ELSEVIER

International Journal of Food Microbiology 58 (2000) 59–63

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.nl/locate/ijfoodmicro

Karyotype of *Penicillium nalgiovense* and assignment of the penicillin biosynthetic genes to chromosome IV

P. Färber, R. Geisen*

Federal Research Centre for Nutrition, Haid und Neustr. 9, 76131 Karlsruhe, Germany

Received 5 July 1999; accepted 3 March 2000

Abstract

The karyotype of *Penicillium nalgiovense* was determined by pulsed-field gel electrophoresis and compared to the karyotype of *P. chrysogenum*. Both species have four chromosomes, but they differ in the size of the chromosomes and in the overall size of the genome. The sizes of the *P. nalgiovense* chromosomes as determined by pulsed-field gel electrophoresis are: 9.1 Mb, 7.9 Mb, 5.4 Mb and 4.1 Mb which gives in summary a genome size of 26.5 Mb. This compares to 34.1 Mb for *P. chrysogenum*. The penicillin gene cluster was located by Southern hybridization on chromosome IV, the smallest chromosome of *P. nalgiovense* compared to chromosome I, the largest chromosome of *P. chrysogenum*.

Keywords: Karyotype; *P. nalgiovense*; Penicillin biosynthetic genes; Fungal starter culture

1. Introduction

Penicillium nalgiovense is a fungal starter culture for the production of fermented foods (Jacobsen and Hinrichsen, 1997). Aroma and texture formation is mainly due to proteolytic (Toledo et al., 1997) and lipolytic activity and to the production of volatiles by the fungus (Jacobsen and Hinrichsen, 1997). It has been shown that *P. nalgiovense* is a producer of penicillin (Andersen and Frisvad, 1994; Färber and Geisen, 1994). According to PCR analysis *P. nalgiovense* carries three genes which are homologous to the penicillin biosynthetic genes of *P. chrysogenum*. With Southern blot experiments using gene probes specific for each of these genes it was shown that all three genes of *P. nalgiovense* are clustered on one 9.1 *Sal*I restriction fragment (Färber, 1999). A detailed analysis of the gene cluster of *P. nalgiovense* was performed by Laich et al. (1999).

P. chrysogenum has four chromosomes of 10.4, 9.6, 7.3 and 6.8 Mb in size (Fierro et al., 1993). The penicillin gene cluster is located on the largest chromosome (chromosome I) of *P. chrysogenum*. Morphological (Samson and van Reenen-Hoekstra, 1988), isoenzyme (Banke et al., 1997) and molecular data (Geisen, 1996) indicate that *P. nalgiovense* is evolutionarily related to *P. chrysogenum*. To compare the locations of the penicillin gene clusters and

*Corresponding author.
E-mail address: rolf.geisen@bfe.uni-karlsruhe.de (R. Geisen)

0168-1605/00/$ – see front matter
PII: S0168-1605(00)00279-8

the karyotype of both species, the chromosomes of *P. nalgiovense* were separated by pulsed-field gel electrophoresis and hybridized to a gene probe, specific for the isopenicillin synthase gene (*ipn*A). The aim of this study was to demonstrate the difference between *P. nalgiovense* and *P. chrysogenum* at the genetic level.

2. Materials and methods

2.1. Strains and culture conditions

The following strains from the culture collection of the Federal Research Centre for Nutrition, Karlsruhe, Germany (BFE) were used in the experiments: *Penicillium nalgiovense* BFE52, *P. nalgiovense* BFE66, *P. nalgiovense* BFE233, *P. nalgiovense* BFE255, *P. chrysogenum* DSM 1075 and *Aspergillus nidulans* BFE145. Strains were routinely grown either on malt extract agar (Merck, Darmstadt, Germany) or liquid medium at 25°C.

