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The application of transcriptomics to understand the ecological reasons of ochratoxin a biosynthesis by *Penicillium nordicum* on sodium chloride rich dry cured foods

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Penicillium nordicum and to some extent also *P. verrucosum* can be found as contaminants on NaCl rich fermented foods like ham, cheeses or vegetables. Both fungal species are able to produce ochratoxin A. Ochratoxin A is a chloride containing mycotoxin which has toxic activities especially against the kidney. Further putative ochratoxin A producing species can be found in this environment. Recent results show that the production of ochratoxin A increases the competitiveness of the producing fungi under salt stress conditions which occur in these types of foods. This increased competitiveness may explain the frequent occurrence of these fungi in salt rich commodities.

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Introduction

Ochratoxin A is an important mycotoxin which can be produced by several species of the genus *Aspergillus* and two species of the genus *Penicillium*. Whereas the Aspergilli like *A. carbonarius*, *A. westerdijkiae*, *A. steynii*, *A. niger* or *A. ochraceus* are mainly responsible for the occurrence of ochratoxin A in food products like grapes, coffee, cocoa or spices (Abarca, Accensi, Bragulat, Castella, & Cabanes, 2003; Thirumala-Devi *et al*., 2001), the two *Penicillium* species occur either in cereals like *P. verrucosum* or in NaCl and protein rich foods like *P. nordicum*. In the latter commodities occasionally also *P. verrucosum* can be found. Ochratoxin A has nephrotoxic, hepatotoxic and immunotoxic activities (Petzinger & Ziegler, 2000), however the kidney is the main target of this toxin. There is a continuing debate whether ochratoxin A is involved in the so called Balkan nephropathy, an endemic kidney disease in a certain region of the Balkan (Vrabcheva *et al*., 2000), however the involvement of ochratoxin A in the Danish porcine nephropathy seems unambiguous (Krogh, 1987). Ochratoxin A is rated as a group II carcinogen by the WHO/FAO and for that reason any contamination of foods and feeds must be avoided. For several food commodities statutory limits have been set in the EU (EU Commission Regulation No 1881/2006 and 105/2010). In fact the Italian Ministry of Health recommend a maximal amount of ochratoxin A in dry cured ham of 1 μg/kg (Dall'Asta *et al*., 2007). It has further been shown, that a high proportion of certain human populations of EU countries contain measurable amounts of ochratoxin A in their blood serum (Rosner, Rohrmann, & Peiker, 2000). The effect of this occurrence however currently cannot be assessed. At the molecular level ochratoxin A seems to be an inhibitor of the formation of phenylalanine-t-RNA, which at the end leads to an inhibition of protein translation (Höhler, 1998; Varga, Rigó, Téren, & Mesterházy, 2001) with the effects described above.

Some foods like dry cured meat products, cheeses or fermented olives contain high concentrations of NaCl due to the production process and are typical habitats of ochratoxin A producing Penicillia. The NaCl on the one hand contributes to the aroma, flavour and characteristics of the products. On the other hand the high NaCl concentration is one of the prerequisites for the microbiological stability of these kinds of foods by reducing the water activity and increasing the chloride concentration in the product. The

water activity is a numerical value given by the amount of free available water in a matrix and is calculated by dividing the hydrostatic pressure over pure water by the hydrostatic pressure over the matrix. High water activities (>0.98) are usually favourable for the growth and physiological activity of microorganisms, including ochratoxin A biosynthesis. Most pathogenic or spoilage bacteria are quite sensitive to high concentrations of NaCl. Sodium chloride rich products may contain up to 20% salt (Arnau, Guerrero, Casademont, & Gou, 1995; Vestergaard, Erbou, Thauland, Adler-Nissen, & Berg, 2005). At the same concentration NaCl has less influence on the water activity compared to other solutes, like for example glycerol. This situation indicates that the antimicrobial activity of NaCl is not only related to the decrease of the water activity but also to the nature of the salt itself. Recently Samapundo, Deschuyffeleer, Van Laere, De Lyn, & Devlieghere (2010) analysed 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 *P. roqueforti* and *A. niger* compared to $MgSO_4$. This was the case with the same molarities of all analysed salts and indicates that high concentrations of chloride ions obviously have growth inhibiting effects. In an analysis of the influence of different preservatives at different water activities on the growth of *P. verrucosum*, it could be shown that both, growth and the expression of an ochratoxin A biosynthesis gene were more inhibited by NaCl than by glycerol at the same water activity (Schmidt-Heydt, Baxter, Geisen, & Magan, 2007). This again is a strong indication, that not only the reduction of water activity influences growth and secondary metabolite production by the fungus, but that also the type of the osmolyte has an influence, with NaCl being generally more inhibiting compared to others. Bacteria are more sensitive to high NaCl concentrations compared to fungi, but also in this case the higher inhibitory effect of NaCl compared to other solutes can be seen. The minimum water activity for *Clostridium perfringens* for example was 0.95 when NaCl was used as an osmolyte and 0.93 when glycerol was used (Jay, 2000). A NaCl concentration of 20% kills most food relevant spoilage and pathogenic bacteria. Already at around 10% NaCl the growth capacity of a *Pediococcus* strain at 10 °C was nearly completely abolished (Goldman, Deibel, & Nieven, 1963). In contrast this concentration generally only reduces the growth of filamentous fungi if they are adapted to this environment.

