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Ribotoxins are a more widespread group of proteins within the filamentous fungi than previously believed

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Abstract

α-Sarcin, restrictocin and mitogillin are the best known members of the family of fungal ribotoxins. In recent years, new members of this family have been discovered and characterised. In this work, we study the occurrence of ribotoxins among different species of fungi. The presence of ribotoxins has been identified in some new species by means of genetic studies, as well as expression and activity assays. The ribotoxin genes have been partially sequenced, and demonstrate a high degree of similarity. These studies demonstrate that these toxins are more widespread than previously considered. This is surprising, considering the ribotoxins are such specific and potent toxins, of unknown biological function. These studies confirm the hypothesis that these proteins are naturally engineered toxins derived from ribonucleases of broad substrate specificity.

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1. Introduction

The ribotoxins are a family of ribosome-inactivating proteins (RIP) which display a highly specific ribonucleolytic activity against a single phosphodiester bond in the universally conserved sarcin/ricin domain of 28S ribosomal RNA (Kao and Davies, 1995). The first members of this family (α-sarcin, restrictocin and mitogillin) were discovered in the early 1960s as secreted proteins of *Aspergillus giganteus* and *A. restrictus* with anti-tumor activity (Olson and Goerner, 1965). Later, an allergen from the pathogenic fungus *A. fumigatus* (AspFI) was identified as being a ribotoxin (Arruda et al., 1990) identical to mitogillin. More recently, other ribotoxins from different strains of *A. clavatus* (clavin, c-sarcin) and *A. giganteus* (gigantin) have been characterised, including their primary sequence (Parente et al., 1996; Huang et al., 1997; Wirth et al., 1997). Finally, ribotoxins have been detected in other *Aspergillus* (Lin et al., 1995) and *Penicillium* species (Lin et al., 1997).

All ribotoxins characterised so far share a minimum of 85% amino acid sequence identity. The three-dimensional structure of two of them, α-sarcin and restrictocin, has been determined (Campos-Olivas et al., 1996a,b; Yang and Moffat, 1996), revealing a common folding motif with fungal guanyl-specific or purine-specific ribonucleases such as T1 and U2 RNases. The catalytic mechanism of these ribotoxins has also been studied (Lacadena et al., 1998), showing similarities with that of guanyl-specific RNases. However, ribotoxins are larger proteins, containing extra loops that are not present in guanyl-specific RNases, and which are likely responsible for the different catalytic specificity and their ability to cross membranes. In fact, ribotoxins must enter target cells to exert their toxic activity and this step determines differential resistance found among different cells (Fernández-Puentes and Carrasco, 1980; Fernández-Puentes, 1983; Olmo et al., 1993; Turnay et al., 1993), as all ribosomes tested so far, including those from ribotoxin-producing species such as *A. giganteus* and *A. restrictus*, are susceptible to inactivation by ribotoxins (Hobden, A.N., 1978. PhD dissertation, University of Leicester, UK; Miller and Bodley, 1988). In fact, α-sarcin interacts with negatively-charged lipid bilayers, promoting their fusion, as well as leakage of intravesicular aqueous contents and alterations of the phospholipid phase transition (Gasset et al., 1989, 1990, 1991a,b; Mancheño et al., 1994; Oñaderra et al., 1998) and is able to translocate across model membranes (Oñaderra et al., 1993, 1998).

The study of the evolution of ribotoxins is of particular interest, as they appear to be in a crossroad of different evolution patterns. The high specificity they have acquired in their ribonucleolytic action directed against a single phosphodiester bond in the whole ribosome, as well as their ability to enter the cells, leads them to be considered as naturally engineered targeted toxins (Lamy et al., 1992; Kao and Davies, 1995), perhaps evolving from the family of guanyl-specific fungal ribonucleases.

In this regard, ribotoxins are another example of ribonucleases with special biological actions (RISBASES), as proposed by D'Alessio et al. (1991). These

proteins could have evolved from ancestors shared with other "typical" RNases (the family of pancreatic RNase in animals and guanyl-specific RNases in fungi), the evolution being directed by the new biological activities engineered. A formal similarity can be established, for example, between angiogenin, which shares approximately 35% sequence with RNase A and also shows ribosome-inactivating properties (Shapiro et al., 1986; St. Clair et al., 1988). Angiogenin, however, is less specific than fungal ribotoxins since it digests both 18S and 28S rRNAs.

Thus, the identification of ribotoxin genes among related species, and their comparison, can shed light on natural evolution of these toxins as well as displaying structural elements that determine cytotoxic activity and biological function.

2. Materials and methods

2.1. Strains

Fungal strains used are described in Table 1. They were grown on potato

Table 1
Results of the detection of ribotoxins in different fungal strains (collection numbers are indicated; for *A. nidulans*, the genetic marker of the strain is indicated). nd: not determined

Strain			Southern	PCR	Activity	Immuno
1.	*Aspergillus giganteus*	MDH 18894	+	+	+	nd
2.	*Aspergillus giganteus*	NRRL 10	+	+	+	+
3.	*Aspergillus giganteus*	NRRL 1780	+	+	nd	+
4.	*Aspergillus giganteus*	NRRL 4763	+	+	nd	+
5.	*Aspergillus viridinutans*		+	+	(1)	nd
6.	*Aspergillus fumigatus*		+	+	+	nd
7.	*Aspergillus spp.*		+	+	nd	nd
8.	*Aspergillus spp.*		+	+	nd	nd
9.	*Aspergillus fischeri*		+	+	nd	+
10.	*Aspergillus restrictus*	MDH 13462L	nd	+	nd	nd
11.	*Aspergillus glaucus*		+	+	nd	+
12.	*Neosartorya glabra*		+	+	nd	+
13.	*Aspergillus nidulans*	(CS2008)	–	–	nd	(2)
14.	*Aspergillus flavus*		–	–	nd	nd
15.	*Aspergillus oryzae*	NRRL 447	–	–	nd	nd
16.	*Aspergillus niger*		–	–	nd	nd
17.	*Aspergillus alutaceus*	NRRL 398	–	–	–	nd
18.	*Aspergillus terreus*		–	–	nd	nd
19.	*Hemicarpenteles acanthosporus*		–	–	nd	nd
20.	*Penicillium notatum*		–	nd	–	nd
21.	*Penicillium italicum*		–	nd	nd	nd

(1) Presence of non-specific ribonuclease.
(2) High molecular mass bands.

dextrose agar (Difco, Detroit, USA) plates from silica gel or slant preparations and assessed for their morphological characteristics.

