DOI 10.1002/mnfr.200400036 Mol. Nutr. Food Res. 2004, *48*, 532 – 540

Molecular monitoring of environmental conditions influencing the induction of ochratoxin A biosynthesis genes in *Penicillium nordicum*

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A real-time polymerase chain reaction (PCR) system specific for the ochratoxin A polyketide synthase gene (*otapks*PN) of *Penicillium nordicum* has been used to analyze environmental conditions, influencing the induction of that key gene of the ochratoxin A biosynthetic pathway. Generally, the induction of that gene coincides very well with the biosynthesis of ochratoxin A, demonstrating that its induction can be used as a molecular signal to monitor ochratoxin A production. It could be shown, that the expression of the *otapks*PN gene is greatly dependent on the media used. In YES medium expression is highest, followed by minimal medium which support ochratoxin A production and minimal medium which suppresses ochratoxin A production. The amount of ochratoxin A produced shows the same tendency. The amount produced is highest on YES medium and decreases successively to the two minimal media. The system was also used to determine the influence of environmental parameters like temperature, pH and NaCl concentration on the expression of the *otapks*PN gene and on ochratoxin A production in parallel. It could be shown that under acidic conditions, below pH 5.0, the expression of the *otapks*PN gene as well as the ochratoxin A concentration were reduced. In case of salt concentration again both measures coincide, having both highest values at increasing NaCl concentrations. In case of the temperature, however, expression of the *otapks*PN gene was uncoupled to ochratoxin A production. The expression was high at all temperatures tested, however, clear differences in the biosynthesis of ochratoxin A by *P. nordicum* could be observed at the different temperatures, showing highest production at 25°C. The importance of these data are discussed with reference to the natural habitat of *P. nordicum.*

Keywords: Gene expression / Ochratoxin A / *Penicillium nordicum* / Polyketide synthase

Received: June 30, 2004; revised: August 19, 2004; accepted: August 24, 2004

1 Introduction

Ochratoxin A is an important mycotoxin with mainly nephrotoxic but also hepatotoxic, immunotoxic and teratogenic properties [1]. It is regarded as a class II carcinogen. Ochratoxin A was first described by van der Merwe *et al.* [2] isolated as a toxic compound from *Aspergillus ochraceus*. At present many fungal species are known to produce ochratoxin A. The most important are several species of the Aspergilli, in particular *A. ochraceus*, *A. carbonarius*, and *A. niger*. *A. niger* and *A. ochraceus* are the most frequently isolated ochratoxin A producing species from coffee [3, 4], whereas *A. carbonarius* seems to play the dominant role on grapes and dried wine fruits [5, 6]. In general ochratoxin A producing Aspergilli occure more frequently on products from regions of warmer climate. In contrast the only two species of the genus *Penicillium* known to produce ochratoxin A are *P. verrucosum* and *P. nordicum*. Both species are adapted to regions of moderate climate and occupy different habitats. *P. verrucosum* can nearly exclusively be isolated as a storage fungus responsible for the occurrence of ochratoxin A in cereals, whereas *P. nordicum* is adapted to the food environment and can be isolated as contaminant from proteinaceous foods like cheeses or fermented meats [7]. *P. nordicum* has recently been described as a new ochratoxin A producing species clearly separated from *P. verrucosum* at the chemotaxonomical [8] and the genetical level [9]. Generally, *P. verrucosum* is a moderate ochratoxin A producer. This species, however, is able to produce citrinin in addition, another important mycotoxin. *P. nordicum* in comparison is generally able to produce high amounts of ochratoxin, but no citrinin.

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Abbreviations: CCP, critical control point; **HACCP**, Hazard Analysis Critical Control Point; **YES**, yeast extract-sucrose-agar medium

Ochratoxin A is a mycotoxin derived form the polyketide pathway. It consists of an dehydroisocoumarin derivative and the amino acid phenylalanine. The dehydroisocoumarin moiety is chlorinated at position 5 in ochratoxin A. Ochratoxin B is the unchlorinated form and is much less toxic than ochratoxin A. Recently, a part of the gene cluster responsible for the biosynthesis of ochratoxin A has been identified from *P. nordicum* [10]. This gene cluster encompasses a gene for a putative non ribosomal peptide synthethase (*nps*PN, GeneBank accession number AY534879), obviously responsible for the ligation of the amino acid phenylalanine to the polyketide moiety and a gene for a polyketide synthase (*otapks*PN, GeneBank accession number AY196315) responsible for the biosynthesis of the dihydroisocoumarin part of the molecule. Based on the nucleotide sequence of the *otapks*PN gene, a real-time PCR system has been developed, which has been used to study gene expression of the *otapks*PN gene in a food environment. It has been shown that the *otapks*PN gene is induced during growth of *P. nordicum* in a food matrix. About 48 h after induction of this gene, ochratoxin A could be detected in the food matrix [11] indicating that the expression of the *otapks*PN gene might be used as a molecular indicator for the onset of ochratoxin A production.