Another characteristic of these food environments are the high contents of proteins, peptides and amino acids. Häggblom and Ghosh (1985) showed that certain amino acids like glutamic acid and proline can enhance ochratoxin A production of *P. verrucosum* and *A. ochraceus* in liquid culture. These authors also described a positive correlation for both species between the protein content of barley and the production of ochratoxin A. Most of the products mentioned above are also especially rich in protein, peptides and amino acids, which also may influence ochratoxin A biosynthesis by *Penicillium* in this environment. Beside the influence of the substrate also other growth parameters affect ochratoxin A biosynthesis during the ripening and fermentation processes. The most important of these are the temperature and the water activity. This influence was demonstrated by Cairns-Fuller, Aldred, and Magan (2005) and Pardo, Malet, Marin, Sanchez, and Ramos (2006). These factors determine the production window of ochratoxin A which is usually narrower than the growth window of the fungus.

Several of the genes of the ochratoxin A biosynthesis pathway from *P. nordicum* have been identified either by differential display PCR (Färber & Geisen, 2004) or by using heterologous primers, and could be localized adjacent to each other (Karolewiez & Geisen, 2005). A scheme of the current knowledge is depicted in Fig. 1. It has been shown in earlier work, that the expression of the *otapks*PN gene, the gene coding for the ochratoxin A polyketide synthase, is correlated to the production of ochratoxin and can be regarded as a key gene in the production of ochratoxin A. The influence of external factors on the regulation of mycotoxin biosynthesis is exerted mainly via influences on gene transcription (Georgianna & Payne, 2009; Schmidt-Heydt, Magan, & Geisen, 2008). In this work the current knowledge about the interrelationships between the food related environment and the activation of ochratoxin A biosynthesis genes in *Penicillium* is reviewed. This new perspective may help to understand the ecological role of ochratoxin A biosynthesis in this habitat.

The fungal population of NaCl rich dry cured ham

During the production process dry cured ham is stored for some time in thick layers of NaCl resulting in diffusion of NaCl into the meat and in reducing the water activity. The dry cured ham is then ripened in temperature and moisture controlled rooms for up to 2 years. During this ripening time the concentration of NaCl can vary between 10 and 20% (Arnau *et al.*, 1995). The surface of the hams gets covered by moulds, which are desired and which contribute to the quality of the product. A problem however is the fact that the production of mycotoxins, especially ochratoxin A is also possible by certain species.

Ochratoxin A consists of a polyketide part which is coupled to the amino acid phenylalanine via a peptide linkage (Fig. 1). Ochratoxin A, which is the main end product of the fungus, contains a chloride atom, whereas ochratoxin B, has a hydrogen at this position. The toxicity of ochratoxin B is by a factor of about 100 less than the toxicity of ochratoxin A (Stander *et al.*, 2000). The toxin is produced by several *Aspergillus* and by two *Penicillium* species, in particular *P. verrucosum* and *P. nordicum*.

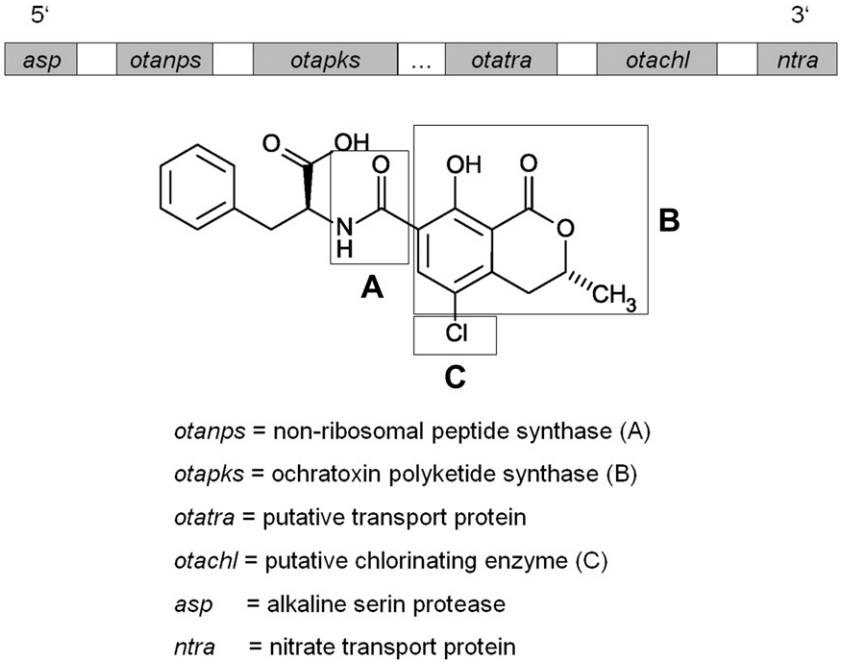

Fig. 1. Chemical structure and known biosynthesis genes of ochratoxin A. In ochratoxin B the chlorine is replaced by a hydrogen. The names of the genes and the abbreviations are indicated. The dotted area indicates that this connection has not been established yet. The reactivities of the enzymes are indicated: A = peptide formation by the product of the *otanps* gene, B = polyketide formation by the product of the *otapks* gene and C = chlorination by the activity of the chlorinating enzyme. The *asp* and the *ntra* genes apparently mark the boundaries of the gene cluster, they are co-regulated (see Fig. 3) but there involvement in the biosynthesis is not known.

