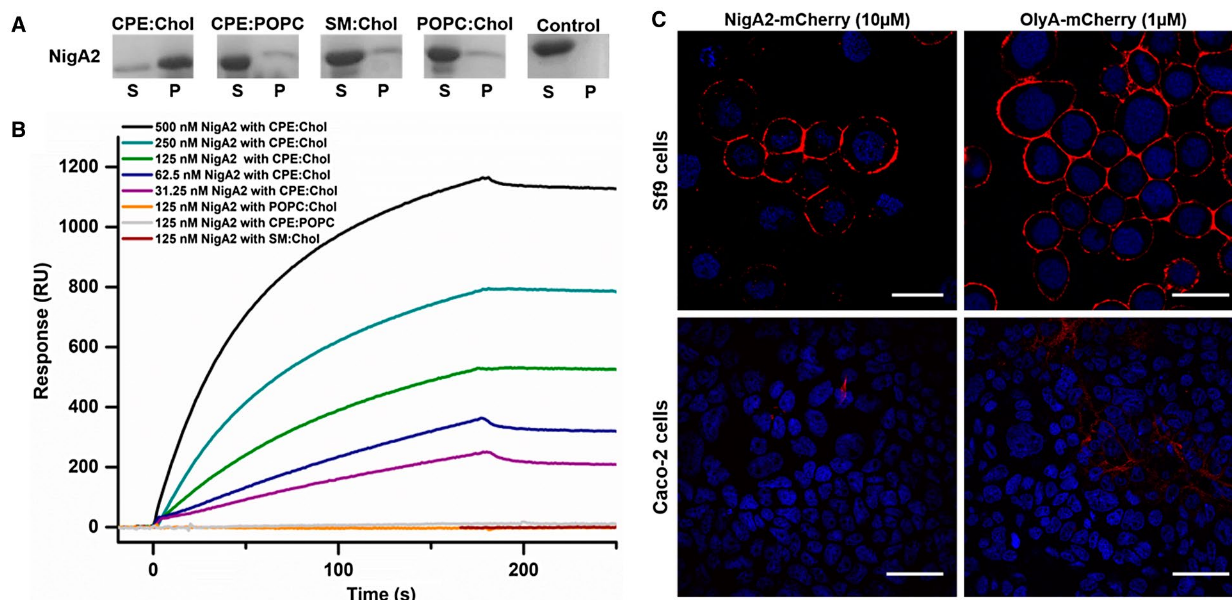


Fig. 3. Aegerolysin NigA2 specifically binds to an invertebrate sphingolipid, ceramide phosphoethanolamine, in combination with cholesterol; and its fluorescent variant can be used for staining of the membranes of Sf9 cells (derived from the moth *S. frugiperda*) that contain this sphingolipid.
A. NigA2 was incubated with multilamellar vesicles composed of binary mixtures of lipids (1:1, mol:mol), as described in the Methods section, and its binding (protein detected in the pellet) was confirmed with sedimentation assay. The control experiment without multilamellar vesicles showed no sedimentation of NigA2.
B. Sensorgrams showing interaction of NigA2 with large unilamellar vesicles composed of binary mixtures of lipids (1:1, mol:mol) as determined with surface plasmon resonance. Binding of one (125 nM) or different concentrations (31.25–500 nM) of NigA2 to large unilamellar vesicles is given as response in time.
C. Staining of the membranes of the insect Sf9 cells and mammalian Caco-2 cells (i.e. human epithelial colorectal adenocarcinoma cells) with NigA2-mCherry (10 µM) as observed with a confocal microscope (blue, DAPI; red, mCherry). Aegerolysin ostreolysin A-mCherry from *P. ostreatus* (1 µM, Skočaj et al. 2014) was used in parallel. Staining, fixation and microscopy were performed as described in the Methods section. Scale, 20 µm (Sf9 cells) or 50 µm (Caco-2 cells). CPE, ceramide phosphoethanolamine; Chol, cholesterol; SM, sphingomyelin; POPC, palmitoyl-oleoyl-phosphatidylcholine; S, supernatant; P, pellet.

proteins against organisms containing CPE/cholesterol in their membranes, as showed for aegerolysin/MACPF complexes deriving from *Pleurotus* mushrooms (Panevska *et al.*, 2019). Our preliminary experiments on insect larvae *Aedes aegypti* and nematodes *Caenorhabditis elegans* (Markus Künzler, personal communication) as well as *Meloidogyne incognita* showed no toxic effect of NigA2 up to 1 uM, however the toxicity of NigA2/NigB1 complexes was not assayed yet.

Conclusions

Even though biological role(s) of aegerolysins (and MACPF-like proteins) in fungi remain not fully explained, two general hypotheses have been established based on known characteristics of these proteins: (i) they are involved in growth and development of the producing organisms, or (ii) they have a role in defence and survival of the organisms. Results obtained for aegerolysin and MACPF-like proteins in filamentous fungus *A. niger* show, that the expression of genes encoding these proteins, and later on production of the proteins, is linked to sporulation, possibly having protective role in this sensitive period of fungal development. Moreover, expression can also be affected by different abiotic and biotic factors of the environment. Deletion of either of the aegerolysin genes has no effect on survival, development, growth dynamics and phenotype of the fungus in the conditions tested. From previous reports and this study, we know that aegerolysins of *A. niger* and at least one of the MACPF-like proteins (Braaksma *et al.*, 2010) are being secreted from the fungus. Furthermore, aegerolysin NigA2 can specifically interact with artificial and biological membranes that contain CPE, a sphingolipid that can be found in the membranes of different invertebrate groups, including those sharing a common ecological niche with *A. niger* (e.g. mites and insect larvae). Many of the observed characteristics for aegerolysin proteins in *A. niger* resemble those described for secondary metabolites and lectins in fungi who act as defence molecules in organisms that produce them (Calvo *et al.*, 2002; Mylonakis *et al.*, 2007; Künzler, 2015; Sabotič *et al.*, 2015). Therefore, this leads us to believe

that the main role(s) of aegerolysins in *A. niger* are linked to the defence/protection of the fungus during the sensitive period of its development, rather than to growth and development of the fungus.

Experimental procedures

Strains and storage, cell lines and chemicals

Aspergillus niger strain N402, a mutant with short conidiophores (*cspA1*) that was derived from the wild-type strain (CBS 120.49, ATCC 9029) was used in this study (Bos *et al*., 1988). All of the other fungal and bacterial strains are listed in Table S5.

Fungal spores or mycelia and bacterial spores or cultures were stored over the long term at –80°C in 20% and 25% (v/v) glycerol, respectively. For spores preparation, *A. niger* was grown in the dark on solid minimal medium (Punt and van den Hondel, 1992) with 1% (w/v) glucose, at 30°C for 4 days, and the *Streptomyces* strains were grown on solid GYM medium (0.4% [w/v] glucose, 0.4% [w/v] yeast extract, 1% [w/v] malt extract, 0.2% [w/v] $CaCO_3$, 1.2% [w/v] agar), at 30°C for up to 7 days. The spores were collected in sterile 0.9% (w/v) NaCl, filtered through Miracloth (Merck, DE), and then stored in a fridge for no more than 3 months. For the bacterial plate cultures, *Pseudomonas* and *Bacillus* long-term stock cultures were streaked on nutrient agar (0.8% [w/v] nutrient broth, 0.5% [w/v] NaCl, 1.5% [w/v] agar) and grown at 30°C for 1 day. The plates were then stored in a fridge for no more than 1 month.

