

RAPD analyses of recombination processes in the entomopathogenic fungus *Beauveria bassiana*

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To understand the nature of recombination processes in *Beauveria bassiana*, double-auxotrophic complementary mutant strains were used to produce six heterokaryons by three different methods. Conidia from these heterokaryons were plated on selective media and stable haploid (but not diploid) recombinants were isolated. Single colony recombinants were recovered with both parental and non-parental random amplified polymorphic DNA (RAPD) profiles. These results suggest that a range of different recombination mechanisms may be occurring in *B. bassiana*.

INTRODUCTION

The biocontrol agent *Beauveria bassiana* is an entomopathogenic fungus isolated from various insect species in different parts of the world (Khachatourians 1986). A variety of techniques have shown that there is much variation among *B. bassiana* strains, including esterase polymorphism (Paccola-Meirelles & Azevedo 1990, St Leger *et al.* 1992a), electrophoretic protein fraction patterns (Gorstein *et al.* 1996), mitochondrial DNA (Hegedus & Khachatourians 1993), random amplified polymorphic DNA (RAPD) markers (Bidochka *et al.* 1994, Urtz & Rice 1997, Berretta *et al.* 1998, Castrillo, Wiegmann & Brooks 1999), pulsed field gel electrophoresis (Pfeifer & Khachatourians 1993), and ITS sequencing and PCR-RFLP (Coates, Hellmich & Lewis 2002). Variation is possibly due to parasexual recombination, which has been demonstrated under laboratory conditions (Paccola-Meirelles & Azevedo 1991, 1994, Coutealdier, Viaud & Riba 1996). In *Metarhizium anisopliae*, genetic exchange has been demonstrated between strains co-infecting an insect host (Leal-Bertioli *et al.* 2000), and it is possible that this event could also occur in *B. bassiana*. Li *et al.* (2001) described the teleomorph *Cordyceps bassiana* associated to a *B. bassiana* anamorph, suggesting that genetic

variation could also be increased by sexual recombination in this entomopathogenic species.

The diploid stage, which occurs during the classical parasexual cycle, has not been observed in *B. bassiana* with haploid recombinant conidia being isolated directly from the heterokaryons (Paccola-Meirelles & Azevedo 1991, Bello & Paccola-Meirelles 1998). Similar results have been described for other fungi, and the term parameiosis has been proposed for this special variation of the parasexual cycle (Bonatelli Azevedo & Valent 1983). One possible explanation for parameiosis is that the diploid stage is very unstable, with haploidization occurring inside the hyphae. However, other mechanisms besides the occurrence of transient diploids have been postulated to explain the emergence of recombinants directly from heterokaryons without diploid production (Stasz & Harman 1990, Bonatelli & Azevedo 1992).

In the present study, heterokaryons between complementary mutants were obtained and recombinant conidia were recovered. These recombinants were evaluated by RAPD analysis and the stability of molecular markers and genetic exchange between parental strains during heterokaryon formation were studied. The results suggest that different possible mechanisms exist for the emergence of *B. bassiana* parasexual recombinants.

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Table 1. Genetic markers and RAPD profiles of *Beauveria bassiana* strains used in the crosses and RAPD analyses.

Strains ^a	Isolated/derived from ^b	Genetic markers ^c	RAPD profile ^d	Reference
A (E3A1/1)	WT-1	<i>bio 4, cys 3</i>	I	This study
B (E8A1/1)	WT-1	<i>cyt 1, nia 1</i>	I	This study
C (196A11/3)	WT-2	<i>met 1, bio 1</i>	II	Bello & Paccola-Meirrelles (1998)
D (196W1/6)	WT-2	<i>pab 1, nic 3</i>	III	Bello & Paccola-Meirrelles (1998)
E (252J4/1)	WT-3	<i>nic 5, thi 3</i>	II	Paccola-Meirrelles (unpubl.)

^a All strains used are permanently preserved in the fungal collection of the Laboratory of Microorganism Genetics (ESALQ/USP, Piracicaba).