However the ochratoxin biosynthesis genes have also been detected in *P. nalgiovense* (Bogs, Battilani, & Geisen, 2006) and in an as yet non-characterized *Penicillium* sp. *P. nalgiovense* is usually used as a starter culture for the production of mould fermented dry cured meats. It is also very well adapted to this environment. Many other species found in this habitat belong to the genus *Penicillium*. Comi, Orlic, Redzepovic, Urso, and Lacumin (2004) mainly isolated *Penicillium* and *Aspergillus* species from Istrian ham. The *Penicillium* isolates belonged to the species: *P. verrucosum, P. citrinum*, *P. chrysogenum, P. commune,* and *P. expansum*. Núnez, Rodriguez, Bermúdez, Córdoba, and Asensio (1996) also mainly found *Penicillium*, *Aspergillus* and *Eurotium* strains as contaminants of Iberian ham. Among these *P. citrinum*, *P. expansum, P. chrysogenum* and *P. viridicatum* could be isolated again. Among other Penicillia, Battilani *et al.* (2007) again identified *P. chrysogenum, P. citrinum, P expansum, P. nalgiovense* and *P. nordicum* in dry cured ham manufacturing plants from Italy. The presence of *P. nordicum* was confirmed by Bogs *et al.* (2006), using molecular methods. During this work 18% of the isolated *Penicillium* species could be identified as *P. nordicum*. Sørensen, Jacobsen, Nielsen, Frisvad, & Granly Koch (2008) also analysed the fungal population of the surface of dry cured ham and found up to 38% of Penicillia in the whole population with *P. commune, P. solitum* and *P. chrysogenum* among them, depending on the time of year and the production plant. Asefa *et al.* (2009) isolated *P. chrysogenum, P. citrinum, P. expansum, P. solitum*, and *P. nalgiovense*. A population analysis which was made within the PathogenCombat project came to very similar results, in which *P. nordicum, P. verrucosum, P. solitum, P. allii* and *P. citrinum* could be isolated. In addition several strains of the non-characterized *Penicillium* sp. could be identified.

The interesting conclusion which can be deduced from these analyses is the fact that in most analysed fungal populations isolated from the surfaces of dry cured meat products, ochratoxin A producing species or species which at least carry the ochratoxin A genes occur. These species are predominately *P. nordicum, P. verrucosum, P. nalgiovense* and a non-characterized *Penicillium* sp. This is a strong indication that this special environment and the frequent occurrence of potential ochratoxin A producing species in this special environment have ecological reasons. In fact reports about the occurrence of ochratoxin A in dry cured ham or salted olives can be found increasingly in the recent literature (Chiavaro *et al.*, 2002; El Adlouni, Tozlovanu, Naman, Faid, & Pfohl-Leszkowicz, 2006; Ghitakou, Koutras, Kanellou, & Markaki, 2006; Pietri, Bertuzzi, Gualla, & Piava, 2006).

A biological understanding of the toxin biosynthesis and a profound knowledge about the conditions which favour ochratoxin A biosynthesis during the production processes would help to develop novel approaches to prevent its production.

Relationship between environment and ochratoxin biosynthesis

Ochratoxin A, like all other secondary metabolites of fungi is not permanently synthesised, but is regulated in response to the environment. Several parameters have a great influence on the amount of ochratoxin A produced, which can be in the range between high concentrations down to analytically non-detectable levels, despite the fact that growth of the fungus might not be influenced under these different conditions at all. The most important parameters in relation to this aspect are the substrate, the temperature and the water activity. With respect to ochratoxin A biosynthesis it could be shown that YES medium is a very supportive medium for ochratoxin A biosynthesis (Skrinjar & Dimic, 1992). This could be confirmed also for *Penicillium* strains isolated from dry cured meat. YES is a very protein and peptide rich medium which resembles the composition of the natural habitats of *P. nordicum* in this aspect. For this reason the production of ochratoxin A by *P. nordicum* strains isolated from meats was compared after growth on YES or meat extract medium. The high production on YES medium was even surpassed by the production on meat extract medium. This result clearly shows that *P. nordicum* strains are adapted to protein-, peptide and amino acid rich environments and that the natural substrate supports ochratoxin A biosynthesis. This induction may be due to the presence of certain amino acids or peptides (Häggblom & Ghosh, 1985). An analysis of the influence of amino acids on ochratoxin A biosynthesis by *P. nordicum* showed that some amino acids can drastically influence the biosynthesis of ochratoxin A. In our analysis especially small and simple amino acids like glycine, leucine, valine and serine have a strong boosting effect on ochratoxin A biosynthesis in minimal medium. Like in the work of Häggblom & Ghosh (1985) a positive effect of proline could be observed, too. Some nitrogen rich amino acids like histidine or arginine however drastically inhibit the biosynthesis, indicating the importance of these substrate compounds on toxin production.