In the current work, this system has been used to study the influence of environmental parameters, like growth substrate, temperature, pH, and NaCl concentration on the expression of the *otapks*PN gene and on ochratoxin A production by *P. nordicum*. The rationale behind this approach was the idea to determine the conditions which allow the induction of the ochratoxin biosynthesis genes as molecular critical control points (CCPs) to be used in a HACCP-like concept to minimize the occurrence of ochratoxin A in foods.

2 Materials and methods

2.1 Strains and culture conditions

The strain *P. nordicum* BFE487 (strain number of the culture collection of the Federal Research Center for Nutrition and Food) was used throughout this study. This strain produces very high amounts of ochratoxin A. The strain was routinely grown in/on malt extract medium/agar (17 g/L malt extract (Merck, Darmstadt, Germany), 5 g/L glucose, 15 g/L agar, pH 6.5). Incubation was at 25°C for 4 – 7 days. For ochratoxin A determination the strains were grown on YES medium (20 g/L yeast extract; 150 g/L sucrose; 20 g/L agar) or in minimal medium (3.8 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \times 7\ H_2O$, 0.1 g/L NaCl, 0.1 g/L $CaCl_2$, 0.7 g/L KOH, 15 g/L agar, pH 6.5) which supports ochratoxin A production (when supplemented with 5.0 g/L glycerol, 2.5 g/L NH_4Cl) or suppresses ochratoxin A production (when supplemented with 5.0 g/L glucose, 1.2 g/L KNO_3).

2.2 Quantitative determination of ochratoxin A by HPLC

For determination of ochratoxin A, *P. nordicum* BFE487 was grown on various media for a certain time period. An agar plug was taken from the colony with the aid of an steril corer. This agar plug with the adhering mycelium was extracted with 600 µL chloroform under extensive shaking for 20 min. After that time the mycelium and the agar plug were removed and the chloroform was evaporated to dryness. The residue was redissolved in methanol and subjected to HPLC according to the method described in the ISO 15141 standard (1998, www.iso.ch).

2.3 Isolation of total RNA

An amount of 0.5 g of the mycelium was frozen in liquid nitrogen and ground to powder in a mortar. This powder (200 mg) was used for isolation of total RNA. For that purpose, the E.Z.N.A. Fungal RNA kit (Peqlab, Erlangen, Germany) has been used according to the recommendations of the manufacturer. A volume of 80 µL of the RNA preparation was treated with 2 µL DNase I (2.5 Kunitz units/µL; Quiagen, Hilden, Germany) for degradation of traces of genomic DNA. The solution was incubated for 60 min at 37°C and subsequently for 10 min at 65°C to inactivate the DNase. An aliquot of the RNA was separated on an agarose gel to check the integrity of the RNA. The RNA gel was prepared as described by Sambrook and Russel [12]. Prior to further experiments, the RNA concentration for each sample was determined spectrophotometrically and brought to an identical concentration.

2.4 cDNA synthesis

For cDNA synthesis, 8 µL of the DNase I-treated total RNA were used along with the Omniscript Reverse Transcription kit (Qiagen). 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.

2.5 Real-time PCR

The real-time PCR reactions were performed in a GeneAmp 5700® Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). The TaqMan® system with two primers and an internal fluorescence-labelled probe 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/probe set had the following nucleotide

sequences: *otapkstaq*1, 5′-CACGGTTTGGAACACCA-CAAT-3′; *otapkstaq*2, 5′-TGAAGATCTCCCCCGCCT-3′; *otapksprobe* 5′-CGTACCAATCCCCATCCAGGGC-TC-3′ (labelled with the fluorescence marker FAM at the 5′-end and with TAMRA at the 3′-end). For the PCR reaction the TaqMan reagent kit (PE Applied Biosystems) was used according to the recommendations of the manufacturer. For each reaction, 1 μL of the DNA sample solution was mixed with 50 μL of the PCR stock solution containing 5 μL 10 × TaqMan™ buffer A, 7 μL 25 mM $MgCl_2$, 1 μL of each dNTP mixture (10 mM dATP, dCTP, dGTP, and 20 mM dUTP), 0.5 μL of the primers and probe (each 0.5 μM), 0.5 μL uracil-*N*-glycosylase (1 u/μL), 0.2 μL AmpliTaq Gold (5 u/μL), and 29.8 μL sterile deionized water. After an incubation of 2 min at 50°C to allow for uracil-*N*-glycosylase cleavage, AmpliTaq Gold polymerase was activated by an incubation step for 10 min at 95°C. All 35 PCR cycles were performed according to the following temperature regime: 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s. To generate the standard curve, a larger PCR fragment of the *otapks*PN gene with the primer *otapks*1 and *otapks*2 as template was used [11]. The concentration of this standard PCR product was determined in a fluorometer (DyNa Quant 200; Pharmacia, Uppsala, Sweden) and the number of copies were 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.