2.2. DNA purification

DNA purification was carried out from potato dextrose broth fungal cultures as previously described (Wirth et al., 1997). Integrity of DNA was assessed by electrophoresis in 0.7% agarose gels.

2.3. Southern blot

DNA digested with *Hin*dIII or *Eco*RI was loaded on 0.8% agarose gels and, after electrophoresis, transferred by alkali blot to nylon membranes. The *Xba*I-*Bam*HI fragment of pINPGαS containing the sequence coding for mature α-sarcin (Lacadena et al., 1994) was labelled by random priming using [α-^{32}P]dCTP and an Oligolabelling Kit (Pharmacia, Uppsala, Sweden). Hybridisation was carried out at 65°C following the membranes manufacturer's instructions and they were washed at 65°C with 1 × SSPE for 15 min and autoradiographed.

2.4. PCR screening

Two primers were designed which correspond to regions conserved within the known sequences of *Aspergillus* ribotoxins. These primers are KQ1 (5′-CCTCTYTCCGACGGCAAGAC-3′) and KQ2 (5′-GTTRGGATARGTRTAGATGACC-3′). They should allow amplifying a fragment of 273 bp, corresponding to amino acid residues 38 to 128 in α-sarcin (Oka et al., 1990). Touchdown PCR (Don et al., 1991; Roux and Hecker, 1997) was carried out, using *Taq* DNA polymerase, on a thermocycler subjecting the samples to denaturation for 5 min at 94°C, 30 cycles of denaturation (90 s at 94°C), annealing (1 min at temperatures lowering from 65 to 50°C) and extension (2 min at 72°C) and 15 more cycles with annealing at 50°C. Aliquots of 5 μl out of 50 were analysed by electrophoresis on 1.5% agarose gels.

2.5. Sequencing

Significant bands were excised from preparative electrophoresis on 1.5% agarose gels and extracted to water with Qiaex II gel extraction kit. Sequence reactions were carried out with the DNA fragments and each primer (KQ1 and KQ2) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. They were analysed on an ABI PRISM 377 DNA Sequencer.

2.6. Spores activity assay

For analysing the specific activity of ribotoxins by means of generating the α-fragment from 28S rRNA (Schindler and Davies, 1977), conidiospore suspensions

were prepared by vortexing a 0.01% Tween-80 solution on fungal cultures grown on potato dextrose agar slants. 3 μl of the supernatants of such suspensions were incubated with 3 μl of rabbit reticulocyte lysate (Promega, Madison, WI, USA) for 5 min at 37°C. After phenol extraction, nucleic acids were loaded on a 2% agarose gel for electrophoresis. A negative and a positive control with 50 ng of mitogillin, purified as described (Kao and Davies, 1995) were also done.

2.7. Ribotoxin production and immunodetection

Fungal strains were grown in the original α-sarcin-producing broth (Olson and Goerner, 1965), which includes 2% peptone, 2% corn starch, 0.5% NaCl and 1.5% beef extract. Two consecutive precultures of 10 ml were grown for 24 h and the second one was used to inoculate duplicate 50 ml cultures, which were incubated at 30°C for 70 h. Aliquots were taken out at different times and, after

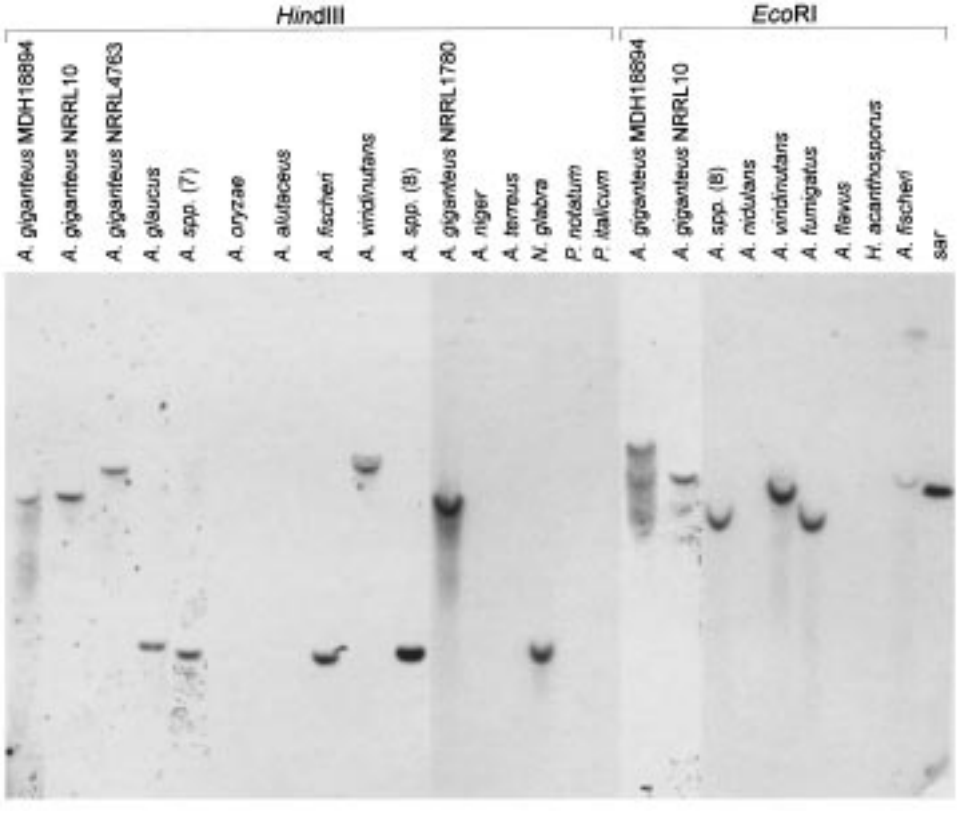

Fig. 1. Southern blot of fungal DNA digested with the restriction enzymes indicated, using as a probe the fragment containing the sequence coding for mature α-sarcin (Lacadena et al., 1994). For the two unidentified *Aspergillus* strains, the numbers in parenthesis are the same as in Table 1. In lane "sar", a plasmid containing the probe was loaded.

