Food Microbiology 29 (2012) 233–241

Contents lists available at SciVerse ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

The biosynthesis of ochratoxin A by *Penicillium* as one mechanism for adaptation to NaCl rich foods

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ARTICLE INFO

Article history:
Received 12 April 2011
Received in revised form
14 July 2011
Accepted 4 August 2011
Available online 22 August 2011

Keywords:
Ochratoxin
Dry cured meat
NaCl
Penicillium nordicum
Penicillium verrucosum
Adaptation

ABSTRACT

Penicillium nordicum is an ochratoxin A producing filamentous fungus, which is adapted to sodium chloride and protein rich food environments like certain cheeses or dry cured meats. *Penicillium verrucosum* usually occurs on cereals but can also be isolated from brined olives. It could be shown that sodium chloride has a profound influence on the regulation of ochratoxin A biosynthesis in both *Penicillium* species. High amounts of ochratoxin A are produced by *P. nordicum* over a wide concentration range of NaCl (5–100 g/l) with a weak optimum at about 20 g/l after growth on YES medium. *P. verrucosum* shifts secondary metabolite biosynthesis after growth on YES medium from citrinin at low to ochratoxin at elevated NaCl concentrations. The ochratoxin A biosynthesis of *P. nordicum* is accompanied by an induction of the *otapks*PN gene, the gene of the ochratoxin A polyketide synthase. A mutant strain unable to produce ochratoxin showed a drastic growth reduction under high NaCl conditions. Determination of the dry weight and the chloride content in the mycelium of the *P. nordicum* wild type strain and a non-ochratoxin A producing mutant strain showed a much higher increase of both parameters in the mutant compared to the wild type. These results suggest, that the constant biosynthesis and excretion of ochratoxin A, which itself contains a chloride atom, ensures a partial chloride homeostasis in the fungal cell. This mechanism may support the adaptation of ochratoxin A producing Penicillia to NaCl rich foods.

1. Introduction

Ochratoxin A is a nephrotoxic, potentially carcinogenic mycotoxin with additional hepatotoxic, teratogenic and immunosuppressive activities. At the molecular level it inhibits protein biosynthesis by competitive inhibition of the phenyl alanyl-t-RNA synthetase (Höhler, 1998). Ochratoxin A is discussed to be involved in the Balkan endemic nephropathy (Vrabcheva et al., 2000). It is produced by several *Aspergillus* and *Penicillium* species (Varga et al., 2001), like *Penicillium nordicum, Penicillium verrucosum, Aspergillus carbonarius* or *Aspergillus westerdijkiae*.

P. nordicum is a typical contaminant of dry cured foods like Italian or Spanish ham, salami or salted cheeses (Lund and Frisvad, 2003). It can occur at a frequency of up to 11% of the fungal population on the surface of dry cured ham (Bogs et al., 2006; Battilani et al., 2007). Dry cured meat products like ham or salami as well as brined olives can contain 6–20% NaCl (Arnau et al., 1995; Vestergaard et al., 2005; Hondrodimou et al., 2011; Collell et al., 2011) depending on the drying status. Most strains of *P. nordicum* are strong and constant ochratoxin A producers (Larsen et al., 2001; Lund and Frisvad, 2003). *P. verrucosum*, the other ochratoxin A producing *Penicillium* species, produces generally less and more inconsistently ochratoxin A. Many strains of this species are also able to produce citrinin. *P. verrucosum* is mainly adapted to cereals, but can occasionally also be found on dry cured ham (Comi et al., 2004; Peintner et al., 2000; Andersen, 1995) and was recently described as a contaminant of brined olives (Heperkan et al., 2009). Ochratoxin A can also be produced by several *Aspergillus* species, like *A. carbonarius*, *Aspergillus niger*, *Aspergillus ochraceus*, *A. westerdijkiae* or *Aspergillus steynii* (Belli et al., 2005; Varga and Kozakiewicz, 2006; Leong et al., 2007). These latter species are adapted to various food commodities like grapes, coffee, cocoa or spices (Bayman and Baker, 2006). Until now none of these species have been described as contaminants of NaCl rich dry cured foods. Ochratoxin A is a secondary metabolite that contains a chloride within its molecule. The structure of citrinin is highly related to the polyketide part of the ochratoxin A molecule, it however does not contain a chloride.

Several hypotheses for the ecological reason to produce secondary metabolites by fungi are being discussed like their putative responsiveness for storage or elimination of “waste”

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doi:10.1016/j.fm.2011.08.003

metabolites, their involvement as substrate for evolutionary processes, or their contribution to the survival of the producer (Roze et al., 2011). According to the last point of view the biosynthesis of mycotoxins may serve as an adaptation to the environment. This view is supported by the fact that the secondary metabolite profile of fungi can vary in response to changes in the external environments. This for example is the case for *AAspergillus parasiticus* which shifts the biosynthesis of aflatoxin B_1 to aflatoxin G_1 or vice versa, when water activity and temperature are changed (Schmidt-Heydt et al., 2010b) or for *P. nordicum* which shifts the production from ochratoxin A to ochratoxin B when illuminated by bright light (Schmidt-Heydt et al., 2010a). It is furthermore important to note, that the biological reason for the biosynthesis of a mycotoxin is often multifunctional. Aflatoxin for example is discussed to be responsible for the removal of acetate, protection against UV damage, reduction of oxidative stresses, protection against insects and regulation of conidiation (Roze et al., 2011).

The current work gives evidence that the biosynthesis of ochratoxin A by *P. nordicum* and *P. verrucosum* increases their fitness under conditions of high concentrations of NaCl, e. g. during growth on dry cured meat products or brined olives.

