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Isolation and Characterization of the Phenotypic Revertants of a *Streptomyces coelicolor abs* Mutant

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We isolated phenotypic suppressors of an *abs*B (antibiotics synthesis suppression) strain. In the *abs*B colonies, all four antibiotics including two pigmented antibiotics were blocked so that no pigmentation could be found. We assumed that in the colonies with the suppressive (or reversive) mutation, both pigmentation would be restored so that the strains with suppressive mutation could be visually detected. Harvested *abs*B spores were treated with chemical mutagen along with electric shock, and were spread on specially formulated minimal medium plates. The pigmented colonies were isolated from the unpigmented majorities. In one candidate strain, the restoration and significant overproduction of actinorhodin and undecylprodigiosin were recognized. In three other candidate strains, the overproduction of actinorhodin and restoration of undecylprodigiosin were observed. The production of the two unpigmented antibiotics (CDA and methylenomycin) were visualized in the tested candidate strains. The strains with suppressive mutations would be very useful in elucidating the regulation network of antibiotics synthesis and overproduction of the antibiotics.

Key words: *abs*, overproduction, phenotypic revertant

Antibiotics are the most important beneficial metabolites produced by *Streptomyces* spp. By inhibiting the growth of other microorganisms, antibiotics may help streptomycetes retain relative advantages to better propagate (8).

However, the production of secondary metabolites like antibiotics might sometimes be a metabolical burden by consuming the nutritional resources in the early exponetial growth. Therefore the mechanism involved in the onset of antibiotics synthesis seems very complicated (5).

Various physiological factors including growth rate, responsiveness to catabolite repression, environmental signals and stresses might influence antibiotic synthesis. Many kinds of regulators are expected to be involved in monitoring many kinds of stimuli (8).

In *S. coelicolor*, where the onset of the synthesis of four antibiotics is coordinately regulated, several types of regulatory genes of antibiotic synthesis have been isolated.

Pathway specific regulators activate only single, specific pathways. For example, ActII/ORFIV (10, 20) activates only actinorhodin biosynthesis. RedD (11) regulator, in a similar fashion transcriptionally activates only undecylprodigiosin biosynthesis.

On the other hand, mutations in global regulators such as the *bld* genes (4, 7) simultaneously inhibit physical differentiation such as the production of aerial mycelium as well as the production of antibiotics.

Other global regulators such as Afs (12) and Abs (2) do not have any effect on physiological differentiation. Their effects are confined only to antibiotic synthesis. AfsR (13, 16, 21) is known to be a protein kinase, and several of its interacting components are now being unveiled. While only pigmented antibiotics synthesis is inhibited in *afs* mutants, pigmented antibiotics synthesis as well as the unpigmented antibiotics synthesis is blocked in *abs*A or *abs*B mutant. The mutational blocks in the *abs* mutants seem less leaky than that in the *afs*R mutant (8).

Because the discoveries of the *abs*A and *abs*B mutations are quite recent, the protein which interacts with the *abs* products has not yet been reported, and it will take sometime before the natures of the AbsA and AbsB products are fully understood (6).

In order to investigate the roles of these genes, we decided to isolate the phenotypical revertants of an *abs*B mutant by introducing a second mutation

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which can occur in unknown regulatory genes as well as be a 2nd site suppression mutation at the *abs*B gene itself. In both cases, we hoped to isolate the overproducers of antibiotics by substituting of regulators.

Materials and Methods

Bacterial strains, the media and the culture techniques

The strains used for genetic analysis were derivatives of *S. coelicolor* A3 (2). The strains used in this study are listed in Table 1. The minimal media and R2YE ,the nutrient agar, were prepared as described by Hopwood *et al* (14). The specially formulated minimal plate used for the selection of revertants had minor modification from the original formula such as the containing of 0.5~3% yeast extracts and 0.01~0.1% of casamino acid. We named it GHUYC.

Mutagenesis and mutant isolation

Spores of C120 (*abs*B) were incubated with a chemical mutagen containing N-methyl N-nitro-N-nitrosoguanidine (NTG) in 20% glycerol solution with 0.01M of $MgCl_2 \cdot 6H_2O$. The poles of an electric shocker charged at 38V AC was submerged in the sample solution while the spores were incubated with NTG. The components and effects of the electric shocker are described in detail elsewhere by Sun *et al.* (23). The NTG was removed from the spore suspension by centrifugation. The mutagenized spores were washed twice with R_2YE liquid medium, and finally resuspended in the 20% glycerol solution.

The mutagenized spores were serially diluted in 20% glycerol solution and 0.1 ml of each diluted sample were spread on the GHUYC plate. If the mutagenized samples had a 0.1~1% survival rate, properly diluted mutagenized spores were spread on 100 GHUYC plates as a single batch, and the rest of the spores were stored at -20°C until the next batch of fresh GHUYC plates were prepared.

Antibiotics assays

For the actinorhodin, and undecylprodigiosin assays, C120 and its phenotypic suppressor strains C 120 SM1, C120 SM7, C120 SM23 and J1501 were streaked on top of cellulose paper placed on GHUYE plates. After 5 days of growth, the cellulose paper with the well-grown mycelia was removed from the plate. The mycelia were then scraped from the cellulase paper and placed on weighing paper. 20 mg of mycelia were solubilized in a 0.5 ml chroloform solution at 30°C for 30 mins. 0.5 ml of 1N NaOH were added, and the two phases were shaken for a while. The aqueous phase was separated from the chlroform phase by centrifugation .The optical density at 590 nm was used to determine the amount of actinorhodin present (15).