electrophoresis in polyacrylamide gels with SDS, they were immunoblotted with antibodies raised up against α-sarcin (Lacadena et al., 1994).

2.8. Phylogenetic tree

The phylogenetic tree was constructed with the DNA sequences obtained in this work and the corresponding fragments from other ribotoxins by the UPGMA method from a Jukes and Cantor distances matrix, using the WET program (J. Dopazo, TDI S.A., Madrid, Spain).

3. Results

Twenty-one strains of species from the *Aspergillus* genus and other related genera (*Penicillium*, *Neosartorya* and *Hemicarpenteles*) were analysed for the presence of ribotoxin genes by means of Southern hybridisation (Fig. 1) and PCR (Fig. 2). Both analyses gave consistent results (Table 1), revealing the presence of a single ribotoxin gene in all strains of species in which ribotoxin production had been already described, such as *A. giganteus*, *A. fumigatus* and *A. restrictus*, as well as in some species in which it has not, such as *A. fischeri*, *A. glaucus*, *A. viridinutans* and *Neosartorya glabra*. The gene was also detected in two *Aspergillus* strains not classified under any species (Table 1, strains 7 and 8). This analysis

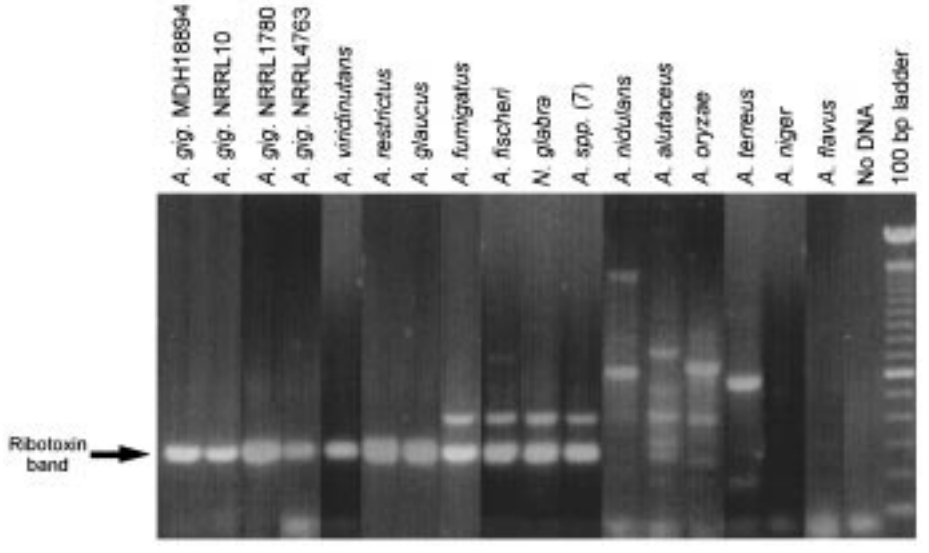

Fig. 2. PCR reactions carried out with fungal DNA as described in Section 2, on 1.5% agarose gel electrophoresis. The band corresponding to the ribotoxin sequences is marked with an arrow. *A. gig.* stands for *A. giganteus*. For the unidentified *Aspergillus* strain, the number in parentheses is the same as in Table 1. DNA size marker is 100 bp ladder (Gibco BRL Life Technologies).

gave a negative result for strains of other nine species of the *Aspergillus*, *Hemicarpenteles* and *Penicillium* genera.

Production of α-sarcin has been associated with conidiophore formation (Brandhorst and Kenealy, 1992; Yang and Kenealy, 1992). Thus, we have used suspensions prepared from sporulated cultures, in which the major component are conidiospores, to detect the presence of specific ribotoxin activity, i.e. production of α-fragment after cleavage of 28S rRNA (Fig. 3). A fragment of molecular weight corresponding to the α-fragment was observed in most of the strains in which the ribotoxin gene has been detected. Appearance of some other bands reveals non-specific hydrolysis of ribosomes. However, only for *A. clavatus*, less-specific extracellular RNases different from ribotoxins have been described (Bezborodova et al., 1983), which may explain the bigger degradation of rRNA observed when reticulocytes are incubated with *A. viridinutans* spores suspension (Fig. 3). Therefore, this assay seems to be a convenient procedure for screening the production of ribotoxins, although it cannot be discarded that some of the α-sarcin analogues have altered specificity, or that the strains produce other ribonucleases. Thus, considering exclusively the appearance of the α-fragment, the