2. Materials and methods

2.1. Strains and growth conditions

P. nordicum BFE487 is a strong ochratoxin A producing strain, *P. nordicum* BFE487 P8 is a mutant strain of BFE487 which produces no detectable amounts of ochratoxin A. *Penicillium expansum* BFE189 is a citrinin producing strain and *P. verrucosum* BFE808 is an ochratoxin and citrinin producing strain. These strains were used as model strains throughout this study. The strains were routinely grown on malt extract agar (MEA, Merck, Darmstadt, Germany) prepared according to the manufacturer's recommendations, except that 5 g/l glucose were added. For ochratoxin A biosynthesis the cultures were incubated for 5 days on YES medium (yeast extract 20 g/l; sucrose 150 g/l; agar 20 g/l) which was supplemented with the respective amount of NaCl (0–100 g/l). To be able to assess the water activity conditions under high NaCl conditions, the water activity of two representative solutions are given: a NaCl solution of 70 g/l has an a_w value of 0.96 and a solution of 100 g/l an a_w of 0.94 according to the Bulletin 03/97 of the BC Center for Disease Control.

Cultures which were taken for RNA isolation were incubated on the respective media as described above, except that before spore inoculation the plates were covered with a sterile cellophane sheet to ensure that the mycelium could be harvested without adhering agar particles.

2.2. Growth assessment

For analyzing the growth rate, the strains were single point inoculated on the agar plates and grown for 5 days under the respective conditions. For this purpose a suspension of 10^7 spores per ml were prepared by harvesting spores of a 7 day old colony with the aid of an inoculation loop and subsequent suspension in TWS solution (25 ml Tween 80 (1% aqueous solution), 8 g NaCl ad 1 l distilled water). The spore number were counted in a Thoma chamber and adjusted by adding additional TWS solution if necessary. An amount of 10 μl of that solution was centrally inoculated on an agar plate. After inoculation of the cultures the diameters of the colonies were measured two times rectangular to each other. All experiments were repeated three times.

2.3. Determination of ochratoxin A by thin layer (TLC) and high pressure liquid (HPLC) chromatography

For determination of ochratoxin A biosynthesis, the strains were grown at 25 °C on NaCl supplemented agar plates. An agar plug (diameter 1 cm) was taken from the colony with the aid of a sterile corer. This agar plug with the adhering mycelium was transferred into 2 ml micro reaction tubes and 1 ml of chloroform was added. The fungal mycelia were extracted for 30 min at room temperature on a rotary shaker; the mycelia were discarded and the chloroform extract was evaporated to dryness in a vacuum concentrator (Speed Vac, Savant Instruments, Farmingdale, USA). The residues were redissolved in 100 μl methanol and 20 μl were spotted onto TLC plates (Silica gel 60, Merck, Darmstadt, Germany). As mobile phase toluol:methanol:acetic acid (90:5:5, [v:v:v]) was used. Pure ochratoxin A (Sigma, St. Louis, USA) was used as standard. The spots were visualized under UV light (366 nm). In case of quantitative determination of ochratoxin A by HPLC, the residues were redissolved in methanol and subjected to HPLC according to the method described in the ISO 15141 standard (1998, www.iso.ch).

2.4. Determination of citrinin by HPLC/LC-MS

HPLC analysis was performed on an HP 1200 system (Agilent Technologies Waldbronn, Germany) equipped with an auto-injector, column oven and fluorescence detector. The auto-injector was set to 10 °C and the column oven to 25 °C. Separation was carried out on a Prontosil C18 (150 mm × 4 mm i.d., particle size 3 μm) reversed-phase column (Bischoff, Leonberg, Germany). Solvent A consisted of 0.1% formic acid in water (pH 3) and solvent B of acetonitrile. A linear gradient was used from 10 to 90% B in 35 min. The flow rate was set to 1.0 ml/min and the injection volume was 60–100 μl. The HPLC system was directly coupled to a hybrid triple-quadrupole/linear ion trap mass spectrometer (QTrap® 186 3200; Applied Biosystems, Darmstadt, Germany) equipped with a TurboIonSpray source. Analytes were detected in the positive ion mode at a vaporizer temperature of 600 °C and ion spray voltage of 5.5 kV. Spectral data were recorded with N_2 (CAD = 4) as collision gas and a declustering potential of 21 V. Data acquisition was performed in the MRM mode monitoring the transition of $[M+H]+$ m/z 250 in Q1 to m/z 233 in Q3 as quantifier and to m/z 205 in Q3 as qualifier. The collision energies were 19 and 35 V, respectively. Quantification was performed by external calibration using the commercially available reference compound. A calibration curve was constructed in the range of 300 pg/ml to 300 ng/ml in which the linearity of the response was given ($R2 = 0.9969$). The limit of detection was 7 pg on column. Data collection and handling was done with Analyst 1.5.

2.5. Isolation of RNA

To perform gene expression experiments, RNA was isolated using the RNAeasy Plant Mini kit (Qiagen, Hilden, Germany). An amount of 1.0 g of the mycelium was ground in a mortar in the presence of liquid nitrogen. For normalization of the subsequent Real Time PCR an amount of exactly 250.00 mg of the mycelium powder determined by a microbalance was used for isolation of total RNA. RNA concentration was determined by a Nanodrop spectrophotometer (Peqlab, Erlangen, Germany). The mycelium was resuspended in 750 μl lysis buffer, mixed with 7.5 μl β-mercaptoethanol and about 100 glass beads with a diameter of 1 mm (B. Braun Biotech International GmbH, Melsungen, Germany) in a RNase free micro reaction tube. The sample was mixed thoroughly and incubated for 15 min at 55 °C and 42 kHz in an S10H ultrasonic bath (Elma, Singen, Germany). All further procedures were essentially the same as suggested by the manufacturer of the kit.

2.6. cDNA synthesis

For cDNA synthesis 12 μl of the DNase I treated total RNA were used along with the Omniscript Reverse Transcription kit (Qiagen, Hilden, Germany). The reaction mixture was composed essentially as described by the manufacturer and incubated at 37 °C for 1 h. The cDNA was either directly used for Real Time PCR or stored at −20 °C.