3 Results

3.1 Kinetics of *otapks*PN expression and ochratoxin A production in YES medium

To get information about the kinetics of the expression of the *otapks*PN gene and the biosynthesis of ochratoxin A, an expression analysis and an analysis of the produced ochratoxin A have been performed during a 10 (ochratoxin production) to 19 day (*otapks*PN gene expression) incubation period. The purpose of these experiments was the identification of the time point under which the *otapks*PN gene is actively expressed. YES medium has been used as a model medium for all other growth conditions.

P. nordicum BFE487 was inoculated in YES medium. Generally, YES medium is the medium which supports ochratoxin A production most [9]. At certain time intervals samples were withdrawn, and the expression of the *otapks*PN gene was quantified by real-time PCR, whereas the ochratoxin A produced was quantified by HPLC. The results are shown in Fig. 1. The first detectable ochratoxin A could be determined after 3 days of incubation. The amount of ochratoxin A increased from day 3 up to day 9. The induction of the *otapks*PN gene on the other hand could be determined 24 h before the first ochratoxin A appeared. The gene showed an increased expression for nine days also. After that time, the determined amount of mRNA decreased rapidly several orders of magnitude and stayed on that very low expression level for the whole observation time. Interestingly, the induction of the *otapks*PN gene showed repeatedly two peaks. One peak at day 5 and another peak between day 7 and day 8. This result corresponds very per-

Table 1. Quantitative data of ochratoxin A production and *otapks*PN gene expression by *P. nordicum* under different conditions

Parameter	Ochratoxin A[a)]	*otapks*PN[b)]
pH		
4.0	4658	693
5.0	10978	2931
6.0	16908	48414
8.0	15965	26801
NaCl concentration (g/L)		
0	16479	140174
10	17620	240586
20	20418	1115288
40	18654	nd
50	14100	nd
Temperature (°C)		
15	77	144506
25	22263	140110
30	2720	88391
Medium		
YES	20398	54067
MM + glycerol/ ammonium	678	3188
MM + glycerol/ nitrate	168	1056
Kinetics (*d*)		
3	4830	5629
4	14315	44947
5	20398	66507
6	18940	36391
7	28740	906049
8	25545	964316
9	30103	640081
10	18475	527769

a) ng/agar plug
b) Copy number of the *otapks*PN cDNA per reaction. For each parameter the expression data are independent and cannot be compared to another parameter as the quantitative correlation between expression and ochratoxin A production has not been determined. The data are relative.

