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P. nalgiovense carries a gene which is homologous to the *paf* gene of *P. chrysogenum* which codes for an antifungal peptide

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Abstract

Penicillium nalgiovense is related to *P. chrysogenum*. *P. chrysogenum* carries the *paf* gene (*Penicillium* antifungal peptide) with homology to the *afp* gene of *Aspergillus giganteus*. This gene codes for a peptide with antifungal activity. Based on the sequence of the published *P. chrysogenum paf* gene primers were generated. By the use of these primers a PCR product of the expected length could be isolated from strains of *P. nalgiovense*. This fragment was sequenced and compared to the sequence of the *paf* gene of *P. chrysogenum*. According to the results *P. nalgiovense* carries a gene (*naf* = *P. nalgiovense* antifungal peptide) which is highly homologous to the *paf* gene of *P. chrysogenum*. The gene also codes for a preproprotein with the same processing sites as the *paf* gene, suggesting that the mature product is also a 55 amino acid (aa) peptide. The *naf* gene has three amino acid exchanges compared to the *paf* gene, which however do not influence the amino acid sequence of the mature peptide. It also carries two introns at the same positions, however, the sequence differences between the introns are higher than between the coding regions. When *P. nalgiovense* were grown on plates containing other food-relevant fungi it showed weak antifungal activity. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Penicillium nalgiovense; Fungal starter culture; Inhibitory peptide; Antagonism

1. Introduction

Penicillium nalgiovense is a fungal starter culture for the production of mould fermented foods. Due to its lipolytic and proteolytic activity and because of the production of volatiles it contributes to the formation of flavour of these type of foods (Ramirez et al., 1995; Jacobsen and Hinrichsen, 1997). During the production of mould fermented foods, the environmental conditions in the ripening chambers are favourable for mould growth. If non-starter fungal strains are present, with higher growth rates than the fungal starter culture a malfermentation can occur leading to spoilage of the product and thereby to economical losses. The presence of undesired strains can not only lead to discolouration or to a negative flavour of the product (Lund et al., 1995), but also potentially to mycotoxin production. Many fungi are able to produce mycotoxins (Frisvad, 1988). Due to this situation fungal fermentations do not always

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result in high quality products. For this reason fungal starter cultures with antagonistic activity against spoilage and toxinogenic moulds would improve the microbial safety of the product.

P. camemberti strains with antibacterial activity have been described by Larsen and Knöchel (1997). Recently Nielsen et al. (1998) described the antifungal activity of species used as fungal starter cultures against spoilage moulds of cheeses. Some of the fungal starter species exhibited antifungal activity. *Geotrichum candidum* showed competition against undesirable fungi in mould fermented cheeses. *P. nalgiovense* showed the strongest inhibitory effect among the *Penicillium* species tested. In particular it had the greatest influence on the reduction of secondary metabolite production of fungal contaminants.

Recently Marx et al. (1995) described an antifungal peptide, which is produced by *P. chrysogenum*. *P. chrysogenum* is related to *P. nalgiovense* as has been shown by enzymological (Banke et al., 1997) as well as molecular data (Geisen, 1996). Because of the taxonomic relatedness of *P. nalgiovense* with *P. chrysogenum* it was possible, that *P. nalgiovense* carries a gene similar to the *paf* gene (Marx et al., 1995) of *P. chrysogenum*. In this work the cloning and sequencing of a gene from *P. nalgiovense* similar to the *paf* gene is described. This gene might be due to the antifungal activity of *P. nalgiovense* described above.

2. Materials and methods

2.1. Strains and culture conditions

P. nalgiovense strains BFE66, BFE67 and BFE474 were used throughout the study. They were routinely grown either in malt extract medium (1.05397.0500, Merck, Darmstadt, Germany) or agar plates at 25°C. The indicator organisms used in this study are indicated in Table 1. Inhibition experiments were also performed on malt extract plates adjusted to pH 4.5.

