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NOTE

Isolation and Characterization of Self-Fertile Suppressors from the Sterile *nsdD* Deletion Mutant of *Aspergillus nidulans*

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To identify downstream and/or interactive factors of the *nsdD* gene, which encodes a positive regulator of sexual development of *Aspergillus nidulans*, suppressor mutants displaying a self-fertile phenotype were isolated from a sterile *nsdD* deletion mutant. At least five different loci (*sndA-E*) were identified and genetically analyzed. In the *nsdD*$^+$ background, most of the suppressors showed a marked increment of sexual development, even under the stress conditions that normally inhibited sexual development. The common phenotype of the suppressor mutants suggested the involvement of the *snd* genes in the negative regulation of sexual development in response to the environmental factors.

***Keywords*:** *Aspergillus nidulans*, *nsdD*, suppressor, *snd*, sexual development

Unlike many other *Aspergillus* species that do not have a sexual development in their life cycle, *Aspergillus nidulans* (teleomorph=*Emericella nidulans*) undergoes sexual development that can be regulated by internal and external stimuli. Environmental conditions are important influences on developmental processes. Several factors including visible light and high osmolarity favor asexual sporulation (Mooney and Yager, 1990; Lee and Adams, 1994; Adams *et al.*, 1998; Han *et al.*, 2003). These factors also affect sexual development, usually in an opposite way. Protection of the culture plate from aeration and light almost completely inhibits asexual sporulation (Han *et al.*, 1990; Mooney and Yager, 1990). Under this condition, sexual differentiation and fruiting body formation can be easily observed. Taking advantage of this condition, we previously isolated and characterized several NSD (never in sexual development) mutants that failed to produce cleistothecia (Han *et al.*, 1994, 2001; Kim *et al.*, 2009). The mutants were divided into four complementation groups: *nsdA*, *nsdB*, *nsdC*, and *nsdD* (Han *et al.*, 1994). Among them, the *nsdD* gene has been isolated and predicted to encode a GATA type transcription factor containing the type IVb Cys_2/Cys_2 zinc finger DNA binding domain (Chae *et al.*, 1995). Deletion of *nsdD* resulted in impaired sexual development and over-expression of *nsdD* induced uncontrolled sexual sporulation, even under conditions that do not allow sexual development. Based on these evidences, it has been proposed that NsdD positively controls the early step of sexual development, and is necessary and sufficient for sexual development of *A. nidulans* (Han *et al.*, 2001).

Understanding the complex regulatory interactions between genes like *nsdD* that function primary in determining sexual sporulation and those that function in activating asexual sporulation will probably provide important clues to understand the mechanisms controlling both processes and their coordination in the *A. nidulans* life cycle. To this end, here we screened suppressor mutants from a *nsdD* deleted strain to study the function of NsdD and the genes that might genetically interact with NsdD. Genetic analysis revealed five complementation groups. The suppressors showed a common phenotype, in which sexual development was induced similar to *nsdD* over-expression mutant, even in the presence of KCl, light and acetate-containing media, which are the repression conditions of sexual development.

All *A. nidulans* strains used in this study are listed in Table 1. FGSC A4, the Glasgow wild type isolate of *A. nidulans*, was used as a *veA*$^+$ wild type. All FGSC strains were obtained from the Fungal Genetics Stock Center. For the convenience of outcross, several *veA*$^+$ strains carrying appropriate genetic markers (LDB115 or LDB302) were constructed in this work. KHH52 (Δ*nsdD*, *veA*$^+$; Han *et al.*, 2001) was used as host strain for isolation of suppressors. Ultraviolet (UV) mutagenesis was carried out as previously described (Han *et al.*, 1990). Construction of heterokaryons, diploid isolation and linkage group determination were performed according to Käfer (1977). Minimal and complete media with appropriate supplements were prepared as described previously (Han *et al.*, 1990). All strains were grown at 37°C unless otherwise described. Mutants were isolated on a complete medium containing 0.01% sodium deoxycholate to reduce colony size. Light illumination was carried out in a growth chamber equipped with white fluorescent and metal SP lamps (max. 4,000 lux) and temperature control system. Growth rate was deter-

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Table 1. Strains used in this study