Plasmids were multiplied in, and isolated from, *Escherichia coli* DH5α cells, while recombinant aegerolysins NigA2 and NigA2-mCherry were expressed in, and isolated from, *E. coli* BL21(DE3) cells. Insect Sf9 cell line (moth *S. frugiperda* pupal epithelial ovarian cells) was from Thermo Fisher Scientific (USA) and mammalian Caco-2 cell line (human epithelial colorectal adenocarcinoma cells) was from American Type Culture Collection (ATCC, USA). The chemicals used were from Merck (DE) or Sigma-Aldrich (USA), unless otherwise stated.

Bioinformatics analysis

To find aegerolysin and MACPF-like protein homologues in *A. niger*, amino acid sequences of three fungal aegerolysins, OlyA from *P. ostreatus*, AspHS and AspHS-like from *A. fumigatus*, and of two fungal proteins with MACPF domain, PlyB from *P. ostreatus* and SpoC1-C1C of *A. nidulans*, were used to search National Center for Biotechnology Information (NCBI), Joint Genome Institute (JGI) Mycocosm (Grigoriev *et al*., 2014) and *Aspergillus* Genome Database (AspGD) (Cerqueira *et al*., 2014) using BLASTP algorithm and then combining BLASTP hits with hits from the Pfam database (PF06355 or PF01823, version 27.0, Finn *et al.* 2016). To confirm the presence of only two aegerolysins coded by the genome of *A. niger* cross-check was performed with all other at least partly studied fungal proteins from aegerolysin family listed in Supplementary figure (Fig. S2).

Growth conditions in quantitative reverse transcription polymerase chain reaction analysis

Different growth conditions of *A. niger* were used according to the experimental investigations undertaken, as defined here.

Growth and development. The spores of *A. niger* N402 were inoculated onto porous cellophane (Hoefer) placed on solid minimal medium with 1% (w/v) glucose. Strain *A. niger* N402 was then incubated in the dark at 30°C for up to 72 h. Every 8 to 16 h, the biomass was collected and the growth was monitored macroscopically (Canon G16 camera) and microscopically (AxioCam mounted on an Axiovert 135 light microscope). The spores were collected after 4 days of growth in the dark at 30°C, as described above.

Physical or genetic prevention of sporulation. To physically prevent sporulation of the fungus, spores of *A. niger* N402 were inoculated onto minimal medium with 1% (w/v) glucose in between two porous cellophane membranes, as in Wösten *et al*. (1991). Strain *A. niger* N402 was then grown, the biomass was collected and the growth was monitored from 24 to 72 h, as described under growth conditions (i) above. The non-sporulating mutants that were derived from the *A. niger* strain N402 were Δ*flbA* and Δ*brlA* (Krijgsheld, Nitsche *et al*., 2013) (genetic prevention of sporulation). Their mycelia were inoculated onto porous cellophane membrane placed on solid minimal medium with 1% (w/v) glucose, and grown in the dark for 7 days, after which their biomass was collected.

Abiotic factors of the environment. All of the media used in these studies originated from minimal medium with 1% (w/v) glucose. When different carbon sources were used, the glucose was substituted by equal amounts of glycerol, arabinose, cellulose, starch, fructose, galactose, glucuronic acid, maltose, inulin or pectin. For the free fatty acids (i.e., oleic, linolenic acids), glucose was supplemented with 0.01 g L^{-1} free fatty acid and 0.05% Tween 80. For the different nitrogen sources, $NaNO_3$ (70 mM) was substituted with equal amounts (70 mM) of NH_4Cl or hydroxyproline. For the osmotic and oxidative stress, 1.5 M NaCl and 15 mM H_2O_2, respectively, were added to the minimal medium with 1% (w/v) glucose. For the pH stress, the pH of the medium was adjusted to pH 3 just before pouring the plates, while for pH 8, the nitrogen stock solution was adjusted to pH 8. For the iron (Fe)-depleted medium, the trace elements were prepared without $FeSO_4$. Spores of strain N402 were inoculated onto porous cellophane membrane (glucose, NH_4Cl, 15°C, 42°C, no oxygen, NaCl, H_2O_2, pH 3, pH 8, +Fe) or PVDF membrane (0.22 μm, diameter 90 mm; Merck, DE; xylose, glycerol, arabinose, cellulose, starch, fructose, galactose, glucuronic acid, maltose, inulin, pectin, hydroxyproline, glucose + oleic acid, glucose + linolenic acid). These were placed upon solid minimal medium with 1% (w/v) glucose, minimal medium with 1% (w/v) glucose without iron (iron repletion), or minimal medium with 1% (w/v) glucose and 0.05% Tween 80 (glucose with oleic or linolenic acid). Strain N402 was then grown in the dark at 30°C for 40 h, after

which time the biomass was either collected (control) or exposed to one of the abiotic factors for 1 h (dark, 30°C), by being transferred onto a different medium, or an atmosphere without oxygen, or to a different temperature. Then, the biomass was collected. To test the effects of the glucose, strain N402 was first grown for 40 h on minimal medium with 1% (w/v) glucose, and then transferred to a fresh plate with the same medium and incubated for 1 h in the dark at 30°C.

Biotic factors of the environment. Strain *A. niger* N402 was co-cultured with *A. brasiliensis*, *A. terreus*, *S netropsis* or *S. anulatus* on solid minimal medium with 1% (w/v) glucose. Co-cultures with *P. aeruginosa*, *P. fluorescens*, *B. cereus* or *B. subtilis* were grown on solid YPD (1% [w/v] yeast extract, 2% [w/v] peptone, 2% [w/v] glucose, 1.5% [w/v] agar). Spores of *A. niger* N402 were inoculated onto PVDF membrane (0.22 µm, diameter 47 mm; Merck, DE) that was placed in the middle of the plate (diameter, 90 mm). The inoculations were carried out all around the PVDF membrane (1 cm from the edge) using spores of the *Aspergillus* or *Streptomyces* species, or overnight liquid cultures of one of the *Pseudomonas* or *Bacillus* species. *Pseudomonas* and *Bacillus* overnight cultures were prepared from bacterial plate colonies that were streaked in 20 ml 0.8% (w/v) nutrient broth and 0.5% (w/v) NaCl and grown in 100 ml Erlenmeyer flasks at 30°C, with agitation at 180 rpm. These co-cultures were incubated in the dark at 30°C for 40 h, after which the *A. niger* biomass was collected. For the controls, only spores of *A. niger* N402 were inoculated onto the PVDF membrane placed on the solid minimal medium with 1% (w/v) glucose, or YPD, and grown in the dark at 30°C for 40 h.