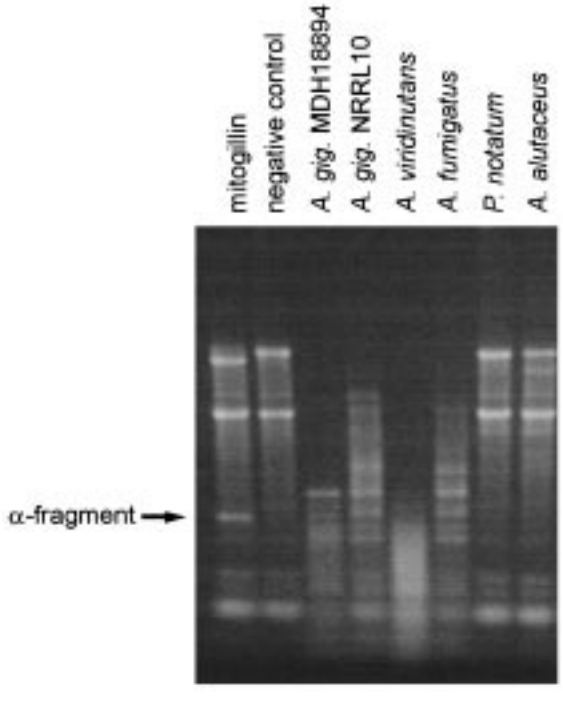

Fig. 3. Spores activity assay. Separation of rRNA after incubation of ribosomes with suspensions of the spores from the strains indicated. The band corresponding to the α-fragment produced specifically by ribotoxins is marked with an arrow. *A. gig.* stands for *A. giganteus*.

results are consistent with the genetic study, revealing expression of the gene and production of ribotoxin by the moulds (Table 1).

We also analysed production of ribotoxins by growing the moulds in liquid cultures, using the conditions described for α-sarcin production. Aliquots of the extracellular media were subjected to SDS-PAGE, electrotransferred to Immobilon membranes, and immunostained with polyclonal antibodies raised in rabbit against α-sarcin. Presence of the ribotoxins was thus detected in *A. giganteus*, *A. fischeri*, *A. glaucus* and *N. glabra*, although levels of production were different among them (Fig. 4, Table 1). Some proteins of higher molecular mass from extracellular medium of *A. nidulans* were also immunostained. As the ribotoxin gene was not detected in this strain, this could be caused by extracellular proteins that share some antigenic similarity with α-sarcin. In a previous study (Lin et al., 1995), cultures of two *Aspergillus* strains gave a faint immunoreactivity against anti-α-sarcin serum but a negative result in gene analysis. A protein of lower molecular mass was also detected in *A. giganteus* NRRL 4763 cultures, which could be a product of partial degradation of the mature ribotoxin.

PCR fragments obtained were sequenced by cycle sequencing with the primers used for amplification. All the ribotoxin sequences obtained were of the same length, as had been found in all the sequences already known. Actually, the known ribotoxin genes were very similar, the only size differences being in the length of the intron and its vicinity (Lin et al., 1995; Wirth et al., 1997). According to their similarities, the ribotoxins can be divided into three groups (Figs. 5–7). One of them would comprise proteins such as restrictocin and

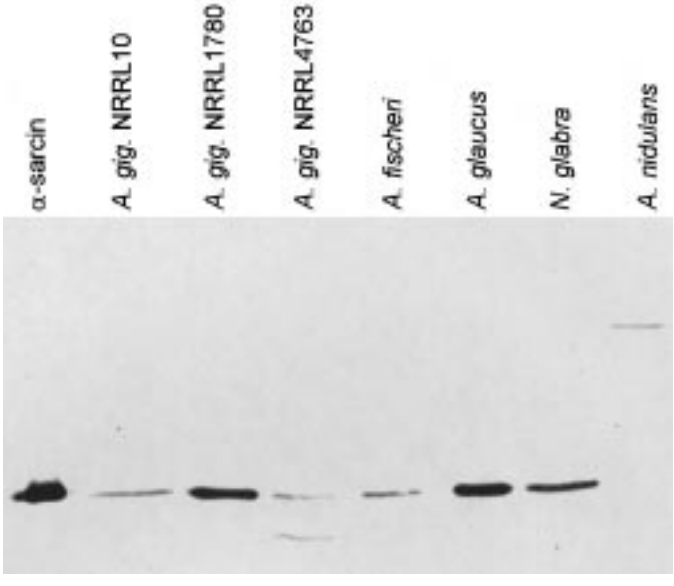

Fig. 4. Immunodetection of ribotoxins in extracellular media of cultures of the strains indicated, with antibodies raised against α-sarcin. *A. gig.* stands for *A. giganteus*.