The influence of temperature or water activity on growth and ochratoxin A biosynthesis at the phenotypical level has been described. Arroyo, Aldred, and Magan (2005) showed that *P. verrucosum* produced high amounts of ochratoxin A at pH 6 and an a_w of 0.93. Cairns-Fuller *et al.* (2005) analysed the interactive effects between a_w and temperature on the production of ochratoxin A by *P. verrucosum*. These authors found a minimal a_w for growth of 0.80. At these conditions no ochratoxin A could be produced any longer. A further analysis by Pardo, Malet, Marin, Sanchis, and Ramos (2006) confirmed these results. These authors also found a minimal a_w at 0.80–0.85 and a maximum a_w at 0.95–0.99 for the growth of *P. verrucosum*.

The described influences of these parameters are at the phenotypical level, thus they are endpoint determinations with respect to ochratoxin biosynthesis. This is in contrast to a transcriptomic approach in which the activity of the ochratoxin A biosynthesis genes are being monitored. Because the gene transcription always precedes phenotypic production, induction of the ochratoxin biosynthesis genes can be regarded as an early alert for toxin biosynthesis. Moreover, if the time window between gene activation and phenotypic toxin production is long enough, predictions can be made and counter active measures can be applied in the best case, before the toxin is produced. Obviously a close relationship between mycotoxin biosynthesis and gene activation exists. A signal from the external parameters to the gene regulation level is forwarded by the action of signalling cascade pathways. It has been shown that several signalling cascade pathways play a role in the activation of mycotoxin biosynthesis genes (Garcia-Rico *et al.*, 2008; Shwab & Keller, 2008), however in the case of increasing amounts of NaCl it can be expected that a HOG-like cascade (high osmolarity glycerol) is involved in signal transduction. HOG-like cascades involved in mycotoxin biosynthesis have been described for trichothecene biosynthesis in *Fusarium graminearum* (Ochiai *et al.*, 2007) or in the stress induced activation of fumonisin biosynthesis in *F. proliferatum* (Kohut *et al.*, 2009). For *P. nordicum* and *P. verrucosum* preliminary data also show, that a HOG-like cascade seems to play an important role in ochratoxin regulation under NaCl stress conditions. These external conditions are recognized by sensor molecules and the signal is transmitted to the gene level by MAP kinases (such as HOG) or heterotrimeric G-proteins, where it activates or represses gene function. Fig. 2 shows a scheme of this relationship. The unravelling of these relationships is currently a very active field of mycotoxin research and in fact MAP kinases from *P. nordicum* and *P. verrucosum* whose activity is related to the NaCl concentration and ochratoxin A biosynthesis have already been identified.

Systematic application of transcriptomic approaches to analyse the influence of temperature × water activity and development of an expression model

A prerequisite for the application of transcriptomic studies is the availability of the ochratoxin A biosynthesis genes. Within PathogenCombat the most important key genes of the ochratoxin A biosynthesis pathway have been identified (Fig. 1, Geisen, Schmidt-Heydt, & Karolewiez, 2006). The expression of one of these genes, the ochratoxin A polyketide synthase (*otapks*PN), is directly coupled to ochratoxin A biosynthesis (Geisen, Mayer, Karolewiez, & Färber, 2004). A high activation of the *otapks*PN gene could be found about 24 h before ochratoxin A could be detected, indicating the predictive nature of these kinds of data. This analysis has been done by Real Time PCR. For all other ochratoxin A biosynthesis genes Real Time PCR systems have been developed, too and differential expression kinetics for the different genes could be found (Geisen, Schmidt-Heydt, & Karolewiez, 2004). By using the transcriptomic approach systematically for the analysis of the influence of temperature and water activity