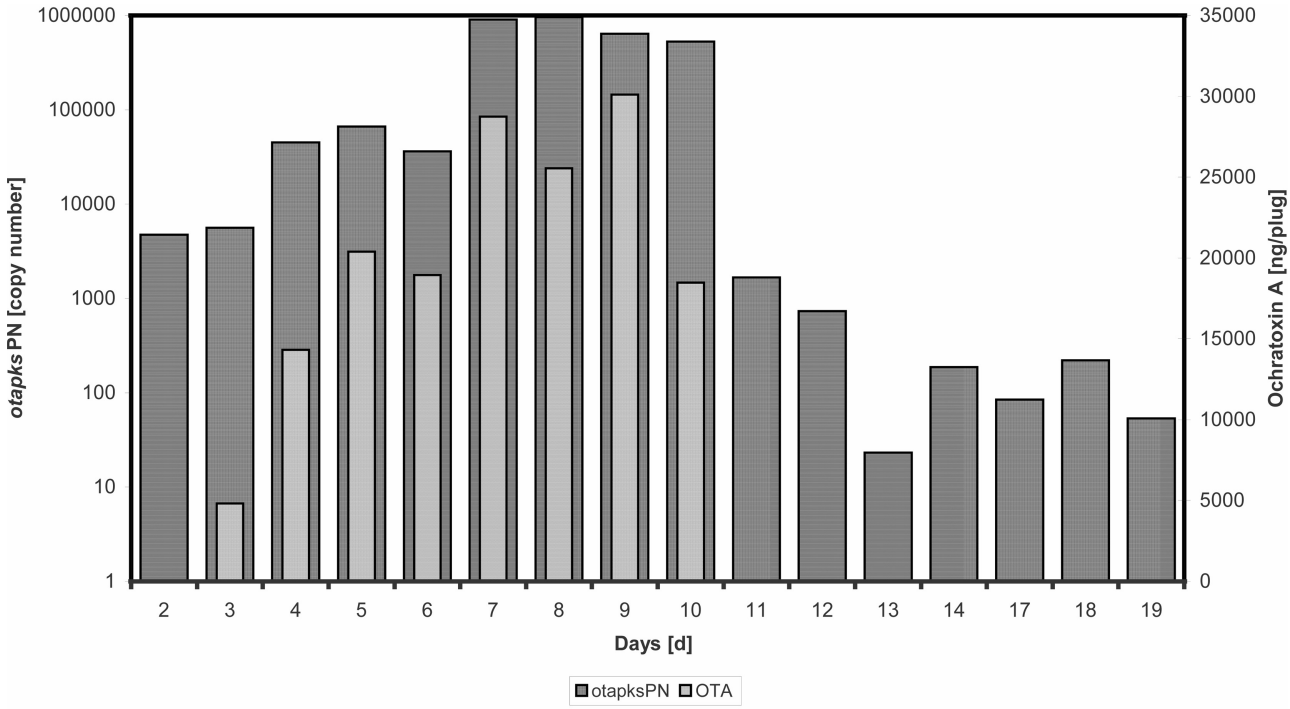

Figure 1. Expression kinetics of the *otapks*PN gene (shaded) compared to the ochratoxin A production kinetics (hatched) of *P. nordicum* BFE487. The ochratoxin A production kinetics was only monitored until day 10.

fectly with the ochratoxin A production data (Table 1, Fig. 1). The biosynthesis of ochratoxin A by *P. nordicum* BFE487 increased from day 3 to day 5, stayed constant until day 6, and again increased until day 9. The results demonstrate the correlation between the expression of the *otapks*PN gene and ochratoxin A biosynthesis during growth of *P. nordicum*. All subsequent analyses concerning the influence of environmental parameters on expression of the *otapks*PN gene have been performed during the first peak after 5 days of incubation.

3.2 Expression of the *otapks*PN gene and ochratoxin A production in different media

To demonstrate the influence of medium composition upon ochratoxin A production by *P. nordicum* at the phenotypic and molecular level the real-time PCR system has been used to study *otapks*PN gene expression and ochratoxin A production on different media. YES medium has been used as a medium which strongly supports ochratoxin A production by *P. nordicum*. YES medium is a rich medium, containing yeast extract as a source of vitamins and peptides. The two minimal media used are of nearly the same composition. The only differences are the carbon- and nitrogen sources. The minimal medium which supports ochratoxin A production contains glycerol as carbon- and NH_4^- as nitrogen source. The minimal medium which suppresses ochratoxin A production contains glucose as carbon- and NO_3^- as nitrogen source. *P. nordicum* BFE487 has been grown on these media for 5 days. After that time the mycelium was harvested, the total mRNA was prepared and subjected to RT real-time PCR. The amount of ochratoxin A produced has been determined in parallel by HPLC. The results are shown in Fig. 2. As expected, the production of ochratoxin A by *P. nordicum* is highest in YES medium, followed by minimal medium with glycerol/NH_4^- and minimal medium with glucose/NO_3^- (Table 1). The expression of the *otapks*PN gene of these cultures showed the same tendency indicating that ochratoxin A production and expression of the *otapks*PN gene is coupled under the observed conditions.

3.3 Expression of the *otapks*PN gene and ochratoxin A production by *P. nordicum* at different parameters

To determine the influence of environmental parameters like temperature, pH or salt concentration upon ochratoxin A production at the phenotypic and molecluar level the same experimental design as above was applied. *P. nordicum* BFE487 was grown in YES medium with different pH values or adjusted to different concentrations of NaCl or incubated at different temperatures. In all experiments, only the influence of a single parameter and no combinations of