2.7. Real Time PCR

The Real Time PCR reactions were performed in a GeneAmp 5700® Sequence Detection System (PE Applied Biosystems, Foster City, USA). The SYBR Green approach was used. The optimal primers and the internal probe used in the reaction were identified within the *otapks*PN gene by the Primer Express 1.0 software (PE Applied Biosystems). The primer set had the following nucleotide sequences: *otapks*PN_for (5′- tcc tcg ctg gtt gct gtt cat ctc g-3′) and *otapks*PN_rev (5′- ata aag cca acc gcg cca gca cca g-3′). For the PCR reaction the SYBR Green Reagent kit (Eurogentec, Liege, Belgium) was used according to the recommendations of the manufacturer. After an activation step of 10 min at 95 °C all subsequent 40 PCR cycles were performed according to the following temperature regime: 95 °C for 20 s, 55 °C for 40 s, and 72 °C for 60 s. A standard curve was generated for absolute quantification of the *otapks*PN mRNA copies. To generate this standard curve a larger PCR fragment of the *otapks*PN gene as template, which was amplified with the primer pair otapks_for (5′-tac ggc cat ctt gag caa cgg cac tgc-3′) and otapks_rev (5′-atg cct ttc tgg gtc cag ta-3′), was used. The concentration of this standard PCR product was determined in a nanodrop spectrophotometer and the number of copies was calculated. These stock solutions were diluted serially by a factor of 10 and an aliquot of the dilutions was used as a copy number standard during each setup of the Real Time PCR reaction. The concentration of unknown samples was calculated by the GeneAmp 5700® system according to the generated standard curve.

2.8. Determination of the cellular chloride content

P. nordicum BFE487 was grown for 7 days on YES agar containing various amounts of NaCl. The agar was covered by a sheet of cellophane to ensure that pure mycelium with no adhering agar could be harvested. The mycelium was transferred to platinum vessels and heated to 500 °C for 5 h. The resulting ash was completely transferred to a 20 ml graduated glass cylinder and filled up with the respective amount of double distilled water. This solution was taken for subsequent chloride determination. For this determination the test kit Chloride CD1 (Tintometer, Dortmund, Germany) was used. This test kit uses a titration principle to determine the chloride concentration. The analysis was essentially been carried out as described by the manufacturer of the kit.

3. Results

3.1. Influence of the NaCl concentration on growth and ochratoxin A biosynthesis by P. nordicum after incubation on YES medium

To analyze the influence of NaCl on growth, ochratoxin A biosynthesis and expression of the *otapks*PN gene, *P. nordicum* BFE487 was single point inoculated on YES medium supplemented with different amounts of NaCl and grown at 25 °C for 7 days. After that time the colony diameter was measured as an indication for the growth rate and samples were withdrawn to analyze the ochratoxin A produced by HPLC and in parallel the expression of the ochratoxin A *otapks*PN gene by Real Time PCR. The results are shown in Fig. 1A–C. Growth of *P. nordicum* is supported by the addition of NaCl to the medium. The growth optimum was observed at 40 g/l NaCl (Fig. 1A). Before and after that concentration the colony diameter gradually decreases. At 80 g/l NaCl the diameter of the colony roughly resembles that of YES medium without added NaCl, indicating the broad growth capacity of *P. nordicum* on NaCl containing substrates. Also the biosynthesis of ochratoxin A was supported by NaCl. After an incubation of 7 days the optimum was around 20 g NaCl per liter, which is aside, but not exactly coincidental with the growth optimum (Fig. 1B). At higher NaCl concentrations the biosynthesis of ochratoxin A gradually decreased up to a NaCl concentration of 80 g/l. At this concentration the ochratoxin A biosynthesis capacity resembles that of YES medium without NaCl. The transcriptional activity of the *otapks*PN gene paralleled that of the biosynthesis (Fig. 1C). In congruence with the biosynthesis a first transcriptional optimum could be found at a NaCl concentration of 20 g/l. However at higher NaCl concentrations an increase of induction of the *otapks*PN gene appeared which is in accordance with a high biosynthesis of ochratoxin after prolonged incubation (more than 10 days, data not shown) under these conditions.

3.2. Influence of increasing amounts of NaCl on the biosynthesis of ochratoxin A and citrinin in P. verrucosum

The influence of increasing amounts of NaCl on ochratoxin A biosynthesis by *P. verrucosum* was also tested. For that purpose *P. verrucosum* BFE808 was grown at 25 °C on YES medium with different concentrations of NaCl. *P. verrucosum* BFE808 is a strain which is capable of producing ochratoxin A and citrinin. After 7 days of incubation samples were withdrawn, extracted and subjected to HPLC analysis for the quantification of ochratoxin and to HPLC/MS analysis for the quantification of citrinin. The results are shown in Fig. 2. Under the conditions analyzed *P. verrucosum* BFE808 was able to synthesize citrinin at low NaCl concentrations in the medium. However after increasing the concentration of NaCl, citrinin biosynthesis gradually decreased. At higher NaCl concentrations only very scarce amounts of citrinin were produced. Very interestingly the biosynthesis of ochratoxin A started at NaCl concentrations when the biosynthesis of citrinin is beginning to be down regulated. *P. verrucosum* BFE808 had a peak of ochratoxin A biosynthesis at 40 g/l, however the amounts produced are lower than that of *P. nordicum* BFE487. Beyond this NaCl concentration the production decreased rapidly but was still detectable up to 100 g/l.

