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Abstract

Generally *niaD* mutants of fungi are selected by spontaneous mutations on appropriate minimal medium supplemented with various concentrations of KClO3 and a nitrogen source (Daboussi *et al.* 1989 Curr. Genet. 15:453-456; Johnstone et al. 1990 Gene 90:181-192; Malardier et al.1989 Gene 78:147-156; Unkles *et al.*1989 Gene 78:157-166). But in case of entomopathogenic fungi it has been observed that niaDmutants isolated simply by spontaneous mutation on chlorate were not stable, (Table-1). Therefore a method has been developed to isolate stable *niaD* mutants of these fungi by treating protoplasts with ethane methane sulfonate (EMS).

A rapid method for isolation of stable *niaD* and *crnA* mutants of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*

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Generally *niaD* mutants of fungi are selected by spontaneous mutations on appropriate minimal medium supplemented with various concentrations of KClO3 and a nitrogen source (Daboussi *et al*. 1989 Curr. Genet.**15**:453-456; Johnstone *et al*. 1990 Gene **90**:181-192; Malardier *et al*.1989 Gene **78**:147-156; Unkles *et al*.1989 Gene **78**:157-166). But in case of entomopathogenic fungi it has been observed that *niaD*mutants isolated simply by spontaneous mutation on chlorate were not stable, (Table-1). Therefore a method has been developed to isolate stable *niaD* mutants of these fungi by treating protoplasts with ethane methane sulfonate (EMS).

The method reported here is advantageous as it yielded more mutants when compared to the method of spontaneous mutation. Secondly, mutants obtained by EMS treatment were more stable than spontaneous *niaD* mutants. Their reversion frequency was less than one in 10^7 viable conidia, whereas comparatively high reversion frequency was obtained, 100 and 60 in 107 viable conidia of spontaneous mutants of *Metarhizium anisopliae* and *Beauveria bassiana*, respectively. Protoplasts of *B. bassiana* and *M. anisopliae* obtained by the method of Shimizu, 1986 J. Seric. Sci. Japan **5**:510- 517,(approximately 10^6) were incubated with 15 ul ethane methane sulfonate (d=1.17g, Sigma) in 1 ml of stablizing medium (0.02M phosphate buffer pH 7.2 containing 0.6M KCl and 2mM $MgCl_2$) at 28 +/- 1 C for 1 h. These protoplasts were then washed twice with 10 ml of stablizing medium by centrifuging at 1,000 X g for 10 minutes, diluted and then spread on plates containing a regeneration medium (2% sucrose; 1% peptone; 0.5% NaCl; 3% yeast extract and 2% agar agar, pH 7.0). Conidia from surviving colonies were harvested and suspended in Tween 80 aqueous suspension (50 ul/100 ml distilled water). Dilutions of this suspension were plated again on a minimal medium (1% glucose; 0.1% K_2HPO_4; 0.5% $MgSO_4$; 0.5% KCl; 0.001% $FeSO_4 \cdot 7H_2O$; 0.005% EDTA disodium salt, pH 6.5) containing 10 mM glutamate as sole source of nitrogen and 470 mM Chlorate. These plates were incubated at 28 C +/-1 C for five days.

It was found that mutants arose at a frequency of 10 and 8 in 10^6 viable conidia of *B. bassiana* and *M. anisopliae* respectively. These mutants were isolated and purified further on a minimal medium containing chlorate and glutamate. The ability of these mutants to grow on nitrate, nitrite, ammonium, hypoxanthin, proline and glutamate as sole source of nitrogen was assessed (Table 2). Of the 18 mutants tested, two each of *B. bassiana* and *M. anisopliae* had phenotypes indicative of *niaD*$^-$. Two were found to be *crnA*$^-$ of *M. anisopliae* while one *crnA*$^-$ of *B. bassiana* was noticed. It was found on testing two *niaD* mutants

(Figure 1) designated *niaD-1* and *niaD-4* of *B. bassiana* and *M. anisopliae* respectively, that reversion to nitrate prototrophy was less than one in 10,sup>7 viable conidia, whereas reversion frequencies of spontaneous mutants was more than 100 and 60 in 10^7 viable conidia of *B.bassiana* and *M. anisopliae*, respectively. Hence EMS treated *niaD*$^-$ mutants should be suitable for future experiments involving protoplast fusion and transformation.

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Table 1. Reversion frequencies of mutants of *B. bassiana* and *M. anisopliae* to wild type growth.[a]

Fungus	Number of mutants isolated		Reversion frequencies	
	Spontaneous[b]	EMS	Spontaneous	EMS
B. bassiana	4	10	>100	<1
M. anisopliae	2	8	>60	<1

[a]Reversion frequencies in 10^6 viable conidia.

[b]Method of Malairdair *et al*. 1989 (Gene **78**:147-156) was followed.

Table 2. Properties of *B. bassiana* and *M. anisopliae* mutants defective in nitrate assimilation.

Gene[a]	Chlorate resistance[b]	Utilization of sole nitrogen source[c]						Summary[d]
		A	B	C	D	E	F	
niaD-	R	-	+	+	+	+	+	Nitrate Reductase structural gene
crnA-	R	+	+	+	+	+	+	encodes nitrate uptake in young cells

[a]The minus superscript denotes loss of functional mutations.
[b]R denotes resistance to chlorate.
[c]Symbol (+) denotes wild type level of growth and (-)denotes poor growth. A = nitrate; B = nitrite; C = ammonium; D = hypoxanthine; E = proline; F = glutamate.
[d] Summary of the role of the genes.

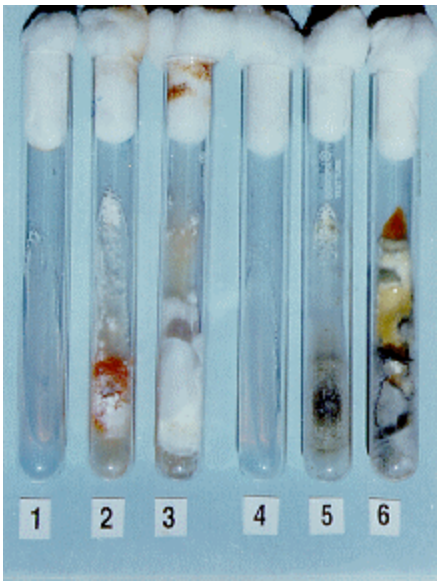

Figure 1. *niaD*-mutants of *Beauveria bassiana* (# 1-3) and *Metarhizium anisopliae* (# 4-6).#1 & 4: *niaD*-mutants not growing on minimal medium with nitrate as sole source of nitrogen; #2 & 5: growth on minimal medium containing chlorate and glutamate; #3 & 6: growth on complete medium.

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