2.2. Pulsed-field gel electrophoresis

For the preparation of chromosomal DNA suitable for pulsed-field gel electrophoresis, protoplasts were produced from mycelium grown for 36 h under shaking conditions at 25°C. The mycelium was harvested by filtration, washed twice with water and the cell wall degraded by treatment for 1.5–2 h at 37°C with lytic enzymes in isotonic medium which contained 0.8 M KCl; lysing enzymes from *Trichoderma harzianum* (Sigma, St. Louis, MO, USA), 10 mg ml^{-1}; β-glucuronidase (Sigma), 10 μl ml^{-1}. The protoplasts produced were separated from residual mycelium by filtration through glass wool. Two volumes of STC buffer (1.2 M sorbitol; 10 mM Tris; 10 mM $CaCl_2$; pH 7.5) were added and the protoplasts precipitated by centrifugation at 5000 rev./min for 5 min. The pellet was washed twice in 500 μl STC buffer, then the protoplasts were resuspended in 100 μl STC buffer. The protoplasts were fixed in 0.6% InCert agarose plugs (FMC, Rockland, USA) and the protoplasts lysed at 54°C for 48 h under gentle shaking in lysis buffer (0.5 M EDTA; 1% sodium laurylsarcosinate; 5 mg ml^{-1} proteinase K, pH 8.0). Agar plugs were stored in 0.05 M EDTA solution. For pulsed field gel electrophoresis (Schwartz et al., 1983) the agar plugs were equilibrated in electrophoresis buffer (0.5 × TBE) for 1 h and embedded in 0.6% FastLane agarose (FMC). A four phase electrophoresis run was carried out at 12°C in a Gene Navigator electrophoresis system (Pharmacia, Uppsala, Sweden): phase 1, pulse duration 180 min for 100 h; phase 2, pulse duration 120 min for 90 h; phase 3, pulse duration 90 min for 60 h; phase 4, pulse duration 60 min to 50 h. The electrophoresis was carried out at a constant voltage of 28 V and at a current of 30 mA. As molecular weight standard, *Schizosaccharomyces pombe* chromosomes were used (Biorad, Hercules, USA). The results were documented and evaluated with a Biorad GS-670 densitometer and the image analysis program Molecular Analyst (Biorad).

2.3. Direct alkaline DNA transfer

To achieve high transfer rates, the DNA was transferred to nylon membranes under alkaline conditions. The separated chromosomal DNA was nicked by irradiation with UV light (302 nm) for 120 s. The DNA was subsequently blotted by capillary transfer in alkaline transfer buffer (0.4 M NaOH; 1.5 M NaCl).

2.4. Hybridization

Hybridization was carried out under standard conditions according to Maniatis et al. (1989). As a probe a digoxygenin labeled 547 bp internal fragment of the *ipn*A gene (positions 801–1348, according to the published sequence of the homologous gene of *P. chrysogenum* (Barredo et al., 1989)) was used. The hybridization of the probe was performed under stringent conditions at 65°C. The hybrid DNA was visualized with alkaline phosphatase coupled antibodies against the labeled probe according to the suggestions of the manufacturer of the Digoxygenin Detection and Labeling kit (Boehringer, Mannheim, Germany).

3. Results

3.1. Chromosome number and genome size of Penicillium nalgiovense

Protoplasts of four strains of *P. nalgiovense* were produced and lysed in agar plugs as described in

Section 2. The released chromosomes were separated and the molecular weight of the bands calculated by comparison with a molecular weight standard of *Schizosaccharomyces pombe* chromosomes. The molecular weight calculations were carried out by densitometric analysis. Fig. 1 shows the result of that analysis. The genomic DNA of all four *P. nalgiovense* strains separated into four distinct bands. Two of them had a much higher molecular weight than the standard bands of *S. pombe*, whereas the other two bands are in the same size range as the *S. pombe* chromosomes. The weak fifth band, of much lower molecular weight, is apparently mitochondrial DNA as it separates to the same position as the mitochondrial DNA of *S. pombe*. All *P. nalgiovense* strains showed the same banding pattern, indicating similar or identical chromosomes with respect to size. Table 1 shows the results of the molecular weight estimation of the four chromosomes. According to the densitometric estimation of the molecular weights, the total size of the *P. nalgiovense* genome is 26.5 Mb.