3.3. Comparison of the growth of the P. nordicum wild type strain with a mutant not able to produce ochratoxin A at increasing levels of NaCl

In order to compare the growth rate of a constantly ochratoxin A producing *P. nordicum* strain (BFE487) and a mutant of this strain (BFE487 P8) unable to produce ochratoxin A, spore suspensions (10^7/ml) of these strains were inoculated on YES medium and incubated at 25 °C for 7 days. After that time the diameter of the colonies were determined and are shown in Fig. 3A. In this experiment a quite constant growth rate for the *P. nordicum* BFE487 wild type with a smooth optimum at 40 g/l NaCl could be observed. Interestingly the wild type sporulates only up to a NaCl concentration of 20 g/l (Fig. 3B). At a NaCl concentration of 100 g/l the growth rate is roughly the same than on plain YES medium. In contrast the growth rate of the mutant strain was a little bit lower than that of the wild type at a NaCl concentration range between 0 and 40 g/l, but runs exactly in parallel (Fig. 3A). However at a concentration range of 40–100 g/l the growth rate drastically decreased. At a NaCl concentration of 100 g/l growth is greatly

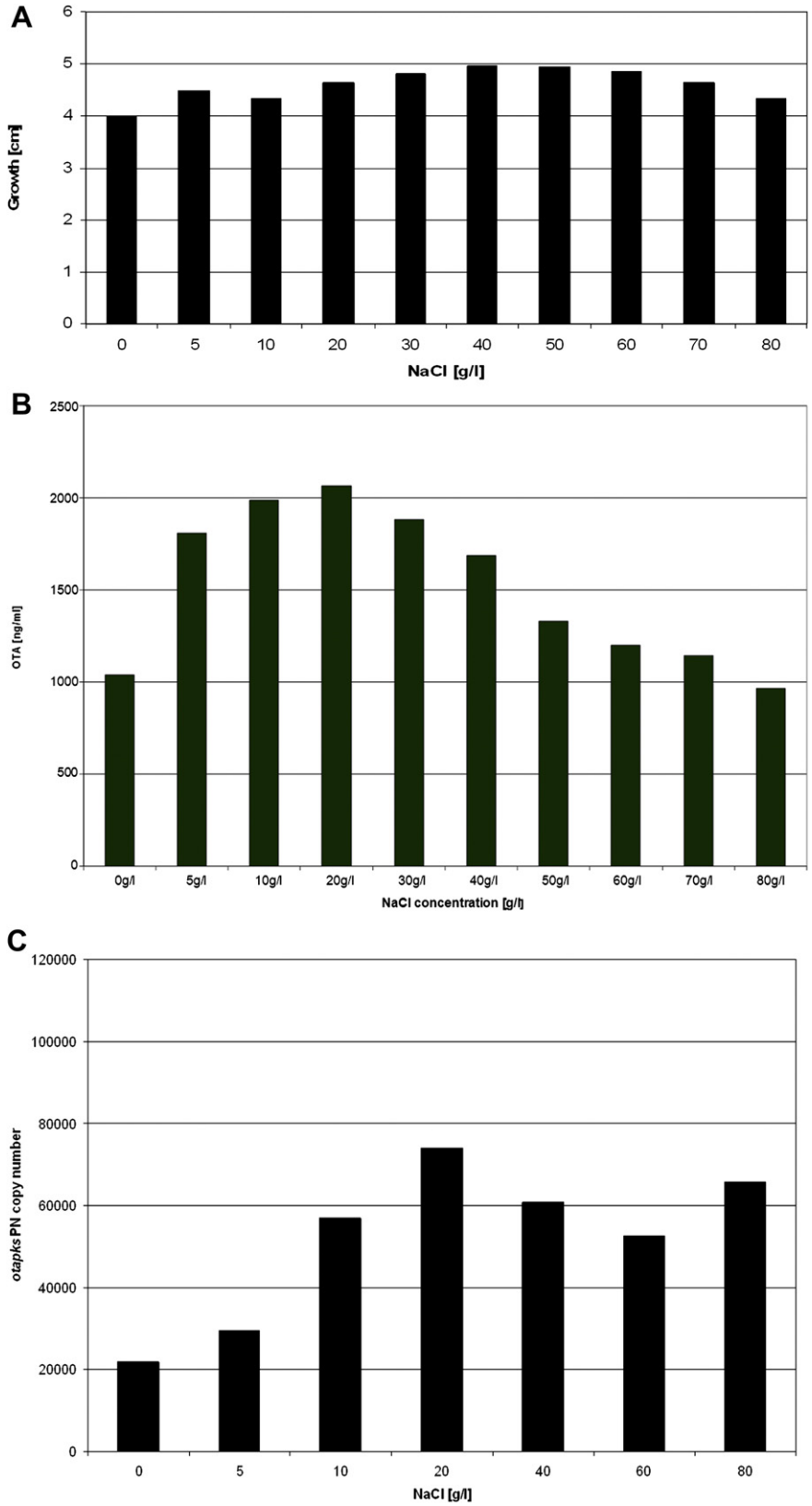


Fig. 1. Growth (A), ochratoxin A biosynthesis (B) and expression of the ochratoxin A polyketide synthase gene (*otapks*PN, C) after incubation on YES medium with increasing amounts of NaCl for 7 days. Growth rates were determined by measuring the diameter of the colony two times in a rectangular fashion. The amount of ochratoxin A produced was determined by HPLC and the expression of the *otapks*PN gene was determined by Real Time PCR. Several sets of experiments which all lead to the same trends have been carried out.