2.2. Isolation of fungal DNA

The isolation of DNA from fungal strains was performed according to a modified method originally described by Yelton et al. (1984). For that purpose 72–96 h old mycelium was harvested from a submersed culture by filtration. The mycelium was transferred to a mortar and frozen in liquid nitrogen. The frozen mycelium was ground and then resuspended in lysis buffer (50 mM EDTA; 0.2% SDS; pH 8.5). This suspension was heated to 68°C for 15 min and then centrifuged for 15 min at $15\,000 \times g$. After centrifugation a volume of 7 ml of the supernatant was transferred to a new centrifuge tube and 1 ml of 4 M sodium acetate was added. This solution was placed on ice for 1 h and centrifuged for 15 min at $15\,000 \times g$. After centrifugation 6 ml of the

Table 1
Inhibition spectrum of *Penicillium nalgiovense*

Indicator strain	*P. nalgiovense* BFE66	*P. nalgiovense* BFE67
Penicillium roqueforti BFE62	++ [a]	+++
Penicillium digitatum BFE152	+	++
Penicillium italicum BFE53	+	+
Aspergillus flavus BFE96	++	+
Mucor sp. BFE206	++	+
Byssochlamys sp. BFE218	+	nd
Geotrichum candidum BFE221	++	nd
Fusarium solani BFE325	−	−
Endomyces lactis	−	−
Zygosaccharomyces rouxii	−	−
Debaryomyces hansenii	−	−
Pichia anomala	−	−

[a] −, No inhibitory activity; +, ++, +++, weak, moderate and strong inhibitory activity based on visual estimation of the area of the inhibition zone.

supernatant was transferred to a fresh tube. The solution was phenol extracted and the isolated DNA was precipitated by the addition of 2.5 volumes of ethanol.

2.3. PCR reactions

PCR reactions for the amplification of the DNA fragment carrying the *naf* gene were performed with primers *paf4* and *paf5*, which were homologous to the 5′ and 3′ end of the published DNA sequence carrying the *paf* gene from *P. chrysogenum*. The primers had the following sequences and covered the region from nucleotide −432 to nucleotide 654 according the published sequence of the *paf* gene (Marx et al., 1995):

*paf*4: CCGAGTGGCCCAGTCACCGTGTC

*paf*5: ACAACCATTTCGTTGATGAGCAAC

The PCR were performed according to the following temperature regime: 95°C, 30 s; 50°C, 30 s; 72°C 60 s; 30 cycles. A typical PCR reaction mixture contained: 5 pg template DNA, 5 μl Taq polymerase buffer (supplied by the manufacturer), 1 μl Taq-polymerase (5 units/μl, 27-0799-01, Pharmacia, Uppsala, Sweden) 8 μl of a mixture of all four nucleotides (each 2.5 mM), 1.25 μl primer (120 pmol/ml, each), 25 μl H_2O.

2.4. Sequencing reactions

For sequencing the 1.3 kb PCR fragment generated with primers *paf4* and *paf5*, template DNA from *P. nalgiovense* was separated from nucleotides by agarose gel electrophoresis. The respective bands were cut out and isolated with the Gene-Out™ Kit from Fischer Scientific (017600, Schwerte, Germany). An amount of 1.5 μg of DNA were used for the sequencing reaction. For the reaction the Cycle Sequencing Kit from Pharmacia (27-1694-01, Uppsala, Sweden) was used according to the suggestions of the manufacturer with the following temperature regime: 95°C, 60 s; 65°C, 30 s; 72°C, 60 s, 45 cycles.

After the reaction the PCR products were heated to 95°C for 5 min and loaded onto a 7% polyacrylamide urea gel according to Sambrook et al. (1989). After electrophoresis the gel was stained with the Silver Sequence™ DNA Staining Reagents of Promega (Q4132, Madison, USA) according to the suggestions of the manufacturer.

For the sequence strategy the walking primer approach has been used, meaning that from the determined sequence new primers were deduced for the next sequencing reaction.

For analysis of the sequence the software DNASIS (version 2.1, Hitachi Software, Yokohama, Japan) was used.

2.5. Southern blotting

Southern blotting was performed essentially as described in Sambrook et al. (1989). The DNA fragment which was used as a probe for the hybridization reaction, was labelled during a PCR reaction. The composition of that PCR reaction mix was identical to the one described above with the following exceptions: instead of the conventional nucleotide mixture the PCR DIG Probe Synthesis Kit (1 636 090, Boehringer, Mannheim, Germany) was used as substrate for polymerisation. The primers *paf4* and *paf5* were used. The digoxigenin labelled nucleotide is incorporated directly into the PCR product during PCR reaction. As template DNA the chromosomal DNA of *P. nalgiovense* BFE67 was used. After hybridization of the probe and binding of the antibody against digoxigenin the signals were visualized by alkaline phosphatase reaction according to the protocols of the DIG-High Prime Labeling and Detection Starter Kit (1 745 832, Boehringer, Mannheim, Germany).