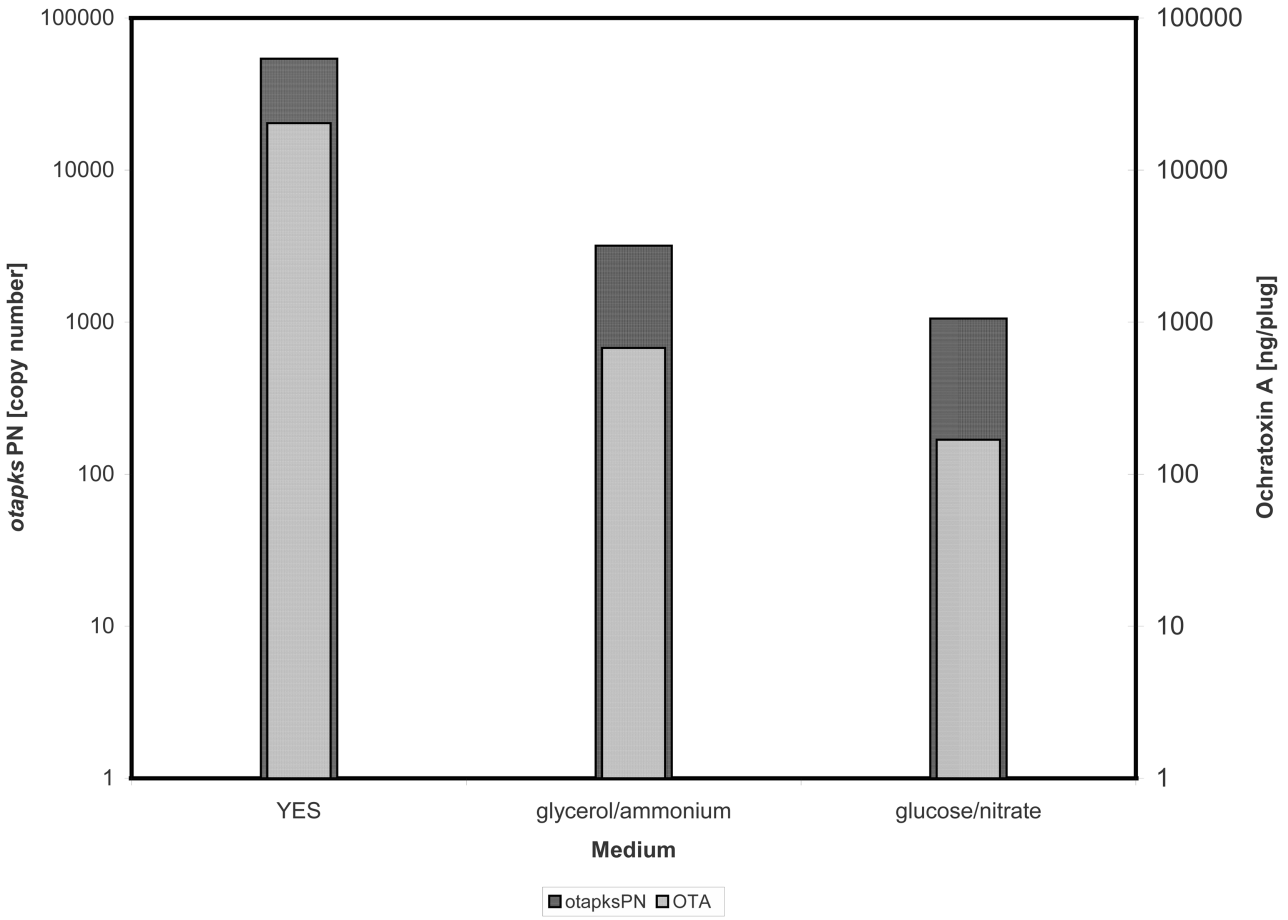

Figure 2. Expression of the *otapks*PN gene (shaded) and ochratoxin A production (hatched) by *P. nordicum* BFE487 after incubation on different media for 5 days at 25°C.

parameters have been analyzed. The transcription of the *otapks*PN gene has been determined by RT real-time PCR and the production of ochratoxin A by HPLC. Figure 3 and Table 1 show the result of this analysis. At the phenotypic level obviously near neutral pH conditions favors the synthesis of ochratoxin A. At more acidic conditions (pH 4) the biosynthesis decreased. This is completely reflected in the expression behavior of the *otapks*PN gene. This gene also shows highest expression around neutral conditions (between pH 6 and 8). At lower pH (pH 4) expression is strongly reduced. The same coincidence could be observed in the case of NaCl concentration as environmental parameter. Highest ochratoxin A production could be demonstrated at a concentration of 20 g/L (Table 1). Up to that point, the expression of the *otapks*PN gene increased steadily, indicating that a certain concentration of NaCl in the medium supports ochratoxin A production. The only parameter which showed no direct correlation between the phenotypic production of ochratoxin A and the molecular expression of the *otapks*PN gene was the temperature. The amounts of ochratoxin A synthesized at the different temperatures differed considerably. It could be clearly demonstrated that 25°C is the optimal temperature for ochratoxin A production among the temperatures analyzed (Table 1). With both other temperatures the amount of ochratoxin A synthesized was drastically reduced. These clear differences were not mirrored in the molecular data. In fact, the expression levels of the *otapks*PN gene under unfavorable conditions (15°C, 30°C) are roughly the same than at optimal conditions (25°C) indicating an upregulation of the *otapks*PN gene under these conditions.