^b WT, wild type: WT-1 was isolated from *Anthonomus grandis* (Coleoptera, Curculionidae); WT-2 was isolated from *Hypothenemus hampei* (Coleoptera, Scolytidae); WT-3 was isolated from *Diatrea saccharalis* (Lepidoptera, Crambidae).

^c Nutritional requirements (bio, biotin; cys, cysteine; cyt, cystine; met, methionine; nic, nicotinic acid; pab, *p*-aminobenzoic acid; and thi, thiamine). The *nia* marker indicates inability to grow on medium with nitrate as the only source of nitrogen.

^d See Fig. 1.

MATERIALS AND METHODS

Strains and growth conditions

Five double-auxotrophic strains (A, B, C, D and E) of *Beauveria bassiana* were used in this work (Table 1). Some mutants were obtained by us following UV irradiation of strains A and B. Mutant strains C, D and E were kindly supplied by Luzia D. Paccola-Meirrelles (UEL, Londrina PR, Brazil). The fungi were grown in minimal (MM) and complete (CM) media (Pontecorvo *et al.* 1953) at 28 °C.

Parasexual crosses

Heterokaryons were obtained by three different methods. In the first method, described by Pontecorvo *et al.* (1953) with modifications for the entomopathogenic fungus *Metarhizium anisopliae* by Messias & Azevedo (1980), about 10⁶ conidia of each of two strains with complementary genetic markers were inoculated into tubes containing 2 ml of liquid MM plus 1% liquid CM. After 7 d of static incubation at 28 °, the mycelial mat, which had formed on the surface of the medium, was teased out onto plates containing thick MM. In the second method, described by Azevedo & Roper (1970), 10⁶ conidia of two complementary strains were inoculated into liquid MM plus 2% CM and then transferred to 1 cm diam wells in MM plates. In the third method, described by Ingle & Hastie (1974), 10⁶ conidia of two strains were inoculated into liquid MM plus 2% CM and individual drops were then transferred to sterile Petri dish lids containing filter paper soaked in distilled water. After incubation for 48 h at 28 °, the mats that had formed on the drops were transferred to MM plates. Irrespective of the method used the plates were subsequently incubated at 28 ° for 20 d.

Detection of recombinants

Samples of about 10⁶ conidia from each heterokaryon were plated onto solid MM, which allowed the growth of possible diploids formed as well as haploid prototrophs. Recombinants carrying auxotrophic markers were selected by plating the same number of conidia

(10⁶) onto MM supplemented with all of the nutritional requirements of the parental strains except one for each parental strain. For example, in cross AD, conidia were plated onto MM + bio + nic; MM + bio + pab; MM + pab + cys and MM + cys + nic. For analysis, colonies that developed on MM and supplemented MM were plated onto MM, MM supplemented with all of the nutritional requirements of the parental strains and MM supplemented with all of the nutritional requirements except for one. Controls were carried out by plating about 10⁷ conidia from each of the parental strains onto the selective media to allow the growth of revertants. Viable counts were made by plating appropriate dilutions onto CM.

RAPD analysis

For DNA extraction, parental and recombinant strains were grown on CM plates for 72 h at 28 °, after which the mycelium formed was transferred to tubes and lyophilized for 48 h, ground in liquid nitrogen and the DNA extracted according Raeder & Broda (1985). RAPD analysis was carried out in 25 µl final volume, containing 45–55 ng of DNA template, 3 mM MgCl₂, 0.5 U Taq DNA polymerase (Life Technologies, Grand Island, NY), 0.4 mM primer (OPX1 – 5'-CTGGGCACGA-3'; OPX2 – 5'-TTCCGCCACC-3'; OPX6 – 5'-ACGCCAGAGG-3'; OPX7 – 5'-GAGCGAGGCT-3'; OPX11 – 5'-GGAGCCTCAG-3'; OPX12 – 5'-TCGCCAGCCA-3'; OPX13 – 5'-ACGGGAGCAA-3'; OPX17 – 5'-GACACGGACC-3' and OPX19 – 5'-TGGCAAGGCA-3' Operon Technologies, Alameda, CA), 0.2 mM of each dNTP in 20 mM pH 8.4 Tris-HCl containing 50 mM KCl. PCR amplifications were conducted with an initial denaturation at 92 ° for 4 min, followed by 40 PCR amplification cycles consisting of denaturing at 92 ° for 1 min, annealing at 37 ° for 1.5 min, and extension at 72 ° for 2 min. The last cycle of extension was extended to 5 min, and the amplification products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. All reactions were repeated at least twice and negative controls that contained water instead of DNA were also used.