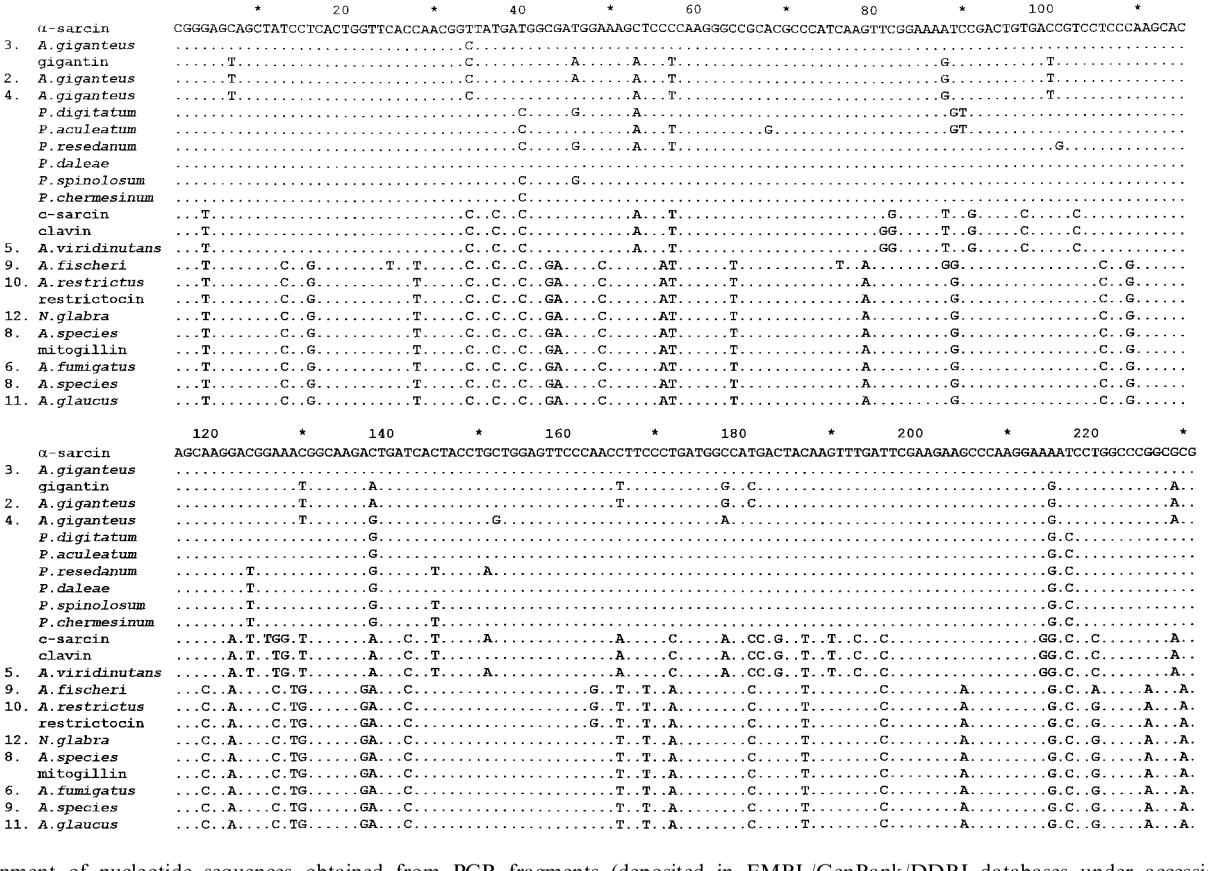

Fig. 5. Alignment of nucleotide sequences obtained from PCR fragments (deposited in EMBL/GenBank/DDBJ databases under accession numbers AJ009764 to AJ009769), and homologous fragments from already published ribotoxin genes (Oka et al., 1990; Lamy and Davies, 1991; Lamy et al., 1991; Parente et al., 1996; Huang et al., 1997; Lin et al., 1997; Wirth et al., 1997). The numbers used for the strains are the same as in Table 1. (.) represents residues that are conserved with respect to the α-sarcin gene.

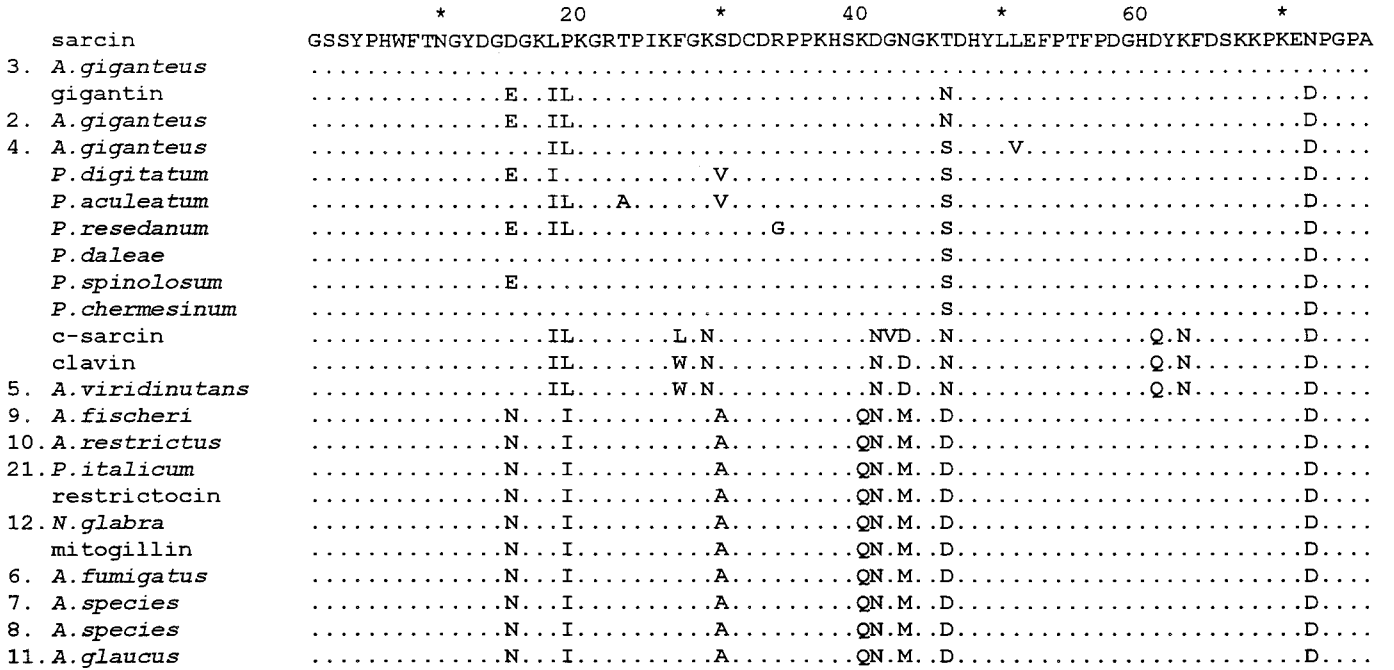

Fig. 6. Alignment of the amino acid sequences deduced from genomic sequences obtained in this work and from the databases (Lin et al., 1997), and homologous fragments from already published ribotoxin sequences (Sacco et al., 1983; López-Otín et al., 1984; Fernández-Luna et al., 1985; Parente et al., 1996; Huang et al., 1997; Wirth et al., 1997). The number used for the strains are the same as in Table 1. (.) represents residues that are conserved with respect to α-sarcin.

mitogillin and new ones from *A. fischeri*, *A. glaucus* and *N. glabra*, which are very similar or identical (*A. glaucus*, *N. glabra*) to mitogillin. A second group of a different sequence pattern is found in the ribotoxins from *A. clavatus*, clavin and c-sarcin (Parente et al., 1996; Huang et al., 1997). The sequence obtained from *A. viridinutans* would fall among this group too. Finally, the third group would be constituted by sequences of ribotoxins produced by different *A. giganteus* strains, α-sarcin and gigantin among them, as well as *Penicillium* ribotoxins described by Lin et al. (1997). *A. giganteus* strains NRRL 1780 and NRRL 4763 are included in this group.