4 Discussion

It was recently shown that *P. nordicum* is besides *P. verrucosum* a second ochratoxin A-producing species within the genus *Penicillium* [8, 9]. Both species are related but occupy different habitats. *P. verrucosum* can predominantly be isolated from cereals, whereas *P. nordicum* is adapted to proteinacious foods like cheeses or fermented meats [7]. It is well-known that the production of mycotoxins is depend-

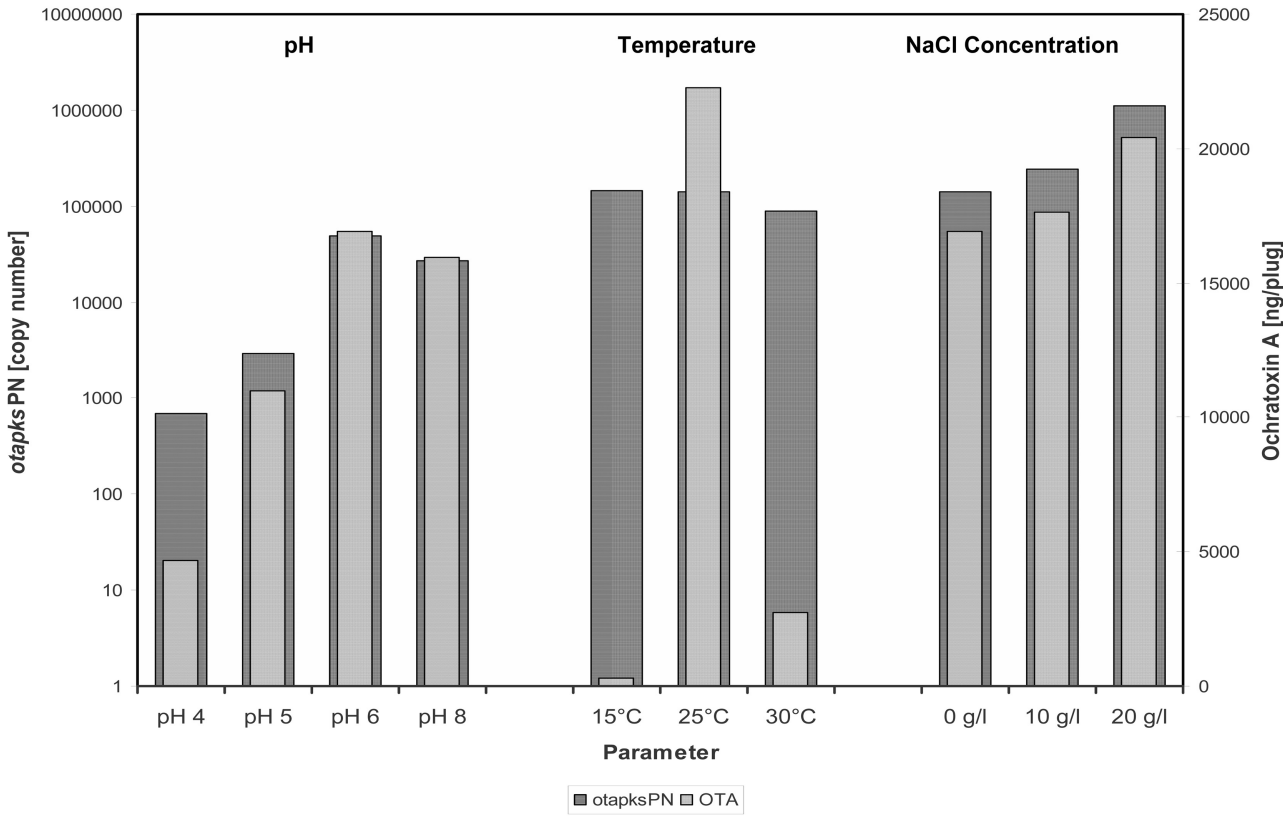

Figure 3. Expression of the *otapks*PN gene (shaded) and ochratoxin A production (hatched) by *P. nordicum* BFE487 after incubation on YES medium for 5 days at different parameters.

ent on the growth substrate and the environmental conditions [13 – 15]. Generally, the parameter window for growth is much broader than for mycotoxin production. This observation is due to the fact that the genes which are responsible for mycotoxin production are regulated [15, 16] and induced only under certain conditions. Up to now, the physiological conditions which support ochratoxin production have been determined only at the phenotypical level, *e. g.*, by analytical determination of the mycotoxin produced at certain growth conditions [17 – 19]. This approach, however, is a monitoring of the situation after the biosynthesis of mycotoxins, whereas the moleclular data give direct information about the environmental conditions which enables the induction of the genes before or during the biosynthesis of mycotoxins. The exact knowledge of the environmental conditions which allows induction of mycotoxin biosynthetic genes can be regarded as molecular CCPs with respect to a HACCP concept to reduce mycotoxin contamination. If these molecular CCPs are known, measures can be adapted to prevent or minimize induction of mycotoxin biosynthetic genes.