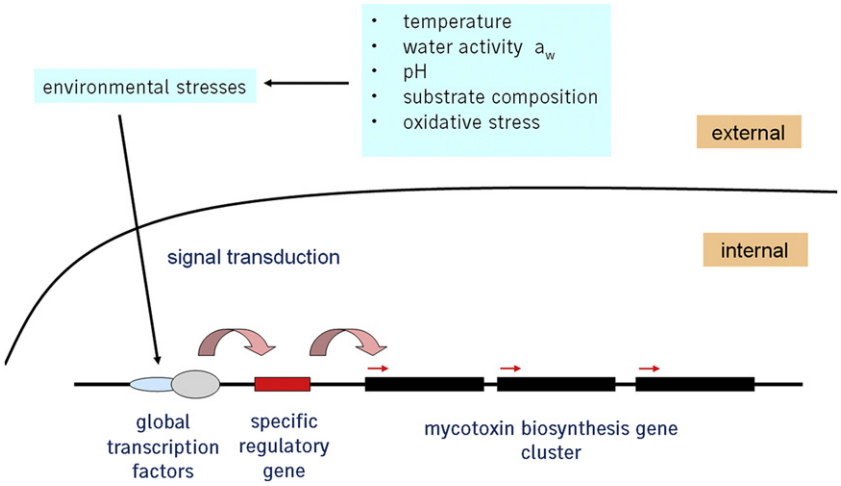

Fig. 2. Scheme of the influence of external stress parameters on the activation of mycotoxin biosynthesis genes via a signal transduction pathway. Generally global regulatory genes are influenced first, which than transmit the signal to more specialized transcription factors, which by itself activate the mycotoxin biosynthetic enzyme structural genes.

on ochratoxin A biosynthesis by *P. nordicum*, clear regularities in the expression of the ochratoxin biosynthesis genes became obvious. For both parameters temperature as well as water activity, the same general expression profile occurred. *P. nordicum* produces ochratoxin A to a high amount at conditions near the growth optimum (a_w 0.99, growth optimum a_w 0.98). However there is a second peak of gene induction at conditions where the growth of the fungus is greatly reduced (a_w 0.93). This second induction suggests that the activation of ochratoxin biosynthesis is stress regulated which could be confirmed in further analysis (Schmidt-Heydt *et al.*, 2008). These typical profiles could be found for all parameters tested (pH, a_w, temperature) suggesting that this is a general regulatory mechanism. It is known that HOG MAP kinase pathways can also be cross activated by other abiotic stresses (Adam, Kohut, & Hornok, 2008) and the described activation at marginal growth conditions might be transmitted via that pathway.

In a further development a microarray was constructed which carries all the biosynthesis genes of the ochratoxin A cluster and additional co-regulated genes (Schmidt-Heydt & Geisen, 2007) not further characterized, but also sub arrays from other known mycotoxin biosynthesis genes. This microarray enabled the monitoring of the transcriptional activation of the whole gene cluster in a more general way. Analysis with this microarray at different combinations of water activity × temperature revealed a very similar expression profile compared to the Real Time PCR data. The expression optimum of the cluster is also near, but not exactly at the parameter combinations for optimal growth and a second peak at the margins of growth could be identified (Fig. 3). The systematic generation of the data described, and the identification of regularities in the activation profile of the ochratoxin cluster enabled the prediction of ochratoxin A gene activation. This was demonstrated after analysis of the fungal population of dry cured ham by Real Time PCR of the *otapks*PN gene. A clear influence of the temperature on ochratoxin A gene expression and phenotypic ochratoxin A production could be shown as predicted before by the *in vitro* expression data. At a ripening stage where the temperature was 8 °C, which lies below the minor induction peak, only a very low copy number of the *otapks*PN transcript and a very low production of ochratoxin A could be observed. However, when the ripening temperature rose to 10–15 °C, which is exactly in the range of the minor gene induction peak, a clear gene activation and an increase in ochratoxin A biosynthesis was seen. This prediction approach has been further developed for the expression of the trichothecene genes of *Fusarium culmorum*, a plant pathogen which leads to the biosynthesis of trichothecenes in wheat. An algorithm was developed, which describes the relation between the expression of the trichothecene genes at various a_w × temperatures to the production of trichothecenes (Schmidt-Heydt, Parra, Geisen, & Magan, 2011). The mathematical predictions, of trichothecene biosynthesis, using this model fits very nicely to the analytical data. A similar model may also be developed from the microarray data generated for the activation of the ochratoxin A cluster.

Transcriptomics to analyse the influence of NaCl on ochratoxin a biosynthesis

Not only environmental parameters like temperature and water activity that are important, but also the substrate plays a role, may be even the major role in determining the amount of ochratoxin A produced. As discussed above NaCl rich substrates seem to determine ochratoxin biosynthesis by the respective fungi.