2.6. Determination of inhibition spectrum

To test the inhibitory activity of *P. nalgiovense* against different fungi a plate inhibition assay was carried out. For that purpose a *naf* producing *P. nalgiovense* strain was grown on malt extract agar for 4 days. With a sterile corer a plug from the actively growing part of the colony was cut out and transferred onto a fresh agar plate. Molten malt extract agar, cooled to 50°C was mixed with spores of the indicator strain and poured onto this agar plate up to the height of the agar plug with the mycelium of *P. nalgiovense*. All media were adjusted to pH 4.5.

It was ensured that the mycelium of *P. nalgiovense* was not covered with agar. The plates were incubated at 25°C and inspected after 24 h for growth of the indicator strains. The intensity of the inhibition was qualitatively estimated according to the area of the inhibition zone around the colony.

3. Results

3.1. Isolation and sequencing of the naf gene of P. nalgiovense

As the primers were located far outside the coding region of the gene, the hybridization step during the PCR reaction were performed at a low temperature. Under these conditions it was possible to amplify a DNA fragment of approximately 1.3 kb. The fragment was purified and used as template DNA for the sequencing reaction. The results of this analysis are shown in Fig. 1. The sequence of the *naf* gene was highly homologous compared to the sequence of the *paf* gene of *P. chrysogenum*. In the 5′ and 3′ non-coding regions and also in the two introns of the gene, the homology between the two sequences was less pronounced. The coding region of the *naf* gene differed only in three point mutations compared to the *paf* gene of *P. chrysogenum*. The first two point mutations are located in the putative signal and prosequences and therefore have no influence upon the amino acid sequence of the mature NAF peptide. The third mutation is located within the coding sequence for the mature peptide. However the mutation is neutral. The codon for the amino acid lysin at position 34 (with respect to the mature peptide) is changed from AAA to AAG both coding for lysin. According to these results none of the mutations change the primary structure of the mature peptide. The *naf* gene shows similar structural features than the *paf* gene, that means that the first 18 amino acids had the characteristic properties of a eukaryotic signal sequence. According to the results of Marx et al. (1995) the next 19 amino acids code for an amino acid prosequence, which is missing in the mature protein. The *naf* gene also carries two introns at the same position as the *paf* gene. All structural features, like the intron sequences, the start and stop codon, etc. were taken from the sequence of the *P. chrysogenum naf* gene (Marx et al., 1995).

3.2. Southern analysis with the cloned 1.3 kb fragment as probe

From three *P. nalgiovense* strains chromosomal DNA was isolated and subjected to Southern blot analysis to analyse if the gene is present in several copies at different locations of the genome. For that purpose the chromosomal DNA of *P. nalgiovense* was digested with *Eco*RI and plotted against the labelled 1.3 kb fragment of *P. nalgiovense*. Fig. 2 shows the result of that analysis. In all analysed strains a single band with a fragment size of about 18 kb appeared, indicating that the analysed strains obviously contained only one copy of the gene at the same position. The possibility that several copies are arranged tandemly however cannot be ruled out by that analysis.

3.3. Inhibitory activity of P. nalgiovense strains

To analyse if the *naf* containing strains of *P. nalgiovense* possess inhibitory activity against other fungi an agar inhibition assay was carried out. The analysed strains of *P. nalgiovense* showed inhibitory activity against a range of different fungi (Table 1). Inhibitory activity was strongest against other Penicillia, like *P. roqueforti* or *P. italicum*. *P. nalgiovense* showed no activity against yeasts. The expression of the inhibitory activity by *P. nalgiovense* was somewhat dependent on the medium composition. *P. nalgiovense* had antifungal activity when grown on malt extract medium, but on minimal medium this phenotypic trait was not expressed (data not shown). This result indicates that either the formation of the inhibitory metabolite is regulated or that specific nutritional compounds are needed for its production. The inhibition of fungal strains by *P. nalgiovense* was transient, e.g. the inhibition zone was visible for about 3 days. After that time the aerial mycelium of the indicator strain overgrows the inhibition zone.