Until now only a few systematical approaches to analyze the physiological conditions for ochratoxin A production in *Penicillium* has been published [19 – 22] dealing all with *P. verrucosum* as the main ochratoxin A-producing species in cereals. Nothing, however, is published about the environmental conditions which enable ochratoxin production in *P. nordicum*. Recently, a real-time PCR system, based on the ochratoxin A polyketide synthase gene, has been developed and applied for the detection of *P. nordicum* and for monitoring the expression of the *otapks*PN gene in a food system [11]. This system has been used to determine the environmental conditions which enable the induction of the *otapks*PN gene and thereby the production of ochratoxin A. During the analysis of the kinetics of the induction of the *otapks*PN gene it became obvious that there is a difference in the expression of this gene in wheat [11] (wheat is not the natural habitat, but was used as a model food system in the previous study) and in YES medium. As expected, the transcription of the *otapks*PN gene can be detected much more earlier in YES medium (2 days) than in wheat (4 days). Under both conditions, the induction of the *otapks*PN gene can be detected earlier than ochratoxin A. The gene is induced until day 9 in both cases. After that time, the transcription level is reduced slowly in the case of YES medium but very rapidly in wheat. Interestingly, the expression kinetics of the *otapks*PN gene seems to have two maxima in

YES medium, one at day 5 and a second at day 8. This is completely reflected in the production kinetics of ochratoxin A by *P. nordicum*. Until day 5 there is a steady increase in the ochratoxin A concentration. After that incubation time, the amount remained constant until day 7 and then the concentration increased again until day 9. The reason for the decrease of the ochratoxin A concentration at day 10 is not clear. With respect to the two-phase induction of the *otapks*PN gene it is possible, that *P. nordicum* undergoes some kind of diauxic growth in YES medium using a certain nutrient in the first phase and after complete utilization another nutrient in the second phase. Both nutrients may support ochratoxin A biosyntheses differently. This behavior was not observed for *otapks*PN expression and ochratoxin A biosynthesis in wheat [11].

It has been shown previously that the production of ochratoxin A is strongly dependent on the medium used [22]. These authors demonstrated that the production is best on YES medium, a complete medium containing yeast extract. During the present analysis this finding could be confirmed. *P. nordicum* BFE487 produced the highest amounts of ochratoxin A on YES medium. In parallel, the expression of the *otapks*PN gene was much higher compared to the minimal media. The minimal media have been used earlier for the analysis of ochratoxin A production by *P. nordicum* [23]. It has been shown during that study, that a replacement of the carbon and nitrogen sources can trigger the activation or repression of the ochratoxin biosynthetic genes. The nutrient combination glycerol/NH_4^- supports ochratoxin A production, whereas the nutrient combination glucose/NO_3^- inhibits ochratoxin A production. This finding could be repeated during the present experiments and the molecular background of this characteristic could be demonstrated. *P. nordicum* grown on glucose/NO_3^- medium showed a very low remaining expression level of the *otapks*PN gene, whereas the expression increased by a factor of 3 in glycerol/NH_4^+ medium. The production of ochratoxin A at the phenotypical level is also different by a factor of about 3 between both media, indicating coincidence between the molecular and phenotypical data. The results clearly demonstrate that glucose has a repressive and ammonium a supporting effect on ochratoxin A biosynthesis. It has been shown also for other mycotoxins, that both nutrients have a regulatory effect upon production. Luchese and Harrigan [24] reported that glucose and glycerol are excellent carbon sources for growth and aflatoxin production by *A. parasiticus* which is contrary to the situation of ochratoxin A. Inhibition of synthesis of penicillin by glucose was also observed for *A. nidulans* [25], indicating that glucose indeed has an influence on the secondary metabolite biosynthesis. On the other hand, regulation by the nitrogen source seems to be similar for aflatoxin and ochratoxin A biosynthesis. Aflatoxin production by *A. flavus* and *A. parasiticus* is also reduced after growth in nitrate-based medium and increased in ammonium salt-based medium [16].

The influence of the environmental parameters temperature, pH, and NaCl concentration on phenotypic production of ochratoxin A and expression differs. Variations in the salt concentration have a relatively moderate influence on the biosynthesis of ochratoxin A. Between 10 and 50 g/L NaCl the amount of ochratoxin A synthesized is in the same order of magnitude, however, with an optimum at 20 g/L (Table 1). Interestingly, the expression of the *otapks*PN gene shows the same tendency. The expression increases from media without added NaCl to media containing up to 20 g/L NaCl. *P. nordicum* is adapted to the food environment like cheeses and fermented meats [7]. Both foods contain relatively high concentrations of salt. During the ripening processes of meats and cheeses, the water content reduces due to drying, and thereby increases the concentration of sodium chloride. Because of its adaptation *P. nordicum* tolerates and even prefers a certain concentration of salt in the medium. This species shows a faster growth rate if some NaCl is added to the medium. The same observation was described for *P. roqueforti* [26]. *P. roqueforti* also adapted to the cheese environment shows a growth stimulation with increasing NaCl concentrations. These facts indicate that certain concentrations of NaCl activate the overal metabolism but also the secondary metabolism of *P. nordicum*.