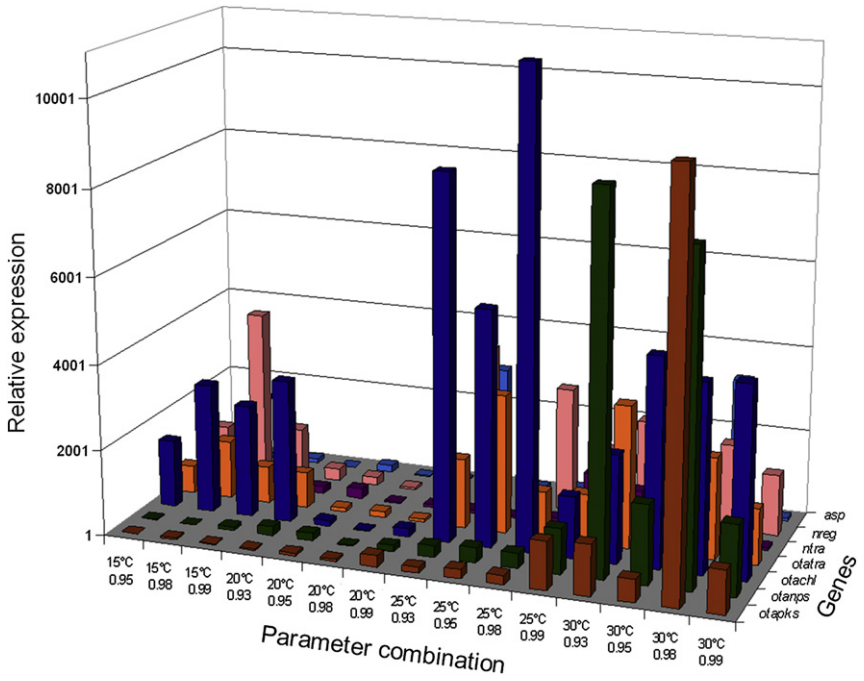

Fig. 3. Expression profile of the whole ochratoxin A gene cluster under various combinations of temperature × a_w. A major peak could be observed near optimal growth conditions and a minor peak at the margins of growth. The genes of the ochratoxin A biosynthetic pathway and associated genes are indicated: *otapks* = ochratoxin A polyketide synthase; *otanps* = ochratoxin A non-ribosomal peptide synthase, *otachl* = putative ochratoxin A chloroperoxidase; *otatra* = putative ochratoxin A transport protein, *asp* = alkaline serine protease and *ntra* = putative nitrate transport protein which are co-regulated with the other ochratoxin A biosynthesis genes and which were identified by differential display PCR.

An analysis whether different NaCl concentrations have an influence on gene activation and ochratoxin A biosynthesis has been carried out. *P. nordicum* was grown on YES medium with increasing concentrations of NaCl and the amount of ochratoxin A produced was followed by thin layer chromatography and HPLC. From the same samples a transcription analysis by Real Time PCR has been performed as well. Based on this analysis it could be shown, that the concentration of NaCl in the medium does indeed have a strong influence on ochratoxin A biosynthesis and gene activation. After an incubation of 7 days the highest production of ochratoxin A was in the range between 10 and 30 g/L NaCl, however after longer incubation, high amounts of ochratoxin A were also produced at high NaCl conditions. This situation again suggests an important influence of sodium chloride rich environments on the secondary metabolism of ochratoxin A producing Penicillia.

A mutant strain of *P. nordicum* which was not able to produce ochratoxin A at high NaCl concentrations showed a changed growth behaviour compared to the wild type. At low NaCl concentrations, at which the mutant still can produce ochratoxin A at reduced amounts, growth is comparable to the wild type strain. However at higher NaCl concentrations when the mutant was not able to produce ochratoxin A (>40 g/L), the growth was drastically reduced, whereas the wild type showed a quite constant growth over the concentration range between 0 and 100 g/L (with an optimum at 40 g/L).

In a growth experiment in which spore suspensions of either the *P. nordicum* wild type or the mutant together with *Alternaria alternata* were co-inoculated on medium containing different NaCl concentrations (Fig. 4) the competitiveness of *P. nordicum* could be demonstrated. *A. alternata* is a very fast growing species in contrast to *P. nordicum*. It can grow at higher NaCl concentrations, but it is not adapted to this environment. Each spore suspension was adjusted to the same concentration for inoculation. This experiment revealed that the fast growing *A. alternata* is more competitive than the *P. nordicum* mutant strain up to 40 g/L NaCl. In contrast the wild type of *P. nordicum* which produced much more ochratoxin A than the mutant already grew better than *A. alternata* at 20 g/L and especially at 40 g/L and could compete against this species and got predominant (Fig. 4). This indicates the ecological advantage of ochratoxin A producing species under these conditions.

Application of transcriptomics for the analysis of the molecular background of ochratoxin a biosynthesis

As mentioned above for each of the ochratoxin A biosynthesis genes Real Time PCR systems have been developed (Geisen *et al.*, 2006). These Real Time PCR systems have been used to analyse the expression kinetics of all putative genes over a time frame of 10 days. Interestingly the expression optima of the different genes occur consecutive in the order of the expected biochemical