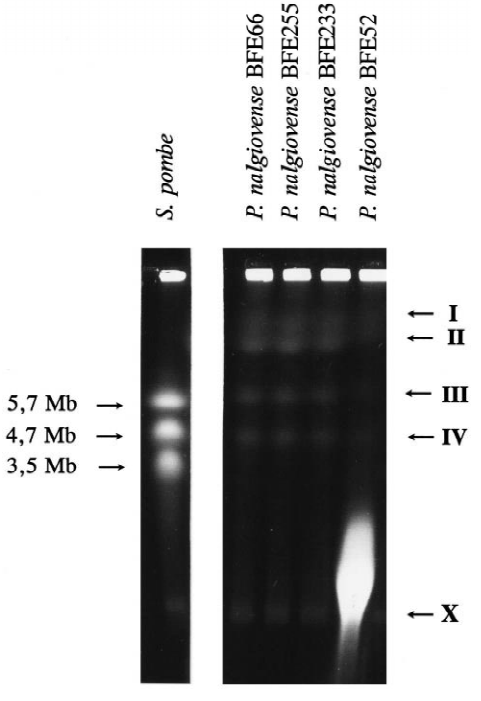

Fig. 1. Pulsed field gel electrophoresis of chromosomal DNA of four *Penicillium nalgiovense* strains. The four chromosomal bands are indicated by roman letters. The *Schizosaccharomyces pombe* chromosomes were taken as molecular weight marker. The sizes of these chromsomes are indicated in Mb. x = mitochondrial DNA.

Table 1
Comparison of the chromosome sizes (Mb) of *Penicillium nalgiovense* and *P. chrysogenum*[a]

Chromosome	*P. nalgiovense*	*P. chrysogenum*
I	9.1	10.4
II	7.9	9.6
III	5.4	7.3
IV	4.1	6.8
Genome size	26.5	34.1

[a] The sizes of the *Penicillium chrysogenum* chromosomes are given according to Fierro et al. (1993).

3.2. Comparison of the chromosomal banding pattern of P. nalgiovense with that of P. chrysogenum

To compare the karyotype of *P. nalgiovense* with that of the related species *P. chrysogenum*, the chromosomes of both species were separated on a pulsed-field gel. *Aspergillus nidulans* was used as a control. Fig. 2 shows the results of this experiment. The karyotype of *P. nalgiovense* is similar to that of *P. chrysogenum*. In general the chromosomes of *P. nalgiovense* are somewhat smaller than the chromosomes of *P. chrysogenum* which is reflected in a genome size difference of 7.6 Mb between the two species. A comparison of the chromosome sizes of both species is depicted in Table 1. As the running conditions were optimized for the separation of large chromosomes, only the three largest chromosomes from *A. nidulans* could be separated. The largest chromsome of *A. nidulans* is 5.0 Mb (Brody and Carbon, 1989) which runs roughly at the position of the smallest chromosome of *P. nalgiovense*.

3.3. Localization of the penicillin biosynthesis gene cluster of P. nalgiovense

It was shown by successive hybridizations of the same membrane with three probes specific for each of the penicillin biosynthetic genes that these genes are clustered on one 9.1 kb *Sal*I fragment in *P. nalgiovense* (Färber, 1999). To determine the chromosomal localization of this cluster, the separated chromosomes of *P. nalgiovense* were transferred after pulsed field gel electrophoresis onto a nylon membrane. The membrane was hybridized with a