4. Discussion

The results presented show that the occurrence of the α-sarcin-like ribotoxins is much wider than expected, involving new *Aspergillus* strains, and another species from a related genus, *Neosartorya*. Although the first ribotoxins were discovered more than 30 years ago (Olson and Goerner, 1965; Olson et al., 1965), the question of the biological goal of ribotoxins has not been elucidated. Their production is associated with the formation of conidiophores (Brandhorst and Kenealy, 1992; Yang and Kenealy, 1992) and deters insect feeding on the fungus (Brandhorst et al., 1996); thus, the ribotoxins may be a component of a natural defence mechanism. In human infections, they are not essential virulence factors (Paris et al., 1993; Smith et al., 1993, 1994), but they seem to be involved in the pathogenicity of allergic bronchopulmonary aspergillosis (Kurup et al., 1994).

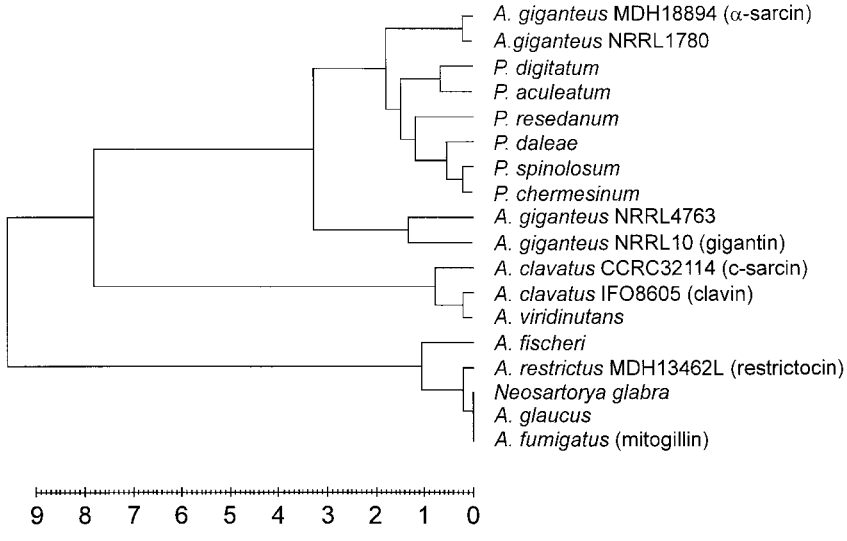

Fig. 7. Phylogenetic tree constructed with nucleotide sequences of Fig. 5, using the WET program (J. Dopazo, TDI S.A., Madrid, Spain). The scale is in percentage of Jukes and Cantor distances.

The wide distribution of these ribotoxins among some filamentous fungi raises interesting questions concerning their evolution. It has been suggested that they are naturally engineered toxins (Lamy et al., 1992; Kao and Davies, 1995), evolved from fungal guanyl-specific ribonucleases, with which they share a common folding motif (Campos-Olivas et al., 1996b; Yang and Moffat, 1996) and catalytic ribonucleolytic mechanism (Lacadena et al., 1998). During evolution, they would have acquired "extra" coding regions that can be responsible for additional molecular features such as specificity against a single phosphodiester bond in the ribosome and ability to translocate across membranes.

The study of the influence of evolution in structure and activity of different RNases families is more advanced in the case of mammalian RNases, where two families can be distinguished, represented by pancreatic RNase A and bovine seminal RNase (Benner et al., 1997). Analysis of evolution between the two families has permitted the identification, by protein engineering, of the sequence determinants for structural and functional difference among the two families (Ciglic et al., 1998; Opitz et al., 1998). This relationship is not so well established for microbial RNases.

Comparison of the primary sequences of ribotoxins obtained in this and previous work permits the identification of several groups according to their phylogeny (Fig. 7). The similarity among these groups is still high (90% for the fragment studied and 85% for the whole protein), presumably due to the high specificity of functions present in such a small structure, but we can clearly define at least three different groups. The first contains the *Aspergillus giganteus* and *Penicillium* strains. The second one includes *A. clavatus* and *A. viridinutans*. The third group contains the fungi producing ribotoxins more similar to mitogillin. The first two groups comprise proteins related to α-sarcin and seem to be phylogenetically more distant from the latter (Fig. 6). Actually, the three groups correspond quite well to morphologically defined groups (Raper and Fennel, 1965), close similarity to α-sarcin of the *Penicillium* ribotoxins (Lin et al., 1997) is perhaps the most striking relationship.

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References

Arruda, L.K., Platts-Mills, T.A.E., Fox, J.W., Chapman, M.D., 1990. *Aspergillus fumigatus* allergen I,

a major IgE-binding protein, is a member of the mitogillin family of cytotoxins. J. Exp. Med. 172, 1529–1532.

Benner, S.A., Ciglic, M.I., Haugg, M., Jermann, T.M., Opitz, J.G., Raillard-Yoon, S.-A., Soucek, J., Stackhouse, J., Trabesinger-Rüf, N., Trautwein, K., Zankel, T.R., 1997. Evolutionary reconstructions in the ribonuclease family. In: D'Alessio, G., Riordan, J.F. (Eds.), Ribonucleases: Structures and Functions. Academic Press, San Diego, pp. 213–243.

Bezborodova, S.I., Khovoda, O.M., Stepanov, V.M., 1983. The complete amino acid sequence of ribonuclease C2 from *Aspergillus clavatus*. FEBS Lett. 159, 256–258.