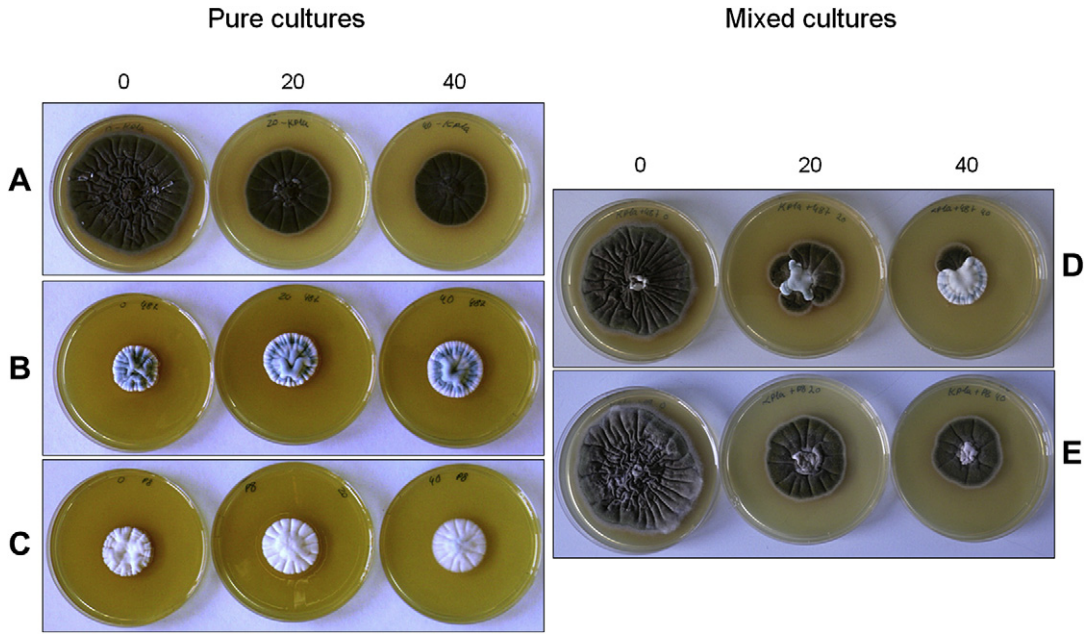

Fig. 4. Growth competition experiment between *A. alternata* and the wild type and mutant strains of *P. nordicum* on YES medium with increasing concentrations of NaCl. The numbers 0, 20 and 40 above the columns indicate the concentration of NaCl in the medium (g/L). Growth of the pure cultures on these increasing amounts of NaCl is seen on the left side of the picture: A = *A. alternata,* B = *P. nordicum* wild type and C = *P. nordicum* mutant. Growth of the mixed cultures is shown at the right side of the picture: *A. alternata* + *P. nordicum* wild type (D); *A. alternata* + *P. nordicum* mutant (E). For the growth experiment with the mixed cultures equal amounts of spores of both strains (10^5 spores/ml) were mixed, one point inoculated on YES agar plates and incubated for 7 days at 25 °C. The *P. nordicum* mutant produces much less ochratoxin A at NaCl concentrations between 0 and 40 g/L. This means that the suggested side effect of the biosynthesis of ochratoxin A, the excretion of chloride out of the cell maintaining chloride homeostasis, is reduced in the mutant. The wild type shows no changes in morphology at all NaCl concentrations analysed, however the mutant shows strongly reduced sporulation and a reduced density of the aerial mycelium at higher NaCl concentrations. As expected for a non-adapted species, *A. alternata* shows slower growth with increasing concentrations of NaCl. In the mixed cultures, the *P. nordicum* wild type is able to establish themselve against the fast growing *A. alternata* at much lower NaCl concentrations (20 g/L), compared to the mutant, indicating that the capacity to produce high amounts of ochratoxin A is coupled with competitiveness of the strain in a mixed culture under high NaCl conditions.

reactions: the polyketide synthase gene, the non-ribosomal peptide synthase, the putative chlorinating enzyme and the putative transport protein. This order of gene activations suggests a certain succession of enzymatic reactions in the biochemical pathway (Fig. 5). Interestingly the putative chlorinating activity and the transport activity seem to be at the very end of the pathway which looks reasonable. Ochratoxin B is non-toxic, also for the production organism itself (Schmidt-Heydt, Bode, Raupp, & Geisen, 2010), however ochratoxin A has toxic activity and seems to be immediately excreted out of the cell. It has been shown, that ochratoxin inhibits translation in eukaryotic (Höhler, 1998; Petzinger & Ziegler, 2000) as well as prokaryotic cells (Heller & Röschenthaler, 1978) by inhibiting the elongation of translation due to competitively inhibiting the amino acetylation of the phenylalanine-t-RNA synthases. Albeit no analysis of the toxicity of ochratoxin A against the production organism itself has been published, it seems likely that ochratoxin A is also toxic against *Penicillium* by the same mechanism. So this organisation of the ochratoxin A biosynthesis in *Penicillium* would ensure self protection of the production organism. However this mechanism leads to another important aspect. The continuous production and excretion of ochratoxin A leads to a continuous flow of chloride out of the cell, which in turn ensures some kind of chloride homeostasis within the cell, especially at higher chloride concentrations. It has been shown earlier, that the amount of ochratoxin A produced by *Penicillium* reaches a certain plateau after prolonged incubation, however this plateau is not static, but the amount of ochratoxin A produced, oscillates in a circadian manner (Schmidt-Heydt *et al*., 2010) around that plateau. Taking this fluctuation in the amount of ochratoxin molecules into account, the chloride excretion takes place continuously even in a stationary culture. This permanent excretion of ochratoxin A accounts for the ability to maintain chloride homeostasis even at high NaCl conditions. Preliminary comparison between the chloride content of the wild type mycelium and the mutant mycelium under high NaCl conditions show that the wild type, e.g. the strain which is able to produce ochratoxin A also under high NaCl conditions, is much more able to keep its cellular chloride content constant than the mutant, which does not produce ochratoxin A under high NaCl conditions. This suggests, that the increased chloride homeostasis, due to the biosynthesis of ochratoxin A of the wild type is the responsible mechanism for the adaptation of ochratoxin A producing Penicillia to high NaCl containing environments.