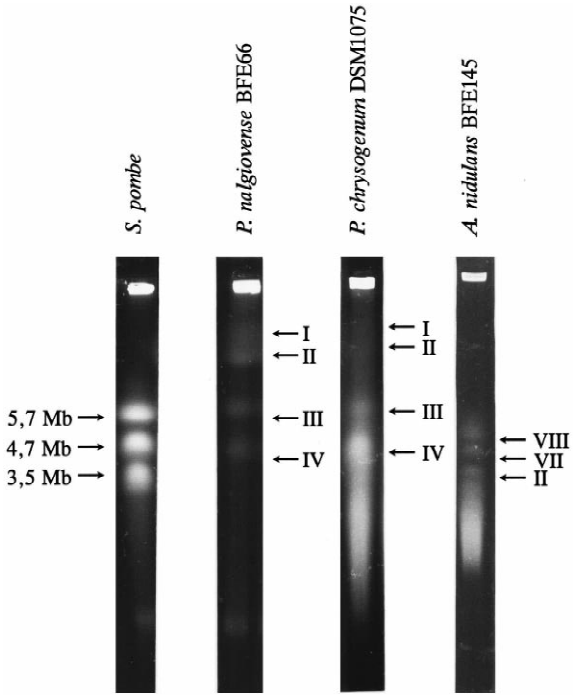

Fig. 2. Pulsed field gel electrophoresis with chromosomal DNA of *Penicillium nalgiovense*, *P. chrysogenum* and *Aspergillus nidulans*. *Schizosaccharomyces pombe* DNA was taken as molecular weight marker. The sizes of the chromosomes are indicated in Mb. The positions of the chromosomes of the analysed *P. nalgiovense*, *P. chrysogenum* and *A. nidulans* strains are indicated by roman letters. The numbering of the *P. chrysogenum* chromosomes is according to Fierro et al. (1993) and of the *A. nidulans* chromosomes according to Brody and Carbon (1989).

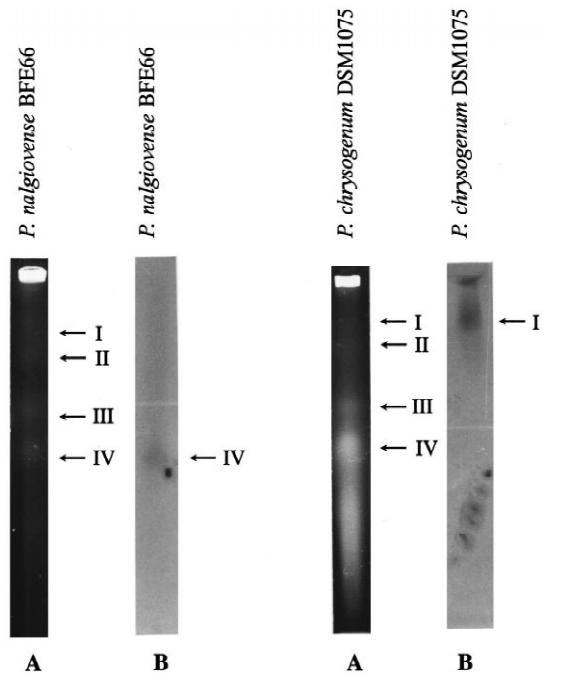

Fig. 3. Direct alkaline blotting of the separated chromosomes of *Penicillium nalgiovense* and *P. chrysogenum* to a DIG-labeled gene probe specific for the *ipn*A gene. The chromosomal DNA of *P. nalgiovense* and *P. chrysogenum* was separated by pulsed-field gel electrophoresis (A) and blotted to positively charged nylon membrane. The blotted DNA was hybridized under stringent conditions to a *ipn*A probe (B). The location of the chromosomes are indicated by roman letters as well as the matching signal after hybridization.

probe specific for the *ipn*A gene. The same analysis was performed for comparison with *P. chrysogenum*. In Fig. 3 the results of the hybridization are shown. Although these two species are related and have the same number of chromosomes, clear differences were seen in the chromosomal location of the penicillin biosynthetic gene cluster. In the case of *P. chrysogenum* the probe hybridized with chromosome I, the largest chromosome of this species, but in *P. nalgiovense* the hybridization signal highlighted at the position for the smallest chromosome (chromosome IV).

4. Discussion

Based on isoenzyme analysis, Banke et al. (1997) suggested that *P. nalgiovense* is distinct from *P. chrysogenum* and more closely related to *P. dipodomyis*, a species which also produces penicillin. The results presented here support this interpretation, as they demonstrate that there are distinct differences between *P. chrysogenum* and *P. nalgiovense* with respect to chromosome size and location of the penicillin biosynthetic genes. Both *P. nalgiovense* and *P. chrysogenum* have a genome consisting of four chromosomes. The chromosomes of *P. nalgiovense* are generally smaller than those of *P. chrysogenum*. *P. chrysogenum* has a genome size of 34.1 (Fierro et al., 1993) while *P. nalgiovense* has an estimated genome size of 26.5 Mb. The sizes of fungal genomes can vary considerably (Kupfer et al., 1997). For *P. janthinellum*, another *Penicillium* species, the genome size has been estimated to range between 39 and 46 Mb (Kayser and Schulz, 1991). The fact that all four analysed *P. nalgiovense* strains showed the same karyotype confirm that it is a

homogenous species, already shown by isoenzyme (Banke et al., 1997) and RAPD analysis (Geisen, 1996).