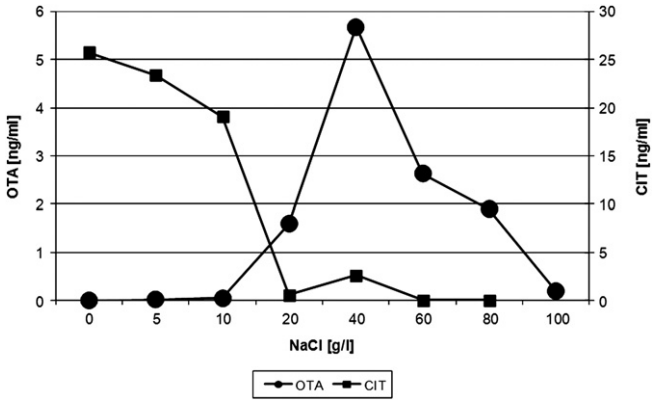


Fig. 2. Biosynthesis profile of citrinin and ochratoxin A after growth of *P. verrucosum* BFE808 on YES medium for 7 days at increasing concentrations of NaCl. The concentrations of citrinin were measured by HPLC/MS, whereas that of ochratoxin by HPLC.

reduced compared to growth on plain YES medium. However taking the diameter as the sole indicator of growth does not really reflect the whole influence of the high NaCl concentration. Fig. 3B shows that in addition to the reduction in diameter the biomass or density of the colony of the mutant strain drastically decreased at NaCl concentrations above 40 g/l resulting in a very poor colony with very little aerial mycelium at a concentration of 100 g/l. This is not the case with the wild type strain. Because *P. nordicum* BFE487 P8 is isogenic to *P. nordicum* BFE487 except for the ability to produce ochratoxin A the results suggest a correlation between the biosynthesis of ochratoxin A and the fitness of the organism at high concentrations of NaCl.

3.4. Differences in the growth responses at high NaCl concentrations between P. nordicum, P. verrucosum and P. expansum

As shown above apparently the biosynthesis of ochratoxin A is correlated to the fitness of the producer at high NaCl concentrations. Because of the fact that the regulation of ochratoxin A biosynthesis is completely different in *P. nordicum* and *P. verrucosum* it is possible that these species have different growth capacities on media with high NaCl concentrations. To demonstrate these possible differences *P. nordicum* BFE487 as a constant ochratoxin A producer, *P. verrucosum* as an adaptive citrinin/ochratoxin A producer and *P. expansum* as an organism producing only citrinin were grown on YES medium with increasing NaCl content and the growth rate was determined (Fig. 4). The growth of *P. nordicum* was quite constant over the whole NaCl concentration range indicating an optimal adaptation of this species. *P. verrucosum* showed an improved growth rate compared to plain YES medium up to 40 g/l. Only at concentrations higher than 50 g/l the growth rate of *P. verrucosum* is lower than on YES medium without additional NaCl. For *P. expansum* a concentration up to 20 g/l did not affect growth rate, however at NaCl concentrations above that value a rapid decline of the growth rate could be measured. This decline was higher than that of *P. verrucosum*.

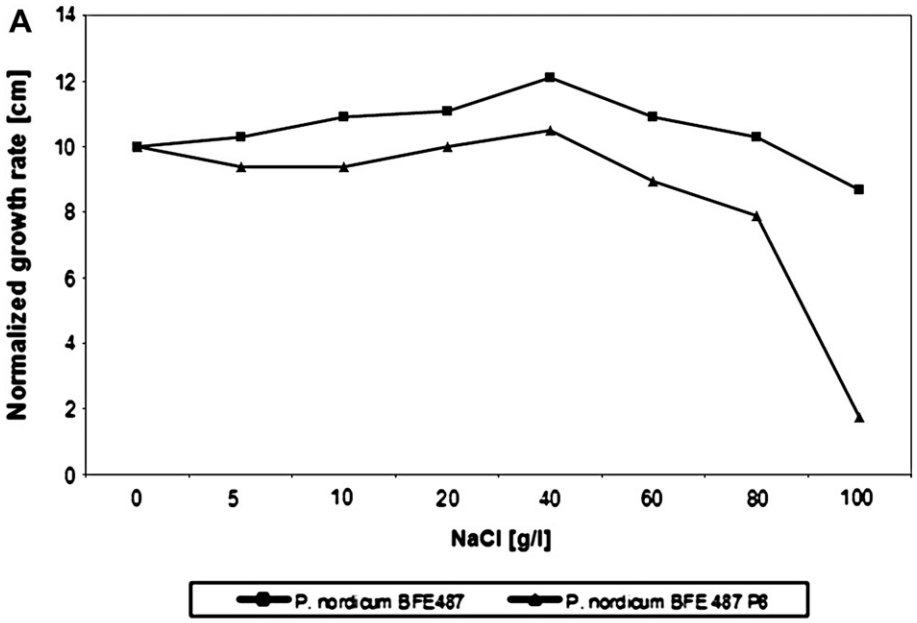
These results are a further hint for the correlation between the capacity to constantly produce high amounts of ochratoxin A and the growth capability at high NaCl concentrations.

3.5. Correlation of ochratoxin A biosynthesis to dry weight and the intracellular chloride content

The results above suggest that the biosynthesis of ochratoxin enhances the growth capacity of the producing strain under high NaCl conditions compared to non-producing strains. To analyze the intriguing hypothesis, that the biosynthesis of ochratoxin A may contribute to the excretion of chloride out of the cell and may thereby ensure some kind of chloride homeostasis within the cell even during growth under high salt conditions, the *P. nordicum* BFE487 wild type which constantly produces ochratoxin A up to 100 g/l NaCl and the *P. nordicum* BFE487 P8 strain unable to produce detectable amounts of ochratoxin A, were grown for 7 days on YES medium containing either no additional NaCl (control), 20 g/l (low NaCl) or 80 g/l (high NaCl). The strains were inoculated by spotting 10 μl of a spore suspension (10^7 spores/ml) onto the respective YES agar plate covered by a wet cellophane sheet. NaCl can freely diffuse through the cellophane foil (LEC 06.11, Determination of Diffusion Potentials, Phywe Series of Publications, Phywe Systeme GmbH, Göttingen Germany). After harvest the mycelium was rinsed with distilled water two times to eliminate external adhering chloride and was used to determine the dry weight and for subsequent chloride analysis. As expected the dry weight is lowest after growth on YES medium without added additional NaCl (Fig. 5A). Both strains show no significant differences. Also at 20 g/l NaCl the dry weight of the strains were the same, but increased compared to the former conditions. At a NaCl concentration of 80 g/l the dry weight of both strains again increased, but the non-ochratoxin A producing mutant had a significant higher dry weight in as compared to the wild type. The chloride content in the fungal mycelia showed the same tendency (Fig. 5B). After growth on YES medium without added NaCl the chloride content of the *P. nordicum* BFE487 wild type and the *P. nordicum* BFE487 P8 mutant were at the same very low level. After increasing the NaCl content of the medium to 20 g/l the mycelial NaCl content in both strains increased compared to the former situation, but was not different. If however the NaCl concentration was higher (80 g/l) there was significant higher chloride content in the *P. nordicum* BFE487 P8 mutant mycelium compared to the *P. nordicum* BFE487 wild type strain.