Quantitative reverse transcription polymerase chain reaction

The RNA isolation, removal of genomic DNA, reverse transcription to cDNA, RT-qPCR, and data analysis were all performed as in Petek *et al.* (2010), with some modifications. The collected fungal biomass was frozen in liquid nitrogen. RNA was isolated from approximately 100 mg fungal tissue that had been ground to a fine powder with a pestle and mortar, and RNeasy Plant Mini Kit was used (Qiagen, DE), following the manufacturer protocol. RLT buffer with 40 mM dithiothreitol was used, and the RNA was eluted from the columns in two steps, using 30 µL and then 25 µL RNase-free water. For samples with low RNA quantities, the amount of RNase-free water used for the elution was adjusted accordingly (e.g., spores, 8, 16, 56, 72, 24 h physical prevention of sporulation, Δ*flbA* and Δ*brlA* mutants). The quantity and quality of the RNA were determined and verified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis. Prior to reverse transcription, the genomic DNA was removed from 1 µg isolated RNA using DNase I (amplification grade; Thermo Fisher Scientific, USA), as recommended by the manufacturer. The RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, USA), following the manufacturer guidelines. Along with random primers, the oligo(dT) primer (AccuScript High Fidelity First Strand cDNA Synthesis kit; Agilent, USA) was also added into 2× RT master mix at a 1:1 molar concentration ratio.

For RT-qPCR, each cDNA sample was tested for the three reference genes (i.e., *actA*, *cox5*, *18S rRNA* or *sarA*) and the four genes of interest (i.e., *nigA1*, *nigA2*, *nigB1*, *nigB2*) (Table S6). The reference genes of *A. niger* for the data normalisation were chosen based on previously published data of Bohle *et al*. (2007). For detection of the reference genes and the genes of interest, TaqMan MGB chemistry was used, and all of the probes were labelled with the FAM reporter dye. Custom TaqMan Gene Expression Assays (primer-probe mix) for the reference genes (except *18S rRNA*) and the genes of interest were designed and synthesised by Thermo Fisher Scientific (USA) (Table S6). The RT-qPCR reactions were performed in 384-well plates using the Light-Cycler LC480 system (Roche, Switzerland). The reactions were prepared in a final volume of 10 µL (2 µL cDNA, 8 µL Master Mix [1× Custom TaqMan Gene Expression Assay, 1× TaqMan Universal Master Mix II with UNG; both from Thermo Fisher Scientific, USA], 2.5 µL water). Using electronic pipettes, the master mixes were pipetted first, followed by the cDNA samples. Each reaction was performed in two dilutions (10^1, 10^2), with two technical replicates per dilution. Also, different cDNA samples were mixed (i.e.; pool) and 10-fold serial dilutions (10^0–10^4, two replicates per dilution step) were prepared, to calculate the standard curves for all of the tested genes, and to follow the amplification efficiency.

After obtaining the quantification cycle (Cq) values using the LightCycler 480 1.5 software, the relative transcript copy numbers of the reference genes and the genes of interest for the samples were calculated using the standard curve method. The relative transcript copy numbers of the genes of interest were then normalised to the geometric mean of the relative transcript copy numbers of two (*actA*, *cox5* for growth and development, prevention of sporulation) or three (*actA*, *cox5*, *sarA* for abiotic and biotic factors of the environment) of the reference genes. The most suitable reference gene for normalisation, or the combination thereof, was determined using the NormFinder software (Andersen *et al.*, 2004). For all of the samples analysed with RT-qPCR, three independent biological replicates were performed. To determine whether the average relative transcript copy numbers between two samples for genes of interest differed significantly, *t*-tests were used to calculate the *p*-values. A *p*-value of 0.05 was used as the cut-off for significance. The fold-changes for the genes of interest were calculated by dividing the average relative transcript copy numbers of the samples by the average relative transcript copy number of a control sample (i.e., calibrator) for that particular gene. A 2-fold increase or decrease in the expression of a gene was used as a cut-off for considerable change in the gene expression.

Construction of aegerolysin gene deletion mutants

The aegerolysin gene-deletion mutants of *A. niger* strain N402, named as Δ*nigA1* and Δ*nigA2*, were constructed following protocols described by Podobnik *et al.* (2008). Primers used are listed in Table S7. Genomic DNA of the wild type strain served as a template DNA. Deletion cassettes for genes were prepared so that part of the gene *nigA1* or the whole gene *nigA2* would be replaced with the hygromycin selection marker in the wild type strain (Fig. S5). For gene *nigA1*, sequences (~500 base pairs) were amplified with

polymerase chain reactions (PCR); one fully upstream of An19g00210 ORF and the other traversing An19g00210 ORF at the 3' end. For gene *nigA2*, sequences, of the same size, upstream and downstream of An01g09980 ORF were amplified. Amplified sequences were inserted to the either side of the hygromycin resistance gene already incorporated into the pBlueScript cloning vector (Podobnik *et al*., 2008). The transformants were selected on solid minimal medium with 1% (w/v) glucose, 1.2 M sorbitol and 100 μg ml^{-1} hygromycin (Invivogen, USA). The deletions of the genes *nigA1* and *nigA2* were confirmed using two-step PCR and RT-qPCR.

Effects of aegerolysin gene deletion on growth and development of A. niger *mutants*

The growth and development of the aegerolysin deletion mutants Δ*nigA1* and Δ*nigA2* were monitored macroscopically and microscopically, as for the wild-type *A. niger* N402 (see growth conditions (i) for RT-qPCR analysis above).

To estimate the sporulation efficiency, the spores of the wild type strain and the Δ*nigA1* and Δ*nigA2* mutants were inoculated onto solid minimal medium with 1% (w/v) glucose, and incubated in the dark at 30°C for 4 days. The spores were collected in sterile 0.9% (w/v) NaCl, filtered through Miracloth (Merck, DE), and counted using a counting chamber (Bürker-Türk; Brand, DE), following the manufacturer instructions. Ten independent biological replicates were performed for each strain.

The growth dynamics and phenotype of the wild type strain and the Δ*nigA1* and Δ*nigA2* mutants were monitored while exposing the strains to different growth factors (see growth conditions (iii) for RT-qPCR analysis above). Point inoculates of the spores (10^4) were prepared on different media. The colony diameters were measured every day for four consecutive days. On days 2 and 4, photographs of the colonies were taken (Canon G16 camera). Three independent biological replicates were performed for each strain.

Secretion of aegerolysins

To follow the secretion of aegerolysins during fungal growth and development on plates, a similar approach was used to that described by Wösten *et al*. (1991). Spores of *A. niger* N402 were point inoculated (3 μL suspension; 10^7 spores ml^{-1}) onto PVDF membrane (0.22 μm; diameter, 47 mm; Merck, DE) placed on solid minimal medium with 1% (w/v) glucose. Strain *A. niger* N402 was incubated in the dark at 30°C for 8, 16, 24, 32, 40, 48, 56, 72, 96 or 120 h. Then, the PVDF membrane was lifted along with the mycelia, and a nitrocellulose membrane (Amersham Hybond ECL, GE HealthCare Life Sciences) was placed in-between the PVDF membrane and the medium. The plates were returned to the incubator at 30°C for another 1.5 h, during which time any proteins secreted from *A. niger* N402 were captured in the nitrocellulose membrane. This procedure was also carried out with the aegerolysin gene deletion mutants, Δ*nigA1* and Δ*nigA2*, except that these strains were incubated in the dark at 30°C for 40 h only.