Table 2. Auxotrophic requirements and RAPD patterns in recombinants from parasexual crosses between different auxotrophic strains of *Beauveria bassiana*.

Heterokaryons	Recombinants	Requirement of recombinants	Number of isolates ^a	RAPD pattern
AD	AD1	cys	22	III
I × III ^b	AD2	pab	1	III
	AD3	nic, cys	1	II
AE	AE1	prototrophic	89	II
I × II	AE2	nic, cys	10	II
	AE3	thi	1	II
	AE4	nic	1	III
	AE5	cys	1	I
	AE6	bio	1	I
BC	BC1	prototrophic	26	III
I × II	BC2	bio	78	II
	BC3	met	2	R1 ^c
BE (I × II)	BE1	nic	8	II
CD	CD1	bio	64	R2 ^d
II × III	CD2	prototrophic	40	R2
	CD3	nic	4	R2
CE	CE1	bio	174	II
II × II	CE2	met	77	R2
	CE3	thi	27	R2
	CE4	nic	1	R2

For abbreviations of nutritional requirements see Table 1.

^a More than one isolate derived from the same heterokaryon was found, 2–5 isolates with the same nutritional requirement(s) were characterized by RAPD.

^b RAPD profiles of the parents used in the cross.

^c R1, recombinant pattern 1.

^d R2, recombinant pattern 2.

RESULTS AND DISCUSSION

Heterokaryon and recombinant production

Using the three methods of producing heterokaryons, six successful instances of heterokaryotic growth were obtained from all of the possible combinations of the strains. All methods resulted in heterokaryon formation but not for all crosses. The only method which produced all six heterokaryotic recombinants was that described by Azevedo & Roper (1970). This methodology was also less time consuming compared to the two others used for obtaining recombinant conidia. Plating of about 10^7 conidia from each parental mutant strain onto selective media produced no revertants. Recombinants were obtained directly from the conidia of the heterokaryons (Table 2). In view of reversion frequencies less than 10^{-7} , the recombinants cannot be explained by back mutations. Large differences in the numbers of different recombinant genotypes are explained by recombination events occurring earlier or later during heterokaryon development.

That diploids were not observed suggests the occurrence of parameiosis, which has already been described in *Beauveria bassiana* by Paccola-Meirelles & Azevedo (1991) and in other fungi such as *Metarhizium anisopliae* (Silveira & Azevedo 1987) and *Trichoderma pseudokoningii* (Furlaneto & Pizzirani-Kleiner 1992, Bagagli *et al.* 1995). Genetic flow in mitospore fungi may occur by several processes (Leal-Bertioli

et al. 2000) including transformation (Stasz & Harman 1990, Harman, Hayes & Ondik 1998) and exchange of extranuclear elements, such as cytoplasmic RNAs (virus-like particles), inverttrons (cytoplasmically transmitted dsDNAs with terminal inverted repeats) and circular mitochondrial plasmids (Bainbridge 1987, Kistler & Miao 1992, St Leger *et al.* 1992b, Harman *et al.* 1998).

RAPD analysis of recombinants

RAPD analysis was conducted of the original double-auxotrophic mutants and the recombinants derived from heterokaryons. Only the well-defined bands were considered. All primers analyzed resulted in polymorphic and scorable bands, which allowed to differentiate the parental and recombinant strains. For ease of understanding, only the RAPD profile generated by the primer OPX7 is shown (Fig. 1). According to Table 1 and Fig. 1, the double auxotrophic strains used showed three RAPD patterns designated I, II, and III. Pattern II from strains C and E was closer to pattern I, while pattern III showed a more divergent band profile (Fig. 1).