Between pH 5 and pH 8 the influence of pH as controlling parameter upon ochratoxin A production is not very pronounced. Only at pH 4 a strong decrease in ochratoxin A biosynthesis could be observed. The expression behavior of the *otapks*PN gene coincides with the phenotype. Highest expression could be observed in the moderate pH range between pH 6 and 8. According to these results it seems that the ochratoxin A biosynthetic genes are upregulated under rather neutral conditions and downregulated at acidic conditions. Generally, pH regulation in filamentous fungi is carried out by a set of regulatory genes acting as transcription factors (*pac*C, *pal*A-I) thereby binding to the promotor regions of the regulated genes [27]. If the *otapks*PN gene also carries a *pac*C binding site has to be shown in future analysis. In contrast to the results with the *otapks*PN gene Keller *et al.* [28] showed that the sterigmatocystin and aflatoxin biosynthetic genes in *A. parasiticus* and *A. niger* are more highly expressed under acidic conditions (pH 4–6) than under neutral to alkaline conditions (pH 7–8).

Temperature was the parameter with the strongest influence on ochratoxin A production. Under the conditions used 25°C was the temperature which supports ochratoxin A production most. At both other two temperatures ochratoxin A biosynthesis was reduced by either one (30°C) or two (15°C) orders of magnitude (Table 1). In contrast to all

other parameters, the temperature apparently had much less influence on the expression of the *otapks*PN gene, resulting in an apparent uncoupling of the expression of the *otapks*PN from ochratoxin A production. The reason for this expression behavior is not clear. It has been shown earlier by gene inactivation experiments [10] that the *otapks*PN gene is indeed involved in the biosynthesis of ochratoxin A. For this reason, it might be speculated that the fungus tries to counteract against the unfavorable conditions for ochratoxin A production by a strong induction of the ochratoxin A biosynthesis genes. However, in this case the reduction of ochratoxin A biosynthesis under nonoptimal temperature conditions must be regulated at another point of the biosynthetic pathway. The results obtained during this study are in good agreement with the results of Häggblom [19]. He found that *P. viridicatum* (which must be *P. verrucosum* from the current taxonomical view) produced most ochratoxin A on wheat at 25°C but only 2.5% of that amount at 10°C.

In this study, a molecular monitoring method has been used to evaluate the conditions of ochratoxin A production by *P. nordicum* at different parameters important for its natural habitat. For all parameters, except the temperature, the expression of the *otapks*PN gene and ochratoxin A production corresponds, indicating that the molecular data can be used under these circumstances to monitor the metabolic activity of *P. nordicum* with respect to ochratoxin A biosynthesis. *P. nordicum* is a contaminating organism of foods like cheeses and fermented meats. Until now, nothing is published if *P. nordicum* is able to produce ochratoxin A under the production or storage conditions of these food commodities. According to the current data, some of the conditions in these respective food commodities allow the expression of ochratoxin biosynthesis genes. Both food commodities represent a rich substrate, with high concentrations of proteins and oligopeptides. In that respect the situation in both substrates can be compared with YES medium as a complete medium. In addition, fungi have a strong proteolytic activity [29], being able to degrade proteins completely up to ammonia to use it as a nitrogen source. As was demonstrated in the present study, the presence of ammonia can activate ochratoxin A biosynthetic genes.

The salt concentrations in both products are in a range supportive for the expression of the *otapks*PN gene suggesting that ochratoxin A production might be possible under these conditions. The pH in fermented meat products is usually in the range between 5 and 5.5 due to the lactic acid produced from glucose by the accompanying lactic acid bacteria. If the pH could be maintained or better reduced, it would be suboptimal for the induction of the *otapks*PN gene according to the described results and would constitute a controlling action at a molecular CCP. The most important parameter with respect to possible ochratoxin A biosynthesis is the temperature. The ripening temperature for fermented meats is usually higher at the first days (up to 22°C) but is than reduced down to 12°C. According to the results obtained, fermentation at this temperature should inhibit ochratoxin A production, albeit at another point in the biosynthetic pathway than at the *otapks*PN gene.

Taken together and according to the results described here, the environmental conditions which allow the induction of ochratoxin A biosynthetic genes, are in the range of the production and storage conditions of the fermented meat products. However, in order to minimize the activation of these genes of *P. nordicum* as a contaminating microorganism for certain fermented meat-type foods, the salt concentration should be minimized, the pH should be kept at values as low as possible, and the temperature should be kept as low as possible.

This work was supported by the EU Project QLK1-CT-1999-01380 (European Commission, Quality of Life and Management of Living Resources Programme (QOL), Key Action 1 on Food, Nutrition and Health, Contract No. QLK1-CT-1999-01380). I would like to thank Nicole Mischke for skillful technical assistance and Marianne Urbanek for the HPLC analysis.

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