Aegerolysins on the nitrocellulose membranes were detected using Western blotting. The nitrocellulose membranes were first blocked for one hour with 5% skimmed milk in Tris-buffered saline, pH 7.4, and 0.1% Tween (TBS-T). The nitrocellulose membranes were then incubated overnight at 4°C with the primary mouse polyclonal antibodies against recombinant NigA2 (1:1000; Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Slovenia), prepared in 5% skimmed milk in TBS-T. This was followed by a 2-h incubation at room temperature with the secondary goat monoclonal anti-mouse antibodies (1:2000; Sigma-Aldrich, USA), also prepared in 5% skimmed milk in TBS-T. The washing of the membranes between the incubation periods was carried out with TBS-T. The proteins were detected using the enhanced chemiluminescence method (Pierce ECL Western Blotting substrate; Thermo Fisher Scientific, USA), according to the manufacturer protocol.

The specificity of the primary mouse polyclonal antibodies against recombinant NigA2 was verified using Western blotting of aegerolysins of different origin (NigA2; RahU from *P. aeruginosa*; OlyA from *P. ostreatus*; homogenate of *P. ostreatus*) (Fig. S6). These proteins were first separated on NuPAGE 4–12% Bis-Tris protein gel (Thermo Fisher Scientific, USA) and transferred to PVDF membrane using iBlot Gel Transfer Device (Thermo Fisher Scientific, USA), all according to manufacturer instructions. Blocking of the membrane and all the following steps of the Western blotting were performed as described above, except the protein detection, which was performed using 4-chloro-1-naphtol (Sigma, USA).

Production and isolation of recombinant aegerolysins NigA2 and NigA2-mCherry

The synthetic construct that encoded recombinant aegerolysin NigA2 (GenScript, USA) was optimised for expression in *E. coli*. This contained the nucleotide sequence of the *nigA2* gene from the publicly available genome of *A. niger* CBS 513.88 (Pel *et al*., 2007), plus the nucleotide sequence encoding the Tev protease cut site (3' end) and three restriction sites for the restriction enzymes *Nde*I (5' end), *Bam*HI and *Xho*I (3' end). Using the *Nde*I and *Xho*I restriction enzymes, this construct was inserted into the pET-21c(+) vector (=*nigA2*/pET-21c[+]), which enabled the production of the C-terminally His-tagged protein NigA2. Using the *BamH*I and *Xho*I restriction enzymes, *mcherry* gene was transferred from *olyA-mcherry*/pET-21c(+) vector (Skočaj *et al*., 2014) into *nigA2*/pET-21c(+) vector, which enabled the production of the fluorescently labelled, C-terminally His-tagged protein NigA2, NigA2-mCherry.

The plasmid vector that contained the construct that encoded recombinant aegerolysin NigA2 or NigA2-mCherry was transformed into BL21(DE3) competent *E. coli* cells using heat shock. After an initial 1-h incubation in Luria–Bertani medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 1% [w/v] NaCl, 1.5% [w/v] agar) at 37°C with agitation, the transformed cells were plated onto selective Luria–Bertani medium containing 0.1 g L^{-1} ampicillin and grown overnight at 37°C. Liquid culture of the transformed bacterial cells was initially prepared in small volume (e.g., 20 ml) of selective Luria–Bertani medium containing 0.1 g L^{-1} ampicillin, and on consecutive day this was used to inoculate larger volume of the same medium for protein production (e.g., 2 L). When this large inoculate had reached OD_{600} of 0.6 to 0.8, expression of the *nigA2* or *nigA2-mcherry* gene was induced by addition

of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). While in liquid culture, the transformed cells were grown at 37°C with agitation at 180 rpm, until the induction with IPTG. Upon induction, the cells were grown overnight at 30°C with agitation at 180 rpm. The bacterial biomass was then collected by centrifugation (10 min, 7 808× *g*, 4°C) and re-suspended in lysis buffer (50 mM $NaH_2PO_4 \times 2H_2O$, 300 mM NaCl, pH 7.5) containing protease inhibitors (0.5 mM PMSF, 1 mM benzamidine) and enzymes (0.5 mg ml^{-1} lysozyme, 5 U ml^{-1} benzonase nuclease, 20 μg ml^{-1} RNase). The bacterial cells were disrupted with 10 min sonication (pulsed, 1 s on, 2 s off; amplitude, 38%) and the homogenate obtained was centrifuged (30 min, 47 850× *g*, 4°C). The recombinant aegerolysins NigA2 and NigA2-mCherry were produced as soluble proteins, and therefore they were isolated from the supernatant from the centrifugation using nickel affinity chromatography (Ni-NTA Superflow nickel-charged resin; Qiagen, DE), according to the manufacturer instructions. After the initial elution of unbound proteins with lysis buffer, non-specifically bound proteins were eluted with wash buffer (lysis buffer with 25 mM imidazole), and then the bound His-tagged recombinant protein NigA2 or NigA2-mCherry was eluted with elution buffer (lysis buffer with 300 mM imidazole). The high imidazole concentration was reduced by overnight dialysis in lysis buffer using 6000 to 8000 Da molecular weight cut-off membrane. This lysis buffer was exchanged for erythrocyte buffer (20 mM Trizma base, 140 mM NaCl, pH 7.4) and the sample was concentrated using centrifugal filtration (NMWL10 or NMWL30; Amicon Ultra 15, Merck, DE). Size-exclusion chromatography was then performed in a column with a volume of 120 ml (HiLoad 16/600 Superdex 75 pg column; GE HealthCare, UK) using a chromatography system (Äkta Purifier; GE HealthCare, UK) and erythrocyte buffer for elution, according to the manufacturer instructions. The recombinant aegerolysins NigA2 and NigA2-mCherry were stored in erythrocyte buffer at –20°C.

Protein sedimentation assay with multilamellar lipid vesicles

Binary multilamellar vesicles were prepared in 20 mM Trizma base, 140 mM NaCl, 5 μM EDTA, pH 8, at the final concentration of 1 mg ml^{-1}, as described previously (Sepčić *et al.*, 2003). These were composed of equimolar ratios of different lipids, including: CPE (Matreya, USA), porcine brain sphingomyelin, wool grease cholesterol, and POPC (all from Avanti Polar Lipids, USA). Aliquots of recombinant NigA2 (25 μg) dissolved in buffer for vesicles (20 mM Trizma base, 140 mM NaCl, 1 mM EDTA, pH 8) were mixed with the different multilamellar vesicles (250 μg) and incubated for 30 min on a rotary shaker (600 rpm/min) at 25°C. Samples were then centrifuged for 60 min at 60 000× *g* and 4°C. The unbound NigA2 in the supernatant was transferred to a clean vial and was precipitated with 20% trichloroacetic acid. After a 10-min incubation on ice, the precipitated protein was pelleted by centrifugation at 14 300× *g* for 12 min at 4°C, and washed twice with 300 μL ice-cold acetone. The pellets containing the free and bound NigA2 were diluted in equal volumes of the non-reducing sodium dodecyl sulphate (SDS) sample buffer (200 mM Tris-HCl, pH 8, 5% [w/v] SDS, 2 mM EDTA, 0.1% [w/v] bromophenol blue), heated to 100°C for 5 min, and applied to homogenous 12% acrylamide gels. The proteins in the gels were stained with Coomassie blue.