Strain	Genotype	Source
A4	Wild type	FGSC[a]
A163	*SuA1adE20*, *yA2*, *adE20*; *AcrA1*; *phenA2*; *pyroA4*; *lysB5*; *sB3*; *nicB8*; *coA1, veA1*	FGSC
A786	*SuA1*, *anA1*, *biA1*; *AcrA1*; *galA1*, *ActA1*; *methG1*; *nicA2*; *sbA3*; *choA1*, *fwA2, veA1*	FGSC
VER7	*pabaA1*, *yA2*; Δ*argB*::*trpC*; *trpC801*	Han *et al*. (2001)
LDB115	*SuA1*, *anA1*, *biA1*; Δ*argB*::*trpC*; *methG1*; *choA1*	VER7 × A786
LDB302	*SuA1*, *anA1*, *biA1*; *galA1*, *methG1*; *sbA3*	VER7 × A786
KHH52	*pabaA1*, *yA2*; Δ*argB*::*trpC*; Δ*nsdD*::*argB*	Han *et al*. (2001)
KHH62	*pabaA1 yA2*; Δ*argB*::*trpC*; *niiA(p)*::*nsdD*	Han *et al*. (2001)

[a] Fungal Genetics Stock Center

mined by measuring single colony size following point-inoculation on solid CM medium containing 0.6 M KCl or 1.2 M sorbitol. The developmental phenotypes of the mutants were observed under the standard culture condition as previously described (Han *et al*., 1990). The conidia were harvested with a 5 mm-diameter cork borer and suspended in 1 ml of 0.8% Tween-80. The number of conidia was measured with a hemocytometer under a light microscope. The cleistothecia were directly scored under a dissecting microscope.

To generate the suppressors, the *nsdD* deletion strain KHH52 was mutagenized by UV irradiation and self-fertile mutants that could develop normal cleistothecia were isolated. Forty eight suppressor mutants, designated as SUNs (Suppressors of Δ*nsdD*), which displayed a similar growth rate to wild type, were obtained and cultured on standard condition to characterize their phenotypic traits. SUN16, SUN47, and SUN76 strains produced mature cleistothecia in the presence of 0.6 M KCl, which completely inhibited sexual development in the wild type, suggesting that these suppressors not only bypassed the requirement of NsdD for sexual development but also hyper-activated sexual development, making cells able to produce cleistothecia regardless of the environmental conditions. However, other SUN strains did not undergo sexual development on 0.6 M KCl-containing complete medium (Table 2).

Table 2. Developmental phenotypes of SUN and SND mutants

Strain	CM		CM+1 M KCl	
	Sex[a]	Asex[b]	Sex	Asex
A4	+	++	-	++++
KHH52	-	++++	-	++++
SUN16	+++	+	++	++
SUN76	+++	+	++	+
SUN47	+++	++	++	+++
SUN10	+	++	-	++
SUN40	+	+	-	++
SUN80	+	+	-	++
SUN12	+	+	-	++
SUN17	+	+	-	++
SUN91	+	+	-	++
SND16	++++	-	++++	-
SND76	++++	-	++++	-
SND47	+++	-	++	+
SND10	++++	-	+/-	++++
SND40	++++	-	+/-	++++
SND80	++++	-	+++	+
SND12	++++	-	+++	+
SND17	++++	-	+++	+
SND91	++++	-	+++	+

Strains were inoculated with 1×10^6 conidia/ml, which were incubated at 37°C for 4-5 days.

[a] Average number of mature cleistothecia per $5^2\pi$ mm^2 of three different areas of plate. -, 0; +, <20; ++, 20-50; +++, 50-100; ++++, >100

[b] Average number of conidia per $5^2\pi$ mm^2 of three different areas of plate. Agar blocks were removed with a cork borer, suspended in 1 ml Tween 80, and scored with a haemocytometer.

-, 0; +, $<1\times10^4$; ++, 1×10^4-1×10^5; +++, 1×10^5-1×10^7; ++++, $>1\times10^7$

To understand the function of the suppressors, it was necessary to know whether the suppression occurred from a single mutation. To this end, 15 randomly selected mutants, (SUN16, 47, 76, 22, 70, 10, 40, 35, 12, 80, 17, 91, 84, 87, and 28) were subjected to outcross with FGSC A786 followed by backcross with Δ*nsdD* strain (see below). While performing outcross, the SUN22, 28, 35, and 87 mutant strains could not generate heterokaryon. In addition, results from the progeny pattern of the heterokaryons revealed that SUN70 and 84 possessed more than two mutations (data not shown). Therefore, among the 15 mutants, six were discarded and, finally, nine strains were chosen for further genetic and physiological analyses. The phenotypes of the nine SUN mutants are summarized in Fig. 1 and Table 2.