The number of isolates showing the same nutritional requirements derived from heterokaryons, varied from just one, such as in recombinants AD2 and AD3 to a maximum of 174 isolates as in recombinant CE1 (Table 2). When more than one isolate derived

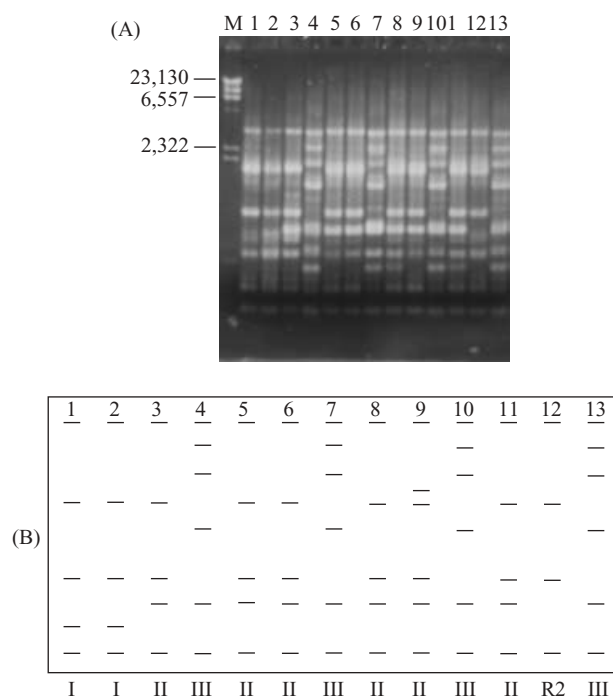


Fig. 1. (A) RAPD profile generated by the primer OPX-07, using genomic DNA from parental and recombinants strains. (B) Graphic representation of bands that were considered for analysis. (1) Strain A (profile I); (2) Strain B (profile I); (3) Strain C (profile II); (4) Strain D (profile III); (5) Strain E (profile II); (6) Rec BE1 (profile II); (7) Rec BC1 (profile III); (8) Rec BC2 (profile II); (9) Rec AE2 (profile II); (10) Rec AE4 (profile III); (11) Rec CE1 (profile II); (12) Rec CD1 (profile R2); (13) RecAD2 (profile III); and M) λ DNA digested with *Hind* III (molecular weights at left; Life Technologies).

from the same heterokaryon had identical nutritional requirements, two to five of them were characterized by RAPD technique. In all cases, the same profiles were found for identical isolates from a given heterokaryon, suggesting that they derived from the same recombination event. Similar results relating to large numbers of identical isolates from the same heterokaryon were already reported in *Beauveria bassiana* (Paccola-Meirelles & Azevedo 1994) and *Metarhizium anisopliae* (Bagagli, Valadares & Azevedo 1991).

Recombinants derived from heterokaryons BC, CD and CE showed not only the RAPD profiles that were the same as those of the parental strains used to produce the heterokaryons, but also novel combinations of parental bands (Table 2). This segregation of parental bands suggests that diploidization followed by recombination occurred during formation of the heterokaryons. In crossings AE and BC, which used parental strains with patterns I and II, recombinant isolates with pattern III were also observed (Table 2), indicating that this profile could be a result of recombination.

Recombinants derived from the heterokaryon BE showed RAPD profiles identical to those observed in parent E (pattern II) while all those obtained

from heterokaryon CD showed recombinant profiles (Table 2). These results suggest that different recombination process could be occurring in these heterokaryons. Besides the parasexual cycle, it is also possible that some transfer of DNA occurred from one strain to the other. Since RAPD bands are usually derived from repetitive DNA (Arnau, Housego & Oliver 1994), these sequences from one strain could find homologous regions in the other strain, allowing DNA recombination.