Brandhorst, T.T., Kenealy, W.R., 1992. Production and localization of restrictocin in *Aspergillus restrictus*. J. Gen. Microbiol. 138, 1429–1435.

Brandhorst, T., Dowd, P.F., Kenealy, W.R., 1996. The ribosome-inactivating protein restrictocin deters insect feeding on *Aspergillus restrictus*. Microbiology 142, 1551–1556.

Campos-Olivas, R., Bruix, M., Santoro, J., Martínez del Pozo, A., Lacadena, J., Gavilanes, J.G., Rico, M., 1996a. ^{1}H and ^{15}N nuclear magnetic resonance assignment and secondary structure of the cytotoxic ribonuclease α-sarcin. Protein Science 5, 962–972.

Campos-Olivas, R., Bruix, M., Santoro, J., Martínez del Pozo, A., Lacadena, J., Gavilanes, J.G., Rico, M., 1996b. Structural basis for the catalytic mechanism and substrate specificity of the ribonuclease α-sarcin. FEBS Lett. 399, 163–165.

Ciglic, M.I., Jackson, P.J., Raillard, S.A., Haugg, M., Jermann, T.M., Opitz, J.G., Trabesinger-Rüf, N., Benner, S.A., 1998. Origin of dimeric structure in the ribonuclease superfamily. Biochemistry 37, 4008–4022.

D'Alessio, G., Di Donato, A., Parente, A., Piccoli, R., 1991. Seminal RNase: a unique member of the ribonuclease superfamily. Tr. Biochem. Sci. 16, 104–106.

Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K., Mattick, J.S., 1991. "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucl. Acids Res. 19, 4008.

Fernández-Luna, J.L., López-Otín, C., Soriano, F., Méndez, E., 1985. Complete amino acid sequence of the *Aspergillus* cytotoxin mitogillin. Biochemistry 24, 861–867.

Fernández-Puentes, C., 1983. Permeability to alpha-sarcin in virus-infected cells. Mol. Cell. Biochem. 50, 185–191.

Fernández-Puentes, C., Carrasco, L., 1980. Viral infection permeabilizes mammalian cells to protein toxins. Cell 20, 769–775.

Gasset, M., Martínez del Pozo, A., Oñaderra, M., Gavilanes, J.G., 1989. Study of the interaction between the antitumour protein α-sarcin and phospholipid vesicles. Biochem. J. 258, 569–575.

Gasset, M., Oñaderra, M., Thomas, P.G., Gavilanes, J.G., 1990. Fusion of phospholipid vesicles produced by the anti-tumour protein α-sarcin. Biochem. J. 265, 815–822.

Gasset, M., Oñaderra, M., Martínez del Pozo, A., Schiavo, G.-P., Laynez, J., Usobiaga, P., Gavilanes, J.G., 1991a. Effect of the antitumour protein α-sarcin on the thermotropic behaviour of acid phospholipid vesicles. Biochim. Biophys. Acta 1068, 9–16.

Gasset, M., Oñaderra, M., Goormaghtigh, E., Gavilanes, J.G., 1991b. Acid phospholipid vesicles produce conformational changes on the antitumor protein α-sarcin. Biochim. Biophys. Acta 1080, 51–58.

Huang, K.-C., Hwang, Y.-Y., Hwu, L., Lin, A., 1997. Characterization of a new ribotoxin gene (c-*sar*) from *Aspergillus clavatus*. Toxicon 35, 383–392.

Kao, R., Davies, J., 1995. Fungal ribotoxins: a familiy of naturally engineered targeted toxins. Biochem. Cell Biol. 73, 1151–1159.

Kurup, V.P., Kumar, A., Kenealy, W.R., Greenberger, P.A., 1994. *Aspergillus* ribotoxins react with IgE and IgG antibodies of patients with allergic bronchopulmonary aspergillosis. J. Lab. Clin. Med. 123, 749–756.

Lacadena, J., Martínez del Pozo, A., Barbero, J.L., Mancheño, J.M., Gasset, M., Oñaderra, M., López-Otín, C., Ortega, S., García, J.L., Gavilanes, J.G., 1994. Overproduction and purification of biologically active native fungal alpha-sarcin in *Escherichia coli*. Gene 142, 147–151.

Lacadena, J., Martínez del Pozo, A., Lacadena, V., Martínez-Ruiz, A., Mancheño, J.M., Oñaderra, M., Gavilanes, J.G., 1998. The cytotoxin α-sarcin behaves as a cyclizing ribonuclease. FEBS Lett. 424, 46–48.

Lamy, B., Davies, J., 1991. Isolation and nucleotide sequence of the *Aspergillus restrictus* gene coding for the ribonucleolytic toxin restrictocin and its expression in *Aspergillus nidulans*: the leader sequence protects producing strains from suicide. Nucl. Acids Res. 19, 1001–1006.

Lamy, B., Mouataouakil, M., Latgé, J.P., Davies, J., 1991. Secretion of a potential virulence factor, a fungal ribonucleotoxin, during human aspergillosis infections. Mol. Microbiol. 5, 1811–1815.

Lamy, B., Davies, J., Schindler, D., 1992. The *Aspergillus* ribonuclelytic toxins (ribotoxins). In: Frankel, A.E. (Ed.), Genetically engineered toxins. Marcel Dekker, New York, pp. 237–258.

Lin, A., Huang, K.-C., Hwu, L., Tzean, S.S., 1995. Production of type II ribotoxins by *Aspergillus* species and related fungi in Taiwan. Toxicon 33, 105–110.

Lin, A., Ciou-Jau, C., Tzean, S.S., 1997. EMBL/GenBank/DDBJ databases, accesion numbers AF012812–AF012817.

López-Otín, C., Barber, D., Fernández-Luna, J.L., Soriano, F., Méndez, E., 1984. The primary structure of the cytotoxic restrictocin. Eur. J. Biochem. 143, 621–634.