Sphingomyelin, cholesterol and POPC concentrations of the multilamellar vesicle suspensions were determined colorimetrically using Free Cholesterol E and Phospholipids C kits (Wako Pure Chemical Industries, Japan). The concentration of CPE was determined according to the amine group concentration (Barenholz *et al.*, 1977).

Surface plasmon resonance

To prepare large unilamellar vesicles (LUVs), multilamellar vesicle suspensions underwent eight cycles of freeze–thawing in liquid nitrogen, followed by extrusion through 0.1-μm polycarbonate filters (Millipore, USA) at 40°C. The sizes of the LUVs were determined using dynamic light scattering (Zetasizer Nano ZSP; Malvern Instruments, Malvern, UK) as described in Vezočnik *et al.* (2015), showing a distribution centred at a diameter between 80 to 120 nm. All of the LUVs used were freshly prepared prior to the SPR.

The NigA2–LUV interactions were monitored on an SPR-based refractometer (Biacore X) using the L1 chip, as described by Beseničar *et al.* (2006) and Hodnik and Anderluh (2010). The LUVs (100 μg lipids ml^{-1}; 600-s injection at 2 μL min^{-1}) were immobilised using running buffer (20 mM Tris, 140 mM NaCl, pH 7.4). The LUVs were bound to the second flow cell of the L1 sensor chip, with a response of approximately 3 000 response units (RU). The first flow cell was left empty and used to control any non-specific binding of proteins to the dextran matrix. To test the interactions between recombinant NigA2 (31.25–500 nM) with the LUVs, the NigA2 was dissolved in running buffer and injected into the refractometer at 10 μL min^{-1}. Regeneration of the system between NigA2 injections was achieved with 1-min injections of 0.5% [w/v] SDS and 40 mM octyl β-D-glucopyranoside, at 10 μL min^{-1}. This study was carried out at 25°C, and the data were processed using the BIAevaluation software (GE Healthcare, UK).

Cell cultures and staining of the cells

Insect Sf9 cells were grown and maintained as suspension culture as described in Naneh *et al.* (2015), whereas mammalian Caco-2 cells were grown and maintained as adherent culture as described in Cajnko *et al.* (2015). In short, Sf9 cells were grown at 28°C with agitation at 140 rpm in Erlenmeyer flasks in the growth medium Insect-XPRESS™ Protein-free Insect Cell Medium with L-glutamate (Lonza, CH). Cells were sub-cultured every 2–3 days. Caco-2 cells were grown at 37°C in the growth medium MEM supplemented with 10% (v/v) fetal bovine serum, 1% L-glutamine and 1% non-essential amino acids, under the atmosphere containing 5% CO2. Cells were sub-cultured every 7 days.

For staining, 90.000 Sf9 cells/well were seeded on chamber slides (iBidi, DE) and cultured overnight at 28°C in the medium Insect-XPRESS™, during which time cells attached to the bottom of the wells, whereas for Caco-2 cells, 60.000 cells/well were seeded and cultured for 7 days at 37°C, under the atmosphere containing 5% CO2, in the test medium DMEM supplemented with 1% glutamine, 1% antibiotic-antimycotic and 10% (v/v) fetal bovine serum. The test medium was changed every 2–3 days. Cells were then washed twice with the medium (Insect-XPRESS™

or DMEM with supplements). Recombinant aegerolysins NigA2-mCherry (10 μM) or ostreolysin A-mCherry from *P. ostreatus* (1 μM, Skočaj *et al.* 2014) were diluted in the corresponding medium and incubated with cells for 15 min at room temperature, upon which time proteins were removed and cells washed three times with phosphate buffer saline (PBS, pH 7.4). Cells were fixed with 4% (m/v) paraformaldehyde for 15 min at room temperature and after two additional washes with PBS permeabilized for 10 min with 0.1% (v/v) Triton X-100 at 4°C. After three more washes with PBS, nuclei of the cells were stained with DAPI (1 μg ml^{-1}) for 3–5 min. Cells were washed again with PBS and observed with inverted confocal microscope Leica TCS SP5 using diode laser (405 nm) and argon laser (543 nm). Fluorescent emission was detected at 430 to 470 nm (DAPI) and 620 to 670 nm (mCherry). Images were captures with 63× objective and LAS AF Lite software and further processed with ImageJ (Abramoff *et al.*, 2004).

Acknowledgements

The authors thank the Slovenian Research Agency for financial support [grants J4-7162, P1-0391 and P1-0207]. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. We would like to acknowledge Prof. Dr. Ronald P. de Vries, Prof. Dr. Arthur F. Ram and Prof. Dr. Nina Gunde-Cimerman for kindly providing us with Aspergillus strains and the A. niger mutant strains (ΔflbA, ΔbrlA) and all of the bacterial strains used in these studies. Anastasija Panevska preformed Western blot included in the supporting information. The authors would also like to thank Dr Chris Berrie for the English language editing.

Author contributions

MN, UČ, VH, MoN, MJ and NK acquired, analysed and interpreted the data; MN, UČ, NK, GA, and KS designed the experiments and wrote the paper.

Data availability statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

References

Abramoff, M.D., Magalhaes, P.J. and Ram, S.J. (2004) Image processing with ImageJ. *Biophotonics International*, **11**(7), 36–42.

Adams, T.H., Boylan, M.T. and Timberlake, W.E. (1988) *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. *Cell*, **54**(3), 353–362.

Anderluh, G., Kisovec, M., Kraševec, N. and Gilbert, R.J.C. (2014) Distribution of MACPF/CDC proteins. In: Anderluh, G. and Gilbert, R. (Eds.) *MACPF/CDC Proteins – Agents of Defence, Attack and Invasion*. London: Springer, pp. 7–31.

Andersen, C.L., Jensen, J.L. and Ørntoft, T.F. (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research*, **64**(15), 5245–5250.

Andersen, M.R., Salazar, M.P., Schaap, P.J., van de Vondervoort, P.J.I., Culley, D., Thykaer, J., *et al.* (2011) Comparative genomics of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88. *Genome Research*, **21**(6), 885–897.

Bando, H., Hisada, H., Ishida, H., Hata, Y., Katakura, Y. and Kondo, A. (2011) Isolation of a novel promoter for efficient protein expression by *Aspergillus oryzae* in solid-state culture. *Applied Microbiology and Biotechnology*, **92**(3), 561–569.