4. Discussion

The high and constant ochratoxin A producing species *P. nordicum* is specifically adapted to NaCl rich foods, like meats and related commodities (Lund and Frisvad, 2003). In these environments also other potential ochratoxin A producing fungi can be identified. *P. verrucosum* for example usually occurs on cereals, but can occasionally be found also on dry cured meat products (Peintner et al., 2000; Comi et al., 2004). Moreover in recent reports the occurrence of ochratoxin A and citrinin in brined olives has been described, which can be attributed to the presence of *P. verrucosum* (El Adlouni et al., 2006; Heperkan et al., 2009; Hondrodimou et al., 2011). *Penicillium nalgiovense*, which is also adapted to the environment of dry cured meats, carries DNA sequences which are homologous to the ochratoxin A biosynthesis genes (Bogs et al., 2006). This situation indicates, that the potential to produce ochratoxin A, may be of ecological advantage to be competitive in this environment. The environment of dry cured ham and olives contains usually quite high amounts of NaCl. (Arnau et al., 1995; Hondrodimou et al., 2011). Solubilized salts of course have an influence on the water activity; however beside this activity the ions of the salt also have a specific activity on the fungus. Recently Samapundo et al. (2010) analyzed the influence of salts with different anion/cation combinations on the growth of various fungi. These authors found that especially the solubilized ions have an inhibitory effect which is separate from the reduction of the water activity. NaCl and $MgCl_2$ for example have a more restrictive influence on the growth of *Penicillium roqueforti* and *A. niger* as compared to $MgSO_4$ at the same water activity. Ayodele



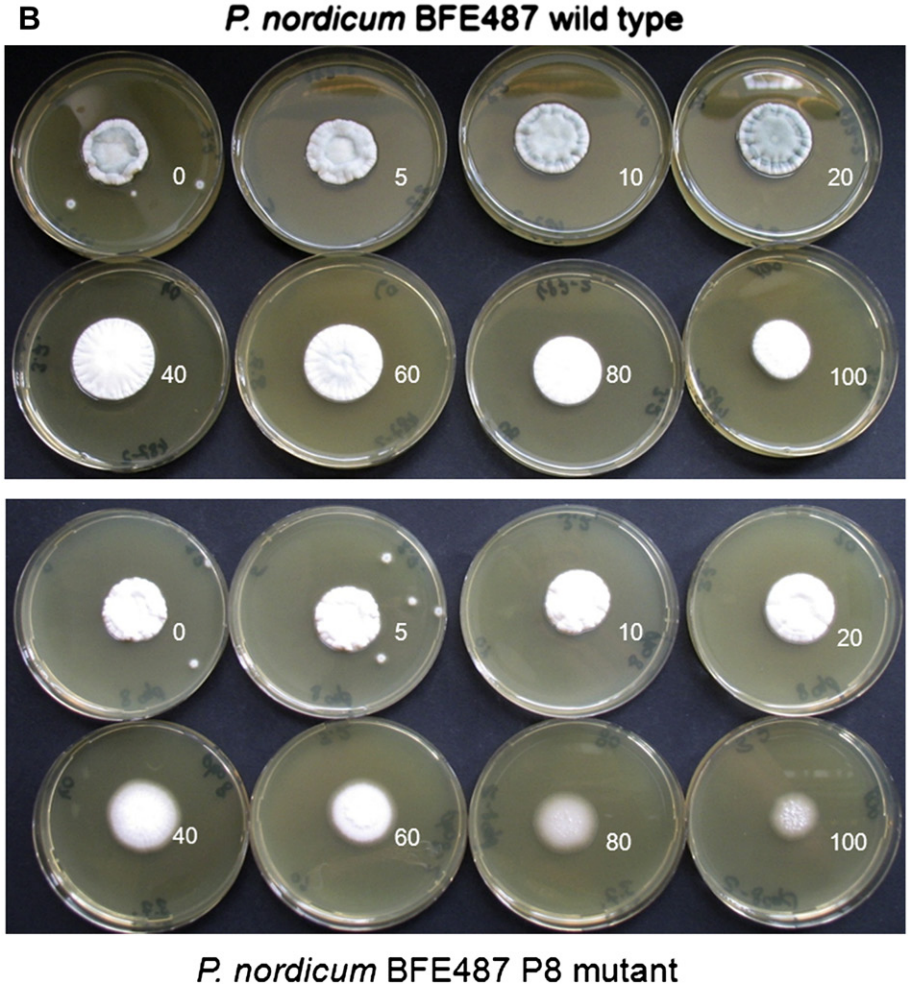


Fig. 3. Comparison of the normalized growth rate of the *P. nordicum* BFE487 wild type with that of the *P. nordicum* BFE487 P8 mutant strain after an incubation of 7 days on YES medium with increasing amounts of NaCl as indicated (A). The growth rate was determined by measuring the diameter of the colony. In (B) the morphology of the cultures grown under the same conditions are shown. The cultures were grown on YES medium with increasing amounts of NaCl (indicated on each petri dish in g/l). Upper two rows show the wild type, the lower two rows show the mutant strain.

and Ojoghoro (2007) also showed that chloride salts and especially sodium chloride have a much higher inhibitory effect on the growth of the fungus *Pleurotus tuberregium* than for example sulfate salts. This again is a strong indication, that not only the reduction of water activity influences growth and secondary metabolite biosynthesis by the fungus, but that also the type of the osmolyte has an influence, with NaCl being generally more inhibiting compared to other salts. Also for many plants a high chloride content of the soil is toxic and leads to necrosis depending on the chloride concentration (Goodrich and Jacobi, 2010). Some plants can increase their salt tolerance by antiport systems (Apse et al., 1999) being able to compartmentalize salt ions from the cytosol.