Recombinants from AE, with one exception, showed RAPD profiles identical to one or other of the parental strains that formed the heterokaryon (Table 2), with predominance of the pattern II suggesting that some selection may be occurring. This possible selection was also observed in the AD cross, since most of the recombinants analysed showed pattern III, which belongs to parent D; only one recombinant presented pattern II, which was not present in the parental strains used in this heterokaryon. The preferential recovery of one of the parental types from a heterokaryon has already been reported in *M. anisopliae* (Bagagli, Valadares & Azevedo 1991) and *B. bassiana* (Paccola-Meirelles & Azevedo 1991, Bello & Paccola-Meirelles 1998). Working with interstrain protoplast fusion in *Trichoderma*, Stasz & Harman (1990) reported that isozyme profiles of the majority of the progeny tested were identical to one parental strain, which they called the 'prevalent phenotype'.

These results suggest that different mechanisms are involved in producing *B. bassiana* recombinants. The RAPD profile found in all or some recombinants from heterokaryons BC, CD and CE suggest transient diploid formation followed by intra-hyphae haploidization. However, recombinants with RAPD profiles identical to one or other of the parental strains were recovered from all, except one of the heterokaryons. It thus appears that recombination can occur not only by diploid formation followed by haploidization and/or mitotic crossing-over, but also by some kind of intra-hyphae transformation process where one parental genome is a receptor for DNA from another parental genome. Cell lysis, which is induced when hyphal fusions occur between strains with heterokaryon incompatibility in filamentous fungi (Glass, Jacobson & Shiu 2000) may release genetic material resulting in intra-hyphae transformation. Similar findings have been reported in some other fungi, with Stasz & Harman (1990), and Harman, Hayes & Ondik (1998) coining the term 'interstrain gene transfer' to explain the process by which some *Trichoderma* recombinants were formed during protoplast fusion. These authors suggest that there were two types of strains, non-prevalent and prevalent. They proposed that some of the nuclei from the non-prevalent strain are degraded into DNA fragments, which insert themselves into the genome of the prevalent strain to produce recombinants without altering the RAPD banding pattern. Viaud *et al.* (1996) also recovered recombinants with

the profile of only one of the parental strains while investigating the protoplast fusion products of two *Beauveria* species.

The insertion of transposable elements (transposons) into a parental genome could also produce recombinants without any detectable modification of RAPD patterns, and the occurrence of about five copies per cell of a transposon (*hupfer*) has been described in *B. bassiana* (Maurer *et al.* 1997). Transposons have also been reported in several other filamentous fungi (Harman *et al.* 1998) and in *Neurospora crassa* at least one transposable element was transmitted between the nuclei in a heterokaryon (Kinsey 1990).

Recombinants with RAPD profiles different from the double-auxotrophic mutants used as parental strains but similar to other parental strains not involved in the heterokaryon occurred in the AD, AE and BC crosses. This may be explained by transient diploid formation. Chromosomal rearrangements may be present in these mutant strains as has been reported by Bello & Paccola-Meirelles (1998). Chromosomal rearrangements may have been produced in the early stages of mutant induction (in this case by γ -irradiation) and account for the differences in RAPD pattern among parental mutant strains. The replacement of part or the entire abnormal chromosome by the normal one from the other parental strain involved in the heterokaryon would explain the return of the original RAPD pattern shown by some recombinant isolates.

Variations in somatic recombination have already been described in several fungi and different mechanisms proposed to explain these variations. In the study reported in the present paper, transfer of DNA by intra-strain transformation or by transposons, as well as production of transient diploids are possible mechanisms to explain the observed parameiotic processes. The data indicate that in *B. bassiana* such mechanisms can occur concomitantly. Although the sexual stage is not present in this species, the high variability among *B. bassiana* strains isolated from nature may indicate that these recombination processes also occur in the field or during co-infection of insect hosts by different strains as shown by Leal-Bertioli *et al.* (2000) in the entomopathogenic fungus *M. anisopliae*.

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