Barenholz, Y., Gibbes, D., Litman, B.J., Goll, J., Thompson, T.E. and Carlson, F.D. (1977) A simple method for the preparation of homogeneous phospholipid. *Biochemistry*, **16**(12), 2806–2810.

Bayry, J., Beaussart, A., Dufrene, Y.F., Sharma, M., Bansal, K., Kniemeyer, O., *et al.* (2014) Surface structure characterization of *Aspergillus fumigatus* conidia mutated in the melanin synthesis pathway and their human cellular immune response. *Infection and Immunity*, **82**(8), 3141–3153.

Berne, S., Križaj, I., Pohleven, F., Turk, T., Maček, P. and Sepčić, K. (2002) *Pleurotus* and *Agrocybe* hemolysins, new proteins hypothetically involved in fungal fruiting. *Biochimica et Biophysica Acta*, **1570**(3), 153–159.

Berne, S., Lah, L. and Sepčić, K. (2009) Aegerolysins: structure, function, and putative biological role. *Protein Science*, **18**(4), 694–706.

Beseničar, M., Maček, P., Lakey, J.H. and Anderluh, G. (2006) Surface plasmon resonance in protein-membrane interactions. *Chemistry and Physics of Lipids*, **141**(1–2), 169–178.

Bhat, H.B., Kishimoto, T., Abe, M., Makino, A., Inaba, T., Murate, M., *et al.* (2013) Binding of a pleurotolysin ortholog from *Pleurotus eryngii* to sphingomyelin and cholesterol-rich membrane domains. *Journal of Lipid Research*, **54**(10), 2933–2943.

Bhat, H.B., Ishitsuka, R., Inaba, T., Murate, M., Abe, M., Makino, A., *et al.* (2015) Evaluation of aegerolysins as novel tools to detect and visualize ceramide phosphoethanolamine, a major sphingolipid in invertebrates. *The FASEB Journal*, **29**(9), 3920–3934.

Bleichrodt, R., Vinck, A., Krijgsheld, P., van Leeuwen, M.R., Dijksterhuis, J. and Wösten, H.A.B. (2013) Cytosolic streaming in vegetative mycelium and aerial structures of *Aspergillus niger*. *Studies in Mycology*, **74**(1), 31–46.

Bohle, K., Jungebloud, A., Göcke, Y., Dalpiaz, A., Cordes, C., Horn, H., *et al.* (2007) Selection of reference genes for normalisation of specific gene quantification data of *Aspergillus niger*. *Journal of Biotechnology*, **132**(4), 353–358.

Bos, C.J., Debets, A.J., Swart, K., Huybers, A., Kobus, G. and Slakhorst, S.M. (1988) Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Current Genetics*, **14**(5), 437–443.

Braaksma, M., Martens-Uzunova, E.S., Punt, P.J. and Schaap, P.J. (2010) An inventory of the *Aspergillus niger* secretome by combining in silico predictions with shotgun proteomics data. *BMC Genomics*, **11**(1), 584.

Butala, M., Novak, M., Kraševec, N., Skočaj, M., Veranič, P., Maček, P., *et al*. (2017) Aegerolysins: lipid-binding proteins with versatile functions. *Seminars in Cell and Developmental Biology*, **72**, 142–151.

Cajnko, M.M., Marušić, M., Kisovec, M., Rojko, N., Benčina, M., Caserman, S., *et al*. (2015) Listeriolysin O affects the permeability of Caco-2 monolayer in a pore-dependent and Ca^{2+}-independent manner. *PLoS One*, **10**(6), e0130471.

Calvo, A.M., Wilson, R.A., Bok, J.W. and Keller, N.P. (2002) Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews*, **66**(3), 447–459.

Cerqueira, G.C., Arnaud, M.B., Inglis, D.O., Skrzypek, M.S., Binkley, G., Simison, M., *et al*. (2014) The *Aspergillus* Genome Database: multispecies curation and incorporation of RNA-Seq data to improve structural gene annotations. *Nucleic Acids Research*, **42**(1), D705–710.

Crone, H. and Bridges, R. (1963) The phospholipids of the housefly, *Musca domestica*. *The Biochemical Journal*, **89**(1), 11–21.

Ebina, K., Yokota, K. and Sakaguchi, O. (1982) Studies on toxin of Aspergillus fumigatus XIV. Relationship between Asp-hemolysin and experimental infection for mice. *Japanese Journal of Medical Science and Biology*, **23**, 246–252.

Ellis, R.T., Stockhoff, B.A., Stamp, L., Ernest, H., Schwab, G.E., Knuth, M., *et al*. (2002) Novel *Bacillus thuringiensis* binary insecticidal crystal proteins active on western corn rootworm, *Diabrotica virgifera* virgifera LeConte. *Applied and Environmental Microbiology*, **68**(3), 1137–1145.

Fernandez Espinar, M.T. and Labarère, J. (1997) Cloning and sequencing of the *Aa-Pri1* gene specifically expressed during fruiting initiation in the edible mushroom *Agrocybe aegerita*, and analysis of the predicted amino-acid sequence. *Current Genetics*, **32**(6), 420–424.

Finn, R.D., Coggill, P., Eberhardt, R.Y., Eddy, S.R., Mistry, J., Mitchell, A.L., *et al*. (2016) The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Research*, **44**(1), D279–285.

Gerbal, M., Fournier, P., Barry, P., Mariller, M., Odier, F., Devauchelle, G., *et al*. (2000) Adaptation of an insect cell line of *Spodoptera frugiperda* to grow at 37 degrees C: characterization of an endodiploid clone. *Vitro Cellular & Developmental Biology - Animal*, **36**(2), 117–124.

Gilbert, R.J.C., Mikelj, M., Dalla Serra, M., Froelich, C.J. and Anderluh, G. (2013) Effects of MACPF/CDC proteins on lipid membranes. *Cellular and Molecular Life Sciences*, **70**(12), 2083–2098.

Grigoriev, I.V., Nikitin, R., Haridas, S., Kuo, A., Ohm, R., Otillar, R., *et al*. (2014) MycoCosm portal: gearing up for 1000 fungal genomes. *Nucleic Acids Research*, **42**(1), D699–704.

Hodnik, V. and Anderluh, G. (2010) Capture of intact liposomes on biacore sensor chips for protein-membrane interaction studies. *Methods in Molecular Biology*, **627**, 201–211.

Itasaka, O., Hori, T., Uno, A. and Iwamori, M. (1973) Occurrence of ceramide phosphorylethanolamine containing hydroxy fatty acid in a bivalve. *Journal of Biochemistry*, **73**(1), 191–193.

Joh, J.H., Lee, S.H., Lee, J., Kim, K.H., Jeong, S.J., Youn, W.H., *et al*. (2007) Isolation of genes expressed during the developmental stages of the oyster mushroom, *Pleurotus ostreatus*, using expressed sequence tags. *FEMS Microbiology Letters*, **276**(1), 19–25.

Kishimoto, T., Ishitsuka, R. and Kobayashi, T. (2016) Detectors for evaluating the cellular landscape of sphingomyelin- and cholesterol-rich membrane domains. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, **1861**(8), 812–829.