4. Discussion

Several antifungal peptides produced by fungi, like the AFP peptide produced by *A. giganteus* (Wnedt et al., 1994) or the PAF peptide produced by

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ATACCAGCCAAGAGGTCCAGTATCCTGAAGTATGTGAGGCATTAATGTCATTGGAGAAGT
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
ATACCAGCCAAGAGGTCCAGTATCCTGAAGTATGTGAGGCATTAATGTCATTGGAGAAGT

CATGCAATCCATAAGCTGCCACCCCCAAGATGACTGCATTGGACCTGAGCATTGTATGTG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CATGCAATCCATAAGCTGCCACCCCCAAGATGACTGCATTGGACCTGAGCATTGTATGTG

TCACCTTTCACACAGAGCTCATGATCTGGTTTATAAAGGCGGCTTCATGACCCTCAATTC
:::X::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TCATCTTTCACACAGAGCTCATGATCTGGTTTATAAAGGCGGCTTCATGACCCTCAATTC

CATATAGTATCACTCCCATCACAGCATTTCGATATC--ACACCACTTTAACCTT---CAG
::::::::::::::::::::::::::::::::::::XXX:::::::::::::::XXX:::
CATATAGTATCACTCCCATCACAGCATTTCGATATCTTCAACCACTTTAACCTTCTCCAG

AGGATCATCATCTCAAGCCCTTCAGAATGCAAATGACCACAGTTGCCCTTTTTGTCTTCG
::::::::::::::::::::::::X:::::::::X::::::::::::::::::X::::::
AGGATCATCATCTCAAGCCCTTCATAATGCAAATCACCACAGTTGCCCTTTTTCTCTTCG

CTGCAATGGGCGGGGTAGCCACCCCCATTGAGTCTGTATCAAACGACCTCGATGCCAGGG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CTGCAATGGGCGGGGTAGCCACCCCCATTGAGTCTGTATCAAACGACCTCGATGCCAGGG

CTGAGGCCGGTGTCCTGGCCAAATACACCGGAgtgagtaaacatcaatatcccatacctg
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CTGAGGCCGGTGTCCTGGCCAAATACACCGGAgtgagtaaacatcaatatcccatacctt

aagtagctcacttgggaatggcgactaacggttcgggaccacaactcagAAATGCACCAA
:::::X::::::::::::::::::::::::::::::::::::::::::::::::::::::
aagta-ctcacttgggaatcgcgactaacggttcgggaccacaactcagAAATGCACCAA

ATCTAAGAACGAATGTAAATACAAGAACGATGCTGGAAAGGACACTTTTATCAAGTGCCC
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
ATCTAAGAACGAATGTAAATACAAGAACGATGCTGGAAAGGACACTTTTATCAAGTGCCC

CAAGTTTGATAACAAAAAGgtaaataccaagtcattcggaagtagccatctgaatcgat
:::::::::::::::X::::::X::::X:::X::::::::::::::::::::::::::::
CAAGTTTGATAACAAGAAGgtagaatatcaa-tcattcggaagtagccatctgaatcgat

ttcgtgctaatctcgctatttttttccagTGCACCAAGGATAATAACAAATGTACCGTCGA
:::::::::::::::::X::::::::::::::::::::::::::::::::::::::::::
ttcgtgctaatctcgctctttttttccagTGCACCAAGGATAATAACAAATGTACCGTCGA

CACCTACAACAACGCTGTCGATTGTGACTAGATGGTCTCTGCGATCACCAGGGCATTTAA
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CACCTACAACAACGCTGTCGATTGTGACTAGATGGTCTCTGCGATCACCAGGGCATTTAA

TGGTTTTTGGTTCCCTTCTTGTTGGTGATATGCGAGATGCCCTGTGATTCTCGAAGC-TA
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::X::
TGGTTTTTGGTTCCCTTCTTGTTGGTGATATGCGAGATGCCCTGTGATTCTCGAAGCTTA

CTACCCTACACCCACAAGGACTCGAACCAAGGACTGCTCGGTGGGTGATACATATACACC
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CTACCCTACACCCACAAGGACTCGAACCAAGGACTGCTCGGTGGGTGATACATATACACC

CAGTATCTATCCAGCTTCAATTTTC-GC-AATTTTATTTCTTATTTCATAAAGACACTCG
:::::::::::::::::::::::::X::X::::::X::::::::::::::::::::::::
CAGTATCTATCCAGCTTCAATTTTCGGCGAATTTTGTTTCTTATTTCATAAAGACACTCG
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Fig. 1. Comparison of the sequence of the *paf* gene (upper rows) and untranslated 5′ and 3′ regions with that of the *naf* gene (lower rows). The double point indicates identical bases, the X different bases and hyphens deletion of bases. The coding region of the gene is indicated in bold letters, the two introns are marked in lower case letters. The start codon and the stop codon are underlined.