The localization of the penicillin biosynthetic gene cluster differs in these penicillin-producing species. In *P. chrysogenum* it is located on chromosome I (10.4 Mb) and in *P. nalgiovense* on chromosome IV (4.1 Mb). Nothing is known about the karyotype of *P. dipodomyis*, a third penicillin-producing species. To our knowledge this is the first time that the differences between the karyotypes of *P. chrysogenum* and *P. nalgiovense* have been reported.

References

Andersen, S.J., Frisvad, J.C., 1994. Penicillin production by *Penicillium nalgiovense*. Lett. Appl. Microbiol. 19, 486–488.

Banke, S., Frisvad, J.C., Rosendahl, S., 1997. Taxonomy of *Penicillium chrysogenum* and related xerophilic species based on isozyme analysis. Mycol. Res. 101, 617–624.

Barredo, J.L., Cantoral, J.M., Alvarez, E., Diez, B., Martin, J.F., 1989. Cloning, sequence analysis and transcriptional study of the isopenicillin N synthase of *Penicillium chrysogenum*. Mol. Gen. Genet. 216, 91–98.

Brody, H., Carbon, J., 1989. Electrophoretic karyotype of *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA 86, 6260–6263.

Färber, P., 1999. Biosynthese des β-lactam Antibiotikums Penicillin durch den filamentösen Pilz *Penicillium nalgiovense*, einer Starterkultur zur Herstellung Schimmelpilz-gereifter Fleischwaren, University of Stuttgart Hohenheim, Germany, Ph.D. Thesis.

Färber, F., Geisen, R., 1994. Antagonistic activity of the food relevant filamentous fungus *Penicillium nalgiovense* is due to the production of penicillin. Appl. Environ. Microbiol. 60, 3401–3404.

Fierro, F., Gutiérrez, S., Díez, B., Martín, J.F., 1993. Resolution of four large chromosomes in penicillin-producing filamentous fungi: the penicillin gene cluster is located on chromosome II (9.6 Mb) in *Penicillium notatum* and chromosome I (10.4 Mb) in *Penicillium chrysogenum*. Mol. Gen. Genet. 241, 573–578.

Geisen, R., 1996. Characterization of the species *Penicillium nalgiovense* by RAPD and protein patterns and its comparison to *Penicillium chrysogenum*. Syst. Appl. Microbiol. 18, 595–601.

Jacobsen, T., Hinrichsen, L., 1997. Bioformation of flavour by *Penicillium candidum*, *Penicillium nalgiovense* and *Geotrichum candidum* on glucose, peptone, maize oil and meat extract. Food Chem. 60, 409–416.

Kayser, T., Schulz, G., 1991. Electrophoretic karyotype of cellulolytic *Penicillium janthinellum* strains. Curr. Genet. 20, 289–291.

Kupfer, D.M., Reece, C.A., Clifton, S.W., Roe, B.A., Prade, R.A., 1997. Multicellular ascomycetous fungal genomes contain more than 8000 genes. Fungal Genet. Biol. 21, 364–372.

Laich, F., Fierro, F., Cardoza, R.E., Martin, J.F., 1999. Organization of the gene cluster for biosynthesis of penicillin in *Penicillium nalgiovense* and antibiotic production in cured dry sausages. Appl. Environ. Microbiol. 65, 1236–1240.

Maniatis, T., Fritsch, E.F., Sambrook, J., 1989. Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbour, NY.

Samson, R.A., van Reenen-Hoekstra, E.S., 1988. Identification of the common food borne fungi. In: Samson, R.A., van Reenen-Hoekstra, E.S. (Eds.), Introduction to Food Borne Fungi, Centraalbureau voor Schimmelcultures, Baarn, Netherlands, pp. 3–219.

Schwartz, D.C., Saffran, W., Welsh, J., Haas, R., Goldenberg, M., Cantor, C.R., 1983. New techniques for purifying large DNAs and studying their properties and packaging. Cold Spring Habor Symp. Quant. Biol. 47, 189–195.

Toledo, V.M., Selgas, M.D., Casas, M.C., Ordóñez, J.A., García, M.L., 1997. Effect of selected mould strains on proteolysis in dry fermented sausages. Z. Lebensm. Unters. Forsch. 204, 385–390.