Krijgsheld, P., Bleichrodt, R., van Veluw, G.J., Wang, F., Müller, W.H., Dijksterhuis, J., *et al*. (2013) Development in *Aspergillus*. *Studies in Mycology*, **74**(1), 1–29.

Krijgsheld, P., Nitsche, B.M., Post, H., Levin, A.M., Müller, W.H., Heck, A.J.R., *et al*. (2013) Deletion of *flbA* results in increased secretome complexity and reduced secretion heterogeneity in colonies of *Aspergillus niger*. *Journal of Proteome Research*, **12**(4), 1808–1819.

Künzler, M. (2015) Hitting the sweet spot-glycans as targets of fungal defense effector proteins. *Molecules*, **20**, 8144–8167.

Künzler, M. (2018) How fungi defend themselves against microbial competitors and animal predators. *PLoS Pathogens*, **14**, e1007184.

Kurahashi, A., Sato, M., Kobayashi, T., Nishibori, K. and Fujimori, F. (2013) Homologous genes, *Pe.pleurotolysin A* and *Pe.ostreolysin*, are both specifically and highly expressed in primordia and young fruiting bodies of *Pleurotus eryngii*. *Mycoscience*, **55**(2), 113–117.

Lee, B.N. and Adams, T.H. (1994) Overexpression of *flbA*, an early regulator of *Aspergillus* asexual sporulation, leads to activation of *brlA* and premature initiation of development. *Molecular Microbiology*, **14**(2), 323–334.

Lee, S.H., Kim, B.G., Kim, K.J., Lee, J.S., Yun, D.W., Hahn, J.H., *et al*. (2002) Comparative analysis of sequences expressed during the liquid-cultured mycelia and fruit body stages of *Pleurotus ostreatus*. *Fungal Genetics and Biology*, **35**(2), 115–134.

Levin, A.M., de Vries, R.P., Conesa, A., de Bekker, C., Talon, M., Menke, H.H., *et al*. (2007) Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. *Eukaryotic Cell*, **6**(12), 2311–2322.

Lu, X., Sun, J., Nimtz, M., Wissing, J., Zeng, A.P. and Rinas, U. (2010) The intra- and extracellular proteome of *Aspergillus niger* growing on defined medium with xylose or maltose as carbon substrate. *Microbial Cell Factories*, **9**, 23.

Lukoyanova, N., Kondos, S.C., Farabella, I., Law, R.H.P., Reboul, C.F., Caradoc-Davies, T.T., *et al*. (2015) Conformational changes during pore formation by the perforin-related protein pleurotolysin. *PLOS Biology*, **13**(2), e1002049.

Marheineke, K., Grünewald, S., Christie, W. and Reilander, H. (1998) Lipid composition of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Tn) insect cells used for baculovirus infection. *FEBS Letters*, **441**(1), 49–52.

McDonagh, A., Fedorova, N.D., Crabtree, J., Yu, Y., Kim, S., Chen, D., *et al.* (2008) Sub-telomere directed gene expression during initiation of invasive aspergillosis. *PLoS Pathogens*, **4**(9), e1000154.

Mylonakis, E., Casadevall, A. and Ausubel, F.M. (2007) Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. *PLoS Pathogens*, **3**(7), e101.

Naneh, O., Zavec, A.B., Pahovnik, D., Žagar, E., Gilbert, R.J.C., Križaj, I., *et al.* (2015) An optimized protocol for expression and purification of murine perforin in insect cells. *Journal of Immunological Methods*, **426**, 19–28.

Nayak, A.P., Blachere, F.M., Hettick, J.M., Lukomski, S., Schmechel, D. and Beezhold, D.H. (2011) Characterization of recombinant terrelysin, a hemolysin of *Aspergillus terreus*. *Mycopathologia*, **171**(1), 23–34.

Nayak, A.P., Green, B.J., Friend, S. and Beezhold, D.H. (2012) Development of monoclonal antibodies to recombinant terrelysin and characterization of expression in *Aspergillus terreus*. *Journal of Medical Microbiology*, **61**(4), 489–499.

Nayak, A.P., Green, B.J. and Beezhold, D.H. (2013) Fungal hemolysins. *Medical Mycology*, **51**(1), 1–16.

Nitsche, B.M., Jørgensen, T.R., Akeroyd, M., Meyer, V. and Ram, A.F.J. (2012) The carbon starvation response of *Aspergillus niger* during submerged cultivation: insights from the transcriptome and secretome. *BMC Genomics*, **13**, 380.

Novak, M., Kraševec, N., Skočaj, M., Maček, P., Anderluh, G. and Sepčić, K. (2015) Fungal aegerolysin-like proteins: distribution, activities, and applications. *Applied Microbiology and Biotechnology*, **99**(2), 601–610.

Ota, K., Leonardi, A., Mikelj, M., Skočaj, M., Wohlschlager, T., Künzler, M., *et al.* (2013) Membrane cholesterol and sphingomyelin, and ostreolysin A are obligatory for pore-formation by a MACPF/CDC-like pore-forming protein, pleurotolysin B. *Biochimie*, **95**(10), 1855–1864.

Ota, K., Butala, M., Viero, G., Dalla Serra, M., Sepčić, K. and Maček, P. (2014) Fungal MACPF-like proteins and aegerolysins: bi-component pore-forming proteins? *Sub-Cellular Biochemistry*, **80**, 271–291.

Panevska, A., Hodnik, V., Skočaj, M., Novak, M., Modic, Š., Pavlic, I., *et al.* (2019) Pore-forming protein complexes from Pleurotus mushrooms kill western corn rootworm and Colorado potato beetle through targeting membrane ceramide phosphoethanolamine. *Scientific Reports*, **9**, 5073.

Pel, H.J., de Winde, J.H., Archer, D.B., Dyer, P.S., Hofmann, G., Schaap, P.J., *et al.* (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature Biotechnology*, **25**(2), 221–231.

Petek, M., Baebler, S., Kuzman, D., Rotter, A., Podlesek, Z., Gruden, K., *et al.* (2010) Revealing fosfomycin primary effect on *Staphylococcus aureus* transcriptome: modulation of cell envelope biosynthesis and phosphoenolpyruvate induced starvation. *BMC Microbiology*, **10**, 159–171.

Pires, A.B.L., Gramacho, K.P., Silva, D.C., Góes-Neto, A., Silva, M.M., Muniz-Sobrinho, J.S., *et al.* (2009) Early development of *Moniliophthora perniciosa* basidiomata and developmentally regulated genes. *BMC Microbiology*, **9**, 158.

Podobnik, B., Stojan, J., Lah, L., Kraševec, N., Seliškar, M., Rižner, T. L., *et al.* (2008) CYP53A15 of *Cochliobolus lunatus*, a target for natural antifungal compounds. *Journal of Medicinal Chemistry*, **51**(12), 3480–3486.

Punt, P.J. and van den Hondel, C.A. (1992) Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *Methods in Enzymology*, **216**, 447–457.