Apparently most of the externally present halogen ions are incorporated into ochratoxin A. Wei et al. (1971) showed by the incorporation of radiolabelled ^{36}Cl into *A. ochraceus*, that the radioactivity concentrated exclusively in the ochratoxin A fraction, indicating a very efficient incorporation of chlorine into ochratoxin A. Simkovic et al. (2004) analyzed an anion transporter of the fungus *Trichoderma viride*, which was specific for chloride and bromide. They show that this transporter is very active in transporting chloride into the cell, but a subsequent efflux could not be determined, indicating a potential imbalance between in- and efflux in this fungus and a potential requirement for an effective efflux mechanism during growth under high NaCl conditions.

According to the results described here NaCl adapted Penicillia have developed an effective system to ensure partial homeostasis of chloride ions under high NaCl conditions. The constant biosynthesis of ochratoxin A ensures permanent excretion of chlorine out of the cell and leads to a partial homeostasis of cellular chlorine. The drastic increase of the dry weight at high NaCl conditions, especially in the non-ochratoxin A producing mutant, reveals that mainly NaCl is responsible for this increase, because only the NaCl concentration has changed in the medium. This demonstrates the importance of a counter acting mechanism. Apparently this mechanism only plays a role at higher NaCl conditions. At concentrations up to 20 g/l the dry weight and the chloride content in both strains were the same. However at NaCl concentrations above the growth maximum of 40 g/l only the ochratoxin A producing strain showed a reduced dry weight, chloride content and a constant growth. Gunde-Cimerman et al. (2009) stressed the importance of ion homeostasis for the adaptation of halophilic fungi. However in their work, the authors are discussing the problem of elevated cation concentrations, e.g. Na^+ and not that of anions like Cl^-.

A conclusion according to which ochratoxin A indeed plays a role in chloride excretion, although at another level and with another mechanism, was described by Geckle et al. (2005) who demonstrated that ochratoxin A obviously interferes with cellular channels of kidney cell membranes of higher eukaryotes involved in electrolyte transport. This interaction leads to an increase in NaCl excretion of these kidney cells.

For a NaCl adapted fungus it is important that this excretion mechanism acts permanently and not only during the main production phase of ochratoxin A. Usually ochratoxin A biosynthesis starts at a certain growth phase and reaches a specific stationary level. The height of this level is dependent on several external parameters. This level however is not static, but rather oscillates in a circadian manner (Schmidt-Heydt et al., 2010a). However not only an oscillation due to a circadian oscillation can be observed, also under constant dark conditions a metabolic oscillation occurs which leads to an alternating production and degradation of ochratoxin (data not shown). This results in an oscillating degradation and re-biosynthesis of ochratoxin A, with the effect that new molecules of ochratoxin A are permanently produced and excreted out of the cell. Such a balance between biosynthesis and degradation was also described by Wei et al. (1971). Moreover degradation of ochratoxin A by fungi and also by producing strains were shown by Varga et al. (2000) and Abrunhosa et al. (2002).

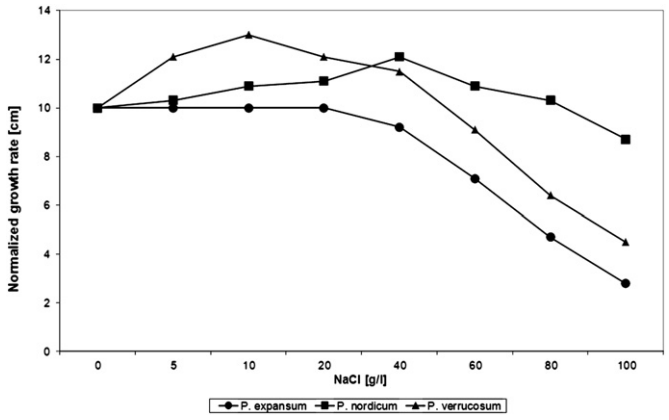


Fig. 4. Plots of the normalized growth rate versus NaCl concentration of the constant ochratoxin A producer *P. nordicum* BFE487, of *P. verrucosum* BFE808 which produces citrinin and ochratoxin and of *P. expansum* BFE189, which only produces citrinin.