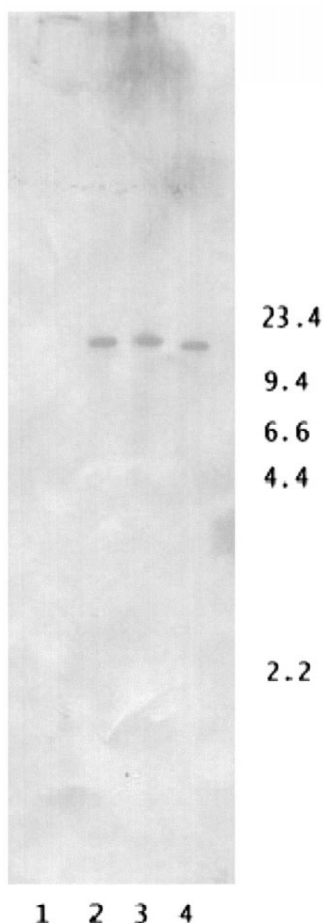

Fig. 2. Agarose gel of the Southern blot of *P. nalgiovense* BFE66, BFE67 and BFE474 probed with a labelled 1.3 kb PCR fragment generated with primers *paf4* and *paf5* and *P. nalgiovense* BFE66 DNA as template. Lane 1, Size standard, unlabelled; lanes 2–4, *P. nalgiovense* BFE66, BFE67 and BFE474. The fragment sizes are indicated in kb.

P. chrysogenum (Marx et al., 1995) have been described. Recently Lee et al. (1999) found a new peptide (ANAFP) produced by *A. niger* with some homology to the two other peptides. Obviously the production of these peptides is more widespread than thought, as for *P. nalgiovense* a gene for a similar peptide could also be identified. Not very much is known about the role and the mode of action of the antifungal peptides produced by fungi. According to Lacadena et al. (1995) the AFP peptide can interact with phospholipids and its cytotoxicity may involve the interaction with membranes. Nakaya et al. (1990) determined the protein sequence of the AFP peptide. They found similarities to phospholipase A_2, which is specific for lectins and removes the unsaturated fatty acid at the C2 position of 3-*sn*-phosphatidylglycerols. Some proteinacious peptides produced by various plant species are also known to have antifungal activity. They are thought to be involved in plant defence against fungal attack. Some of them bind to chitin (Broekaert et al., 1989; van Parijs et al., 1991) and some of them exhibit permeabilizing activity against fungal membranes or cell walls (Roberts and Seltrennikoff, 1990). Bacteria are also able to produce antifungal peptides. The production of iturins, proteinacious peptides with antifungal activity from *Bacillus subtilis* have been described by Gueldner et al. (1988).

The inhibitory spectrum of the *P. chrysogenum* PAF peptide has not been reported. The AFP peptide from *Aspergillus nidulans* could be shown to be active against a range of filamentous fungi from the *Zygomycetaceae* and *Ascomycetaceae* but not against yeast (K. Berghof, personal communication). *P. nalgiovense* shows similar inhibitory activity (Table 1). It is also not active against yeasts, but against a range of different filamentous fungi, which can occur in certain food commodities. The ANAFP peptide in contrast also exhibits inhibitory activity against certain yeasts, however not against bacteria (Lee et al., 1999).

The fact that the inhibitory activity was only transient, could be due to the production of proteases, produced either by *P. nalgiovense* or the indicator strain, which degrades this putative inhibitory peptide. It is known from *P. nalgiovense* that it carries a gene for an active protease (Geisen, 1993). This proteolytic activity maybe due to the inactivation of a proteinacious antifungal peptide.

The results described here show that *P. nalgiovense* carries a gene with high homology to the *paf* gene of *P. chrysogenum* and that *P. nalgiovense* exhibits antagonistic activity against a range of filamentous fungi. It was not explicitly demonstrated that the inhibitory activity was due to the activity of the *naf* gene, for example by gene disruption, however the results suggests that this is the case. Further experiments will be needed to demonstrate this and to analyse the usefulness of this activity for biopreservation purposes.

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