Rao, J., DiGiandomenico, A., Unger, J., Bao, Y., Polanowska-Grabowska, R.K. and Goldberg, J.B. (2008) A novel oxidized low-density lipoprotein-binding protein from *Pseudomonas aeruginosa*. *Microbiology*, **154**, 654–665.

Rementeria, A., López-Molina, N., Ludwig, A., Vivanco, A.B., Bikandi, J., Pontón, J., *et al.* (2005) Genes and molecules involved in *Aspergillus fumigatus* virulence. *Revista Iberoamericana de Micología*, **22**(1), 1–23.

Roberts, R.G., Bischoff, J.F. and Reymond, S.T. (2011) Differential gene expression in *Alternaria gaisen* exposed to dark and light. *Mycological Progress*, **11**(2), 373–382.

Sabotič, J., Ohm, R.A. and Künzler, M. (2015) Entomotoxic and nematotoxic lectins and protease inhibitors from fungal fruiting bodies. *Applied Microbiology and Biotechnology*, **100**(1), 91–111.

Sakaguchi, O., Shimada, H. and Yokota, K. (1975) Purification and characteristics of hemolytic toxin from *Aspergillus fumigatus*. *Japanese Journal of Medical Science & Biology*, **28**(5–6), 328–331.

Sepčić, K., Berne, S., Potrich, C., Turk, T., Maček, P. and Menestrina, G. (2003) Interaction of ostreolysin, a cytolytic protein from the edible mushroom *Pleurotus ostreatus*, with lipid membranes and modulation by lysophospholipids. *European Journal of Biochemistry*, **270**(6), 1199–1210.

Sepčić, K., Berne, S., Rebolj, K., Batista, U., Plemenitaš, A., Šentjurc, M., *et al.* (2004) Ostreolysin, a pore-forming protein from the oyster mushroom, interacts specifically with membrane cholesterol-rich lipid domains. *FEBS Letters*, **575**(1–3), 81–85.

Shibata, T., Kudou, M., Hoshi, Y., Kudo, A., Nanashima, N. and Miyairi, K. (2010) Isolation and characterization of a novel two-component hemolysin, erylysin A and B, from an edible mushroom, Pleurotus eryngii. *Toxicon*, **56**(8), 1436–1442.

Shim, S.M., Kim, S.B., Kim, H.Y., Rho, H.S., Lee, H.S., Lee, M.W., *et al.* (2006) Genes expressed during fruiting body formation of *Agrocybe cylindracea*. *Mycobiology*, **34**(4), 209–213.

Skočaj, M., Resnik, N., Grundner, M., Ota, K., Rojko, N., Hodnik, V., *et al.* (2014) Tracking cholesterol/sphingomyelin-rich membrane domains with the ostreolysin A-mCherry protein. *PLoS One*, **9**(3), e92783.

Stephens, K.E., Miller, K.Y. and Miller, B.L. (1999) Functional analysis of DNA sequences required for conidium-specific expression of the SpoC1-C1C gene of *Aspergillus nidulans*. *Fungal Genetics and Biology*, **27**(2–3), 231–242.

Suh, M.J., Fedorova, N.D., Cagas, S.E., Hastings, S., Fleischmann, R.D., Peterson, S.N., *et al.* (2012) Development stage-specific proteomic profiling uncovers small, lineage specific proteins most abundant in the *Aspergillus fumigatus* conidial proteome. *Proteome Science*, **10**(1), 30.

Teutschbein, J., Albrecht, D., Pötsch, M., Guthke, R., Aimanianda, V., Clavaud, C., *et al*. (2010) Proteome profiling and functional classification of intracellular proteins from conidia of the human-pathogenic mold *Aspergillus fumigatus*. *Journal of Proteome Research*, **9**(7), 3427–3442.

Tomita, T., Noguchi, K., Mimuro, H., Ukaji, F., Ito, K., Sugawara-Tomita, N., *et al*. (2004) Pleurotolysin, a novel sphingomyelin-specific two-component cytolysin from the edible mushroom *Pleurotus ostreatus*, assembles into a transmembrane pore complex. *The Journal of Biological Chemistry*, **279**(26), 26975–26982.

Vacaru, A.M., Tafesse, F.G., Ternes, P., Kondylis, V., Hermansson, M., Brouwers, J.F.H.M., *et al*. (2009) Sphingomyelin synthase-related protein SMSr controls ceramide homeostasis in the ER. *Journal of Cell Biology*, **185**(6), 1013–1027.

Vacaru, A.M., van den Dikkenberg, J., Ternes, P. and Holthuis, J.C.M. (2013) Ceramide phosphoethanolamine biosynthesis in *Drosophila* is mediated by a unique ethanolamine phosphotransferase in the golgi lumen. *Journal of Biological Chemistry*, **288**(16), 11520–11530.

Vezočnik, V., Rebolj, K., Sitar, S., Ota, K., Tušek-Žnidarič, M., Štrus, J., *et al*. (2015) Size fractionation and size characterization of nanoemulsions of lipid droplets and large unilamellar lipid vesicles by asymmetric-flow field-flow fractionation/multi-angle light scattering and dynamic light scattering. *Journal of Chromatography A*, **1418**, 185–191.

Vidic, I., Berne, S., Drobne, D., Maček, P., Frangež, R., Turk, T., *et al*. (2005) Temporal and spatial expression of ostreolysin during development of the oyster mushroom (*Pleurotus ostreatus*). *Mycological Research*, **109**(3), 377–382.

de Vries, R.P., Riley, R., Wiebenga, A., Aguilar-Osorio, G., Amillis, S., Uchima, C.A., *et al*. (2017) Comparative genomics reveals high biological diversity and specific adaptations in the industrially and medically important fungal genus *Aspergillus*. *Genome Biology*, **18**(28).

Wang, M., Gu, B., Huang, J., Jiang, S., Chen, Y., Yin, Y., *et al*. (2013) Transcriptome and proteome exploration to provide a resource for the study of *Agrocybe aegerita*. *PLoS One*, **8**(2), e56686.

Wartenberg, D., Lapp, K., Jacobsen, I.D., Dahse, H.M., Kniemeyer, O., Heinekamp, T., *et al*. (2011) Secretome analysis of *Aspergillus fumigatus* reveals Asp-hemolysin as a major secreted protein. *International Journal of Medical Microbiology*, **301**(7), 602–611.

Wieser, J., Lee, B.N., Fondon, J.W. and Adams, T.H. (1994) Genetic requirements for initiating asexual development in *Aspergillus nidulans*. *Current Genetics*, **27**(1), 62–69.

Wösten, H.A.B., Moukha, S.M., Sietsma, J.H. and Wessels, J.G. (1991) Localization of growth and secretion of proteins in *Aspergillus niger*. *Journal of General Microbiology*, **137**(8), 2017–2023.

Yamaji-Hasegawa, A., Hullin-Matsuda, F., Greimel, P. and Kobayashi, T. (2016) Pore-forming toxins: Properties, diversity, and uses as tools to image sphingomyelin and ceramide phosphoethanolamine. *Biochimica et Biophysica Acta - Biomembranes*, **1858**(3), 576–592.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.