To ascertain whether the suppressors had their own phenotypic traits under the *nsdD*$^+$ background, we isolated recombinant progenies, designated SND strains, carrying only the

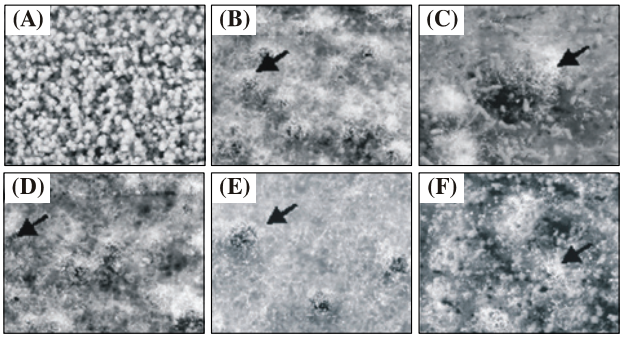

Fig. 1. Phenotypes of Δ*nsdD* suppressor mutants carrying Δ*nsdD* mutation (SUN). (A) KHH52, (B) SUN16, (C) SUN47, (D) SUN10, (E) SUN40, (F) SUN80. Strains were cultured for 5 days on MM plates. Arrows indicate the cleistothecia. Magnification ×40.

Table 3. Characterization of the SND mutants

Strain	Locus/allele	Linkage group	Growth (%)[a]	Dominant/Recessive
SND16	*sndA16*	II	95.7	Recessive
SND76	*sndA76*	II	94.8	Recessive
SND47	*sndB47*	V	80.0	Recessive
SND10	*sndC10*	II	98.3	Recessive
SND40	*sndD40*	V	66.1	Recessive
SND80	*sndE80*	VII	94.8	Recessive
SND12	*sndE12*	VII	94.8	Recessive
SND17	*sndE17*	VII	96.5	Recessive
SND91	*sndE91*	VII	98.3	Recessive

[a] Relative radial growth rate compared with wild type.

suppressor mutation without Δ*nsdD* mutation. This recombination experiment was performed as follows. All SUN strains were crossed with A786 and the segregants were obtained. From this first meiotic progeny, we selected auxotrophic *argB*$^-$ strains. Since the Δ*nsdD* locus is linked with wild type *argB* gene, *argB*$^-$ strains have to carry *nsdD*$^+$. To ascertain whether the first progenies of *argB*$^-$ were carrying *snd*$^+$ or *snd*$^-$, each of the first progeny was backcrossed with KHH52. From these second meiotic progenies, we selected *argB*$^+$ strains, which carried Δ*nsdD*, and checked that the segregation pattern that SUN phenotype (fertile) and Δ*nsdD* phenotype (sterile) was a 1:1 ratio, indicating their parent strain contained a *snd*$^-$ mutation. The isolated SND strains showed very similar phenotype of the *nsdD* over-expression mutant that exuberantly produced fruiting bodies but fewer conidia under normal conditions (Table 2).

The nine *snd*$^-$ strains were selected and counter-crossed with each other. According to the diploid phenotype they were divided into five complementation groups. SND16 and SND76, which did not complement each other, were grouped into *sndA* and their alleles were named *sndA16* and *sndA76*, respectively. Other mutant alleles were grouped and named in same way (Table 3). The mutants were crossed with FGSC A163 or A786 and heterozygous diploids were isolated. All of the heterozygous diploids exhibited same development phenotype as wild type diploid, indicating that the nine mutant alleles were all recessive (Table 3). The haploidization of the heterozygous diploids was induced by 1.7 ppm benomyl and confirmed by serial isolation on benomyl containing plates. The linkage of the *snd* genes was identified by analyzing the mitotic segregation patterns of *snd*$^-$ mutations (Table 3). The *sndA* and *sndC* genes were located on linkage group II, *sndB* and *sndD* on linkage group V and *sndE* on linakge group VII.