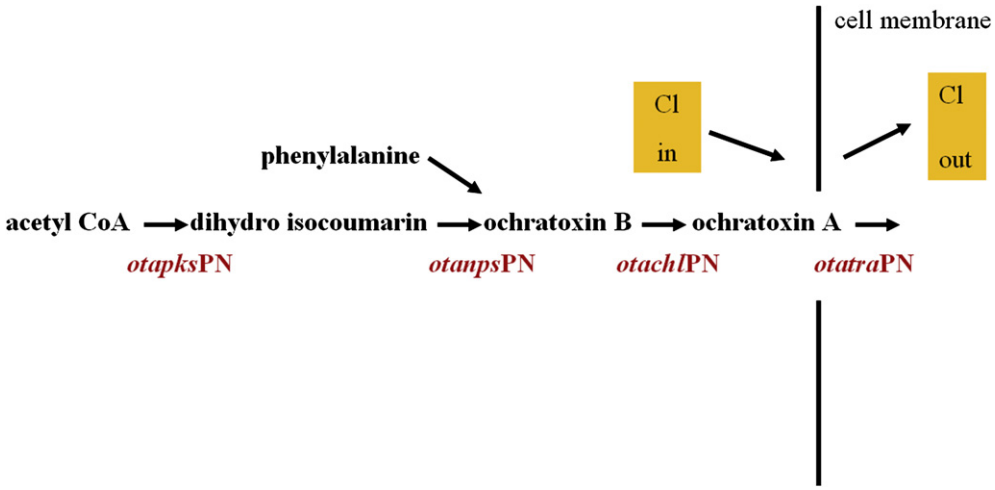

Fig. 5. Suggested pathway of ochratoxin A biosynthesis in *Penicillium*. The order of the reactions are deduced from the succession of the expression optima of the different genes. Chlorination and excretion seem to be at the very end of the ochratoxin A biosynthesis pathway, providing some kind of self protection mechanism. As discussed in the text the permanent production of ochratoxin A, even in a static culture has the effect that chloride is permanently excreted out of the cell.

It has to be kept in mind however that the production of mycotoxins can have several ecological functions (Roze, Chanda, & Linz, 2011) and to ensure chloride homeostasis in NaCl adapted Penicillia, might not be the only one. Aspergilli, which also can produce ochratoxin A, usually do not occur in dry cured foods. A strain of *A. carbonarius* for example was only able to produce ochratoxin A up to a concentration of 40 g/L, indicating the ecological reason for the production of ochratoxin A in *Aspergillus* might be another compared to *Penicillium*.

Conclusions

Ochratoxin A producing Penicillia can be found frequently in NaCl rich fermented foods. Several ochratoxin producing and putatively producing species have been isolated from this environment. The results demonstrated here suggest, that at least one reason for their occurrence in this habitat is the ochratoxin A production itself, which obviously ensures some kind of chloride homeostasis and increases the compatibility in this environment. Increasing NaCl concentrations but also the composition of the natural substrate (proteins, peptides amino acids) induces ochratoxin A biosynthesis which obviously lead to a better adaption to the dry cured food environment due to the chloride homeostasis.

However this maintenance of Cl^- homeostasis is apparently only one ecological reason for the biosynthesis of ochratoxin A. Ochratoxin A is also produced under conditions when the NaCl concentration is low, for example via cross activation by abiotic factors. Moreover ochratoxin A biosynthesis in *A. carbonarius* is not activated by increased NaCl conditions. There are also further hints that under certain environmental conditions which pose oxidative stress to the fungus much more ochratoxin B is produced. Ochratoxin B is non-toxic and contains a dehydroisocoumarin moiety. According to recent data (Fylaktakidou, Hadjipavlou-Litina, Litinas, & Nicolaides, 2004) coumarin derivates could serve as antioxidants, so ochratoxin B may have counteracting effects towards these oxidative stress reactions. Taken together ochratoxin A seems to be a multifunctional metabolite which seems to increase competitiveness under certain stress conditions.

From the food safety point of view these results are of course discouraging, because of the fact that these fungi are optimally adapted to this environment and hardly can be combated by changing the technology of food production, which was the assumption at the beginning of the Pathogen-Combat project. However in further research it was shown that light, a parameter neglected until now can have a very strong influence on ochratoxin A biosynthesis (Schmidt-Heydt *et al.*, 2010). The analysis of this influence is an object of "MycoRed", an ongoing EU project aiming at the reduction of mycotoxins in certain food chains.

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