Mancheño, J.M., Gasset, M., Lacadena, J., Ramón, F., Martínez del Pozo, A., Oñaderra, M., Gavilanes, J.G., 1994. Kinetic study of the aggregation and lipid-mixing produced by α-sarcin on phosphatidylglycerol and phosphatidylserine vesicles: stopped-flow light-scattering and fluorescence energy transfer measurements. Biophys. J. 67, 1117–1125.

Miller, S.P., Bodley, J.W., 1988. The ribosomes of *Aspergillus giganteus* are sensitive to the cytotoxic action of α-sarcin. FEBS Lett. 229, 388–390.

Oka, T., Aoyama, Y., Natori, Y., Katano, T., Endo, Y., 1990. The complete nucleotide sequence of cDNA for the cytotoxin alpha-sarcin. Nucl. Acids Res. 18, 1897.

Olmo, N., Turnay, J., Lizarbe, M.A., Gavilanes, J.G., 1993. Cytotoxic effects of α-sarcin, a ribosome inactivating protein, in cultured Rugli cells. S.T.P. Pharma Sci. 3, 93–96.

Olson, B.H., Goerner, G.L., 1965. Alpha-sarcin, a new antitumor agent, I. Isolation, purification, chemical composition, and the identity of a new amino acid. Appl. Microbiol. 13, 314–321.

Olson, B.H., Jennings, J.C., Roga, V., Junek, A.J., Schuurmans, D.M., 1965. Alpha-sarcin, a new antitumor agent, II. Fermentation and antitumor spectrum. Appl. Microbiol. 13, 322–326.

Oñaderra, M., Mancheño, J.M., Gasset, M., Lacadena, J., Schiavo, G., Martínez del Pozo, A., Gavilanes, J.G., 1993. Translocation of α-sarcin across the lipid bilayer of asolectin vesicles. Biochem. J. 295, 221–225.

Oñaderra, M., Mancheño, J.M., Lacadena, J., de los Ríos, V., Martínez del Pozo, A., Gavilanes, J.G., 1998. Oligomerization of the cytotoxin α-sarcin associated to phospholipid membranes. Mol. Memb. Biol. 15, 141–144.

Opitz, J.G., Ciglic, M.I., Haugg, M., Trautwein-Fritz, K., Raillard, S.A., Jermann, T.A., Benner, S.A., 1998. Origin of the catalytic activity of bovine seminal ribonuclease against double-stranded DNA. Biochemistry 37, 4023–4033.

Parente, D., Raucci, G., Celano, B., Pacilli, A., Zanoni, L., Canevari, S., Adorate, E., Colnaghe, M.I., Dosio, F., Arpicco, S., Catell, L., Mele, A., De Santis, R., 1996. Clavin, a type-I ribosome-inactivating protein from *Aspergillus clavatus* IFO 8605. Eur. J. Biochem. 239, 272–280.

Paris, S., Monod, M., Diaquin, M., Lamy, B., Arruda, L.K., Punt, P.J., Latgé, J.P., 1993. A transformant of *Aspergillus fumigatus* deficient in the antigenic cytotoxin ASPFI. FEMS Microbiol. Lett. 111, 31–36.

Raper, T.B., Fennel, D.I., 1965. The genus *Aspergillus*. Williams and Wilkins, Baltimore.

Roux, K.H., Hecker, K.H., 1997. One-step optimization using touchdown and stepdown PCR. Methods Mol. Biol. 67, 39–45.

Sacco, G., Drickamer, K., Wool, I.G., 1983. The primary structure of the cytotoxin alpha-sarcin. J. Biol. Chem. 258, 5811–5818.

Schindler, D., Davies, J., 1977. Specific cleavage of ribosomal RNA caused by alpha-sarcin. Nucleic Acid Res. 4, 1097–1100.

Shapiro, R., Riordan, J.F., Vallee, B.L., 1986. Characteristic ribonucleolytic activity of human angiogenin. Biochemistry 25, 3527–3532.

Smith, J.M., Davies, J.E., Holden, D.W., 1993. Construction and pathogenicity of *Aspergillus fumigatus* mutants that do not produce the ribotoxin restrictocin. Mol. Microbiol. 9, 1071–1077.

Smith, J.M., Tang, C.M., van Noorden, S., Holden, D.W., 1994. Virulence of *Aspergillus fumigatus*

double mutants lacking restrictocin and an alkaline protease in a low-dose model of invasive pulmonary aspergillosis. Infect. Immun. 62, 5247–5254.

St. Clair, D.K.S., Rybak, S.M., Riordan, J.F., Vallee, B.L., 1988. Angiogenin abolishes cell-free protein synthesis by specific ribonucleolytic inactivation of 40S ribosomes. Biochemistry 27, 7263–7268.

Turnay, J., Olmo, N., Jiménez, J., Lizarbe, M.A., Gavilanes, J.G., 1993. Kinetic study of the cytotoxic effect of α-sarcin, a ribosome inactivating protein from *A. giganteus*, on tumor cell lines: protein biosynthesis inhibition and cell binding. Mol. Cell. Biochem. 122, 39–47.

Wirth, J., Martínez del Pozo, A., Mancheño, J.M., Martínez-Ruiz, A., Lacadena, J., Oñaderra, M., Gavilanes, J.G., 1997. Sequence determination and molecular characterization of gigantin, a cytotoxic protein produced by the mould *Aspergillus giganteus* IFO 5818. Arch. Biochem. Biophys. 343, 188–193.

Yang, R., Kenealy, W.R., 1992. Regulation of restrictocin production in *Aspergillus restrictus*. J. Gen. Microbiol. 138, 1421–1427.

Yang, X., Moffat, K., 1996. Insights into specificity of cleavage and mechanism of cell entry from the crystal structure of highly specific *Aspergillus* ribotoxin restrictocin. Structure 4, 837–852.