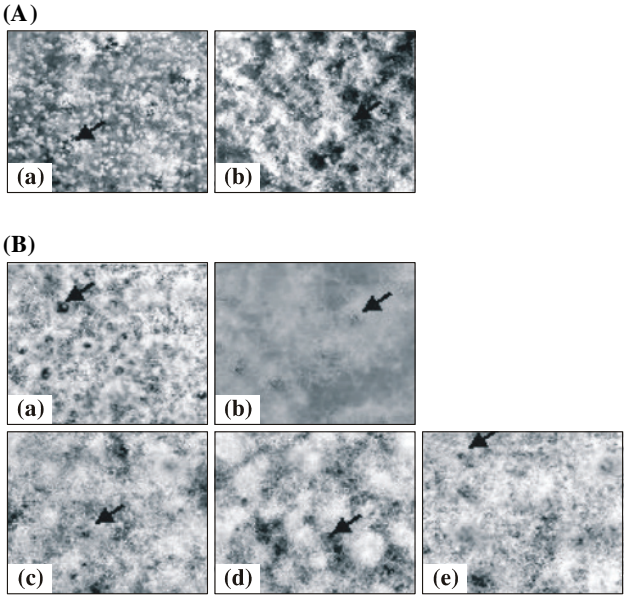

Fig. 2. Phenotypes of Δ*nsdD* suppressor mutants free of Δ*nsdD* mutation (SND). (A) control: (a) A4 (wild type), (b) KHH62 (*nsdD* over expressed strain). (B) SND mutants: (a) SND16, (b) SND47, (c) SND10, (d) SND40, (e) SND80. Strains were cultured for 5 days on MM plates. Arrows indicate the cleistothecia. Magnification ×40.

Most SND strains showed the common phenotype of hyper-activated sexual development similar to the *nsdD* over-expression strain (KHH62) under standard culture condition (Fig. 2). Concurrently, asexual sporulation in all mutants was largely inhibited. SND47 (*sndB47*) developed cleistothecia less than the other four mutants. Cleistothecia of the mutants matured within 4 days to produce wild type-like normal ascospores, except for SND47, which formed mature ascospores in 7 days (data not shown). Most of the mutants except SND47 abundantly produced cleistothecia on acetate medium whereas the wild type could not, indicating that the mutants were able to overcome the unfavorable culture condition for sexual development (Fig. 3). All the mutants also could develop cleistothecia under the visible light. The SND16, SND47, and SND80 mutants overcame the inhibition of sexual development caused by high salt or sorbitol osmotic stress. The overall phenotypes of the mutations, which resulted in the loss-of-function of the genes, strongly suggested that all the suppressor genes were negative controllers of sexual development and, at the same time, were necessary for asexual sporulation. It has been demonstrated that some of genes are also involved in stress-dependent determination of development. The *sndA16* mutation can suppress Δ*nsdC*, another positive regulator of sexual development (Kim *et al*., 2009), indicating that some of the suppressor genes are not specifically related with *nsdD* (data not shown). Only a few genes have been identified as negative regulators of sexual development in *A. nidulans*. The *rosA* (*r*epressor *o*f *s*exual development) gene that encodes a

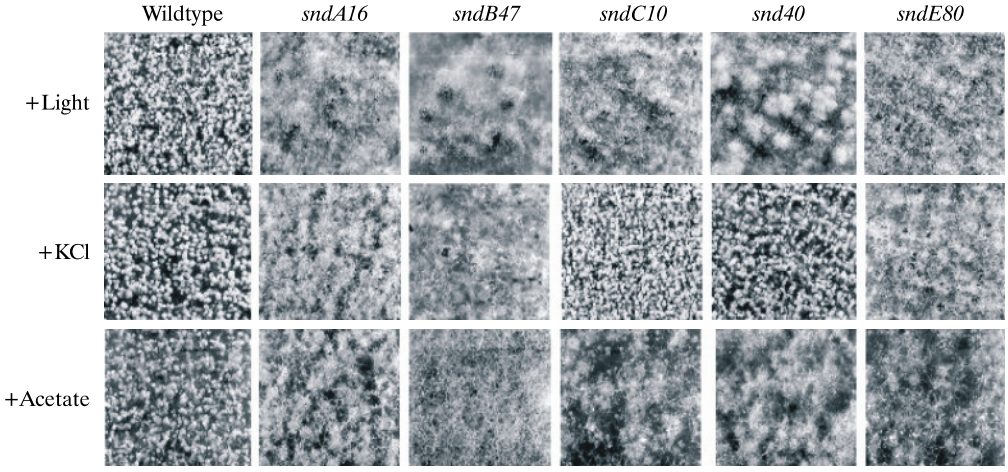

Fig. 3. Phenotypes of Δ*nsdD* suppressor mutants (SND) on various unfavorable conditions for sexual development. The mutants were cultured for 5 days on MM plates. Magnification ×40.

$Zn(II)_2Cys_6$ fungal specific transcription factor is a typical negative regulators (Vienken *et al*., 2005). The genes identified here are potent candidates of additional negative regulators of sexual development. The phenotypes of SUN mutants indicate that the *nsdD* gene is not absolutely required for sexual development. Instead, it may be controlled by or interacts with other development regulators complicatedly in decision of sexual or asexual development in response to various internal or external stimuli. Lack of NsdD prefers asexual development in *snd*⁻ mutants suggesting that the *nsdD* gene controls asexual development negatively. Molecular biological analysis will reveal the detailed function of the *snd* genes and the interaction with known positive regulators including the *nsdD* gene.

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