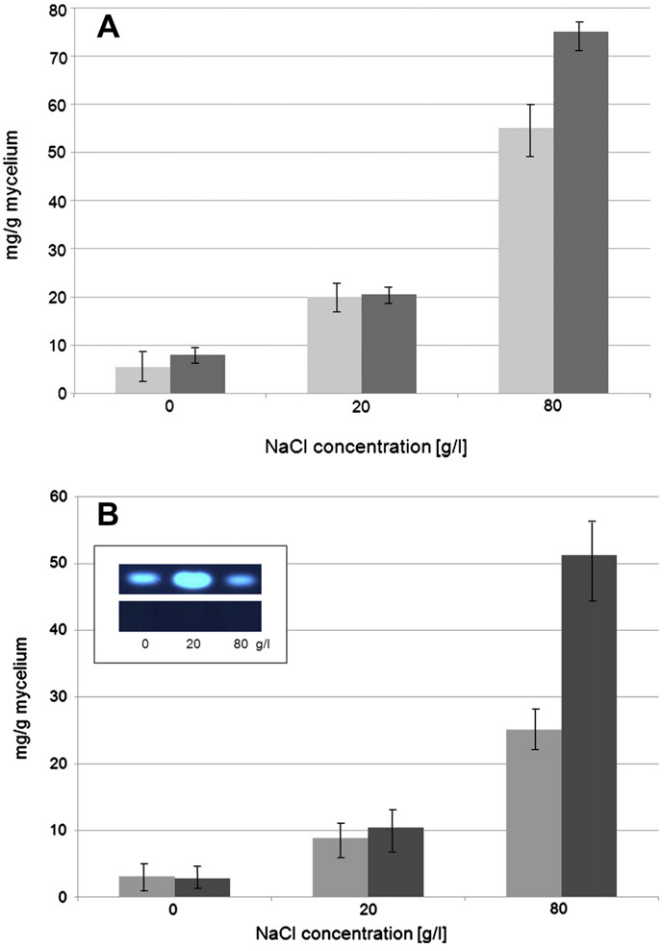


Fig. 5. Dry weight (A) and mycelial chloride concentrations (B) after growth of *P. nordicum* BFE487 wild type (light gray) and *P. nordicum* BFE487 P8 (dark gray) on YES medium for 7 days at 25 °C. The chloride concentration in the mycelium was determined with the Tintometer Chloride Test Kit as described by the manufacturer. The experiment was repeated three times and the mean value is given. The insert in (B) shows a thin layer chromatographic analysis of the production of ochratoxin A by the wild type (upper row) and the mutant (lower row) at the various concentrations of NaCl as indicated.

The biosynthesis of ochratoxin A under high NaCl conditions seems to increase the viability and competitiveness of the fungus under these conditions. This view is confirmed by the fact, that the more constantly a strain or species can produce ochratoxin A, the higher is its growth rate under high NaCl conditions. Further evidence for the correlation between ochratoxin A biosynthesis and adaptation to NaCl rich environments was obtained by comparing the growth capacity of the *P. nordicum* wild type to the mutant strain, isogenic to the wild type except for ochratoxin biosynthesis. This mutant clearly exhibited a reduced growth capacity at high NaCl conditions. In agreement with the results described here Sonjak et al. (2011) very recently identified high cfu numbers (colony forming units) of *P. nordicum* on pure salt and suggested that salt may be a vector for the contamination of meat products by this species.

In contrast to *P. nordicum P. verrucosum* can be found on different habitats and only some of them, which are less important for the fungus (olives, dry cured meat), impose NaCl stress to the fungus. This species obviously has developed a flexible adaptive mechanism to cope with NaCl stress under these particular conditions, by a shift from citrinin to ochratoxin A. *P. verrucosum* usually colonizes cereals, but because of its partial adaptation to NaCl rich environments it can occasionally be found in these products. *P. expansum* which is only able to produce citrinin shows a further reduced growth rate at high NaCl concentrations, compared to *P. verrucosum* and even more to *P. nordicum*. The structure of citrinin is very similar to the dihydroisocoumarin part of the ochratoxin A molecule, but it does not contain a chloride, so it cannot not contribute to a homeostasis mechanism.

It is interesting to note that NaCl has just the opposite effect on the biosynthesis of trichothecenes by *Fusarium graminearum*. Already low amounts of NaCl lead to a complete cessation of the production of trichothecenes by this species (Ochiai et al., 2007).

In fungi osmotic stress is detected and signaled to the transcriptional level by a HOG (high osmolar glycerol) like signal cascading pathway (Hagiwara et al., 2009). Furthermore it has been shown that this signaling pathway also plays a role in the regulation of certain mycotoxins and can either act positively or negatively (Ochiai et al., 2007; Kohut et al., 2009). First results revealed that osmotic sensitive signal cascades (HOG like signal cascades) are also involved in the regulation of ochratoxin A biosynthesis in *Penicillium* and induce ochratoxin A biosynthesis at high NaCl conditions. This NaCl dependent activation of ochratoxin A biosynthesis might not be the case for ochratoxin A producing *Aspergillus* species like *A. carbonarius* because preliminary results show that the regulation of ochratoxin A biosynthesis in relation to NaCl in this species is completely different and not supported by high NaCl. Indeed, in contrast to the Penicillia the HOG pathway does not seem to be activated by NaCl in this species. (data not shown).

During this work evidence is presented that the biosynthesis of ochratoxin A is an adaptive process for *Penicillium* to counteract high osmolarity conditions due to chloride salts. Beside this mechanism probably also other well known osmoadaptive mechanisms, like for example the accumulation of osmoprotectants are playing a role. Moreover the biosynthesis of secondary metabolites may not always be ascribed to only one biological function alone, since ochratoxin A is not solely synthesized under NaCl rich conditions. MAP kinase pathways, like the HOG pathway, are often cross activated by various stressors, which would explain the induction of ochratoxin A biosynthesis also under other non-high sodium chloride conditions. Moreover de Paula et al. (2008) have shown, that circadian clocks and HOG MAP kinase pathways are interconnected, leading to an oscillating expression of certain regulated genes. Results of the current study suggest that this is exactly what can be found with the regulation of ochratoxin A in *Penicillium*.

Acknowledgement

We thank Michaela Ebli and Nicole Mischke for excellent technical assistance. We also thank Sepp Dieterich for his support in the analysis of chloride. This work was supported formerly by the EU-project "Development of cost-effective control and prevention strategies for emerging and future food borne pathogenic microorganisms throughout the food chain" (Pathogen Combat), FOOD-CT-2005-07081 and currently by the continuing EU Project EC KBBE-2007-222690-2 MYCORED.

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