0.5 ml of 1N HCl were added to the remaining chloroform. The two phases were separated after mixing well. The optical density at 530 nm of the aqueous phase was determined for the quantitaion of undecylprodigiosin.

Assay conditions for the calcium-dependent antibiotics were adapted from Lakey *et al.* (18). Briefly, agar plugs of 2-day-old cultures of the test strain which had been grown on Oxoid nutrient agar (ONA) were placed onto plates of ONA with and without added calcium [as $Ca(NO_3)_2$ to 12 mM].

Soft ONA or ONA with Ca^{++} was mixed with CDA-sensetive *Staphylococcus aureus* liquid cultures and overlayed around the plugs. After 8 hours of refrigeration, the plates were incubated overnight at 37°C.

The methylenomycin assay was performed as described by Adamidis *et al.* (2). Methylenomycin production and resistance genes are located on the

Table 1. Strains of *S. coelicolor* used in this study

Strains	Relevant genotype[a]	Source
J1501	*hisA1 uraA1* strA1 SCP1 SCP2 Pgl	K. Chater
J650	cysD18 mthB2 agarA1 NF SCP2$^+$	K. Chater
604	*hisA1 uraA1 strA1 act red* SCP1$^-$ SCP2$^-$ Pgl$^-$	W. Champness
C120	*hisA1 uraA1 strA1 abs*-120 SCP1$^-$ SCP2$^-$ Pgl$^-$	W. Champness
C120 SM1[b]	*hisA1 uraA1 strA1 abs*-120 *abs-SM1* SCP1$^-$ SCP2$^-$ Pgl$^-$	This Study
C120 SM7	*hisA1 uraA1 strA1 abs*-120 *abs-SM7* SCP1$^-$ SCP2$^-$ Pgl$^-$	This Study
C120 SM22	*hisA1 uraA1 strA1 abs*-120 *abs-SM22* SCP1$^-$ SCP2$^-$ Pgl	This Study
C120 SM23	*hisA1 uraA1 strA1 abs*-120 *abs-SM23* SCP1 SCP2 Pgl	This Study
8752	*hisA1 uraA1 strA1 abs-8752* SCP1$^-$ SCP2$^-$ Pgl$^-$	This Study

[a] Abbreviation : SCP1, *S. coelicolor* plasmid 1 (22); SCP2, *S. coelicolor* plasmid 2 (3); NF, SCP1 is integrated into the chromosome at 9 o'clock; pgl$^-$, φC31(9) sensitive.

[b] *abs*-SM stands for the suppressive mutation of *abs*.

plasmid SCP1, so SCP1 carrying *abs* suppressor strains were obtained by crossing the *abs* suppressor isolates with J650 (Table 1) and picking NF abs suppressor recombinants (see below). Methylenomycin production by these strains was tested by cross-streaking them against the methylenomycin-sensitive *S. coelicolor* J1501 SCP1$^-$. Methylenomycin activity was assessed at 3 days of growth at 28°C since methyenomycin is most active against late mycelia.

HPLC assay

25 µl of the first aqueous phase described in the antibiotics assay section was injected into a micro-Bondapak C18 (3.9 mm×300 mm) HPLC column. The flow rate was 0.5 ml/min. Water-acetonitrile (1:1) was used as a solvent. A wavelength of 254 nm was used to detect actionrhodin.

Results and Discussion

The selection principle we applied in finding the second site suppressor was that the particular candidates should be restored to produce the pigment antibiotic and, consequently, to form pigmented colonies so that we could visually select the candidates among the mutagenized colonies.

Theoretically, visualiy selecting the suppressors (or revertants) should be easier than selecting the *abs* mutants because the pigmented colonies can be easily distinguished from the unpigmented majorities. However, in practice, we encountered problems. Most of the mutagenized colonies could not grow properly on the minimal medium plate (on which the *abs*B does not produce any pigment). On the R_2YE plate, (16) which supports growth of most of the mutagenized colonies, absB mutants still produced 10~20% of the pigments that were produced in the J1501 abs$^+$ mother strain. Therefore, we developed a specially formulated minimal medium, namely GHUYC, in order to overcome this problem.

We also developed a low voltage electric shock (the detailed method will be reported elsewhere) method to facilitate the chemical mutagenesis (23). Most of the phenotypic revertants characterzed in this study were obtained through this method. As can be seen in Fig. 1, the survival rate from the chemical mutagenesis of *abs*B colonies with electric shock was reduced to half of that without electric shock.

Another problem we encountered was the frequent appearance of chloramphenicol-sensitive pigmented colonies. It has often been noticed that the deletion of some part of the chromosomal DNA containing a natural chloramphenicol-resistant determinant causes overproduction of pigmented antibiotics (Champness, W. personal communication). Some gene in the deleted DNA fragment may have suppressive effects on the overproduction of the pigments. It is reasonable to regard the chloramphenicol-sensitive pigmented colonies as a product of this deletion mutation, as the frequency of obtaining both phenotypes by base substitution would be rare. It might be interesting to look into the effects of this deletion. However, because we decided to concentrate on the point mutations in this study, we excluded the chloramphenicol sensitive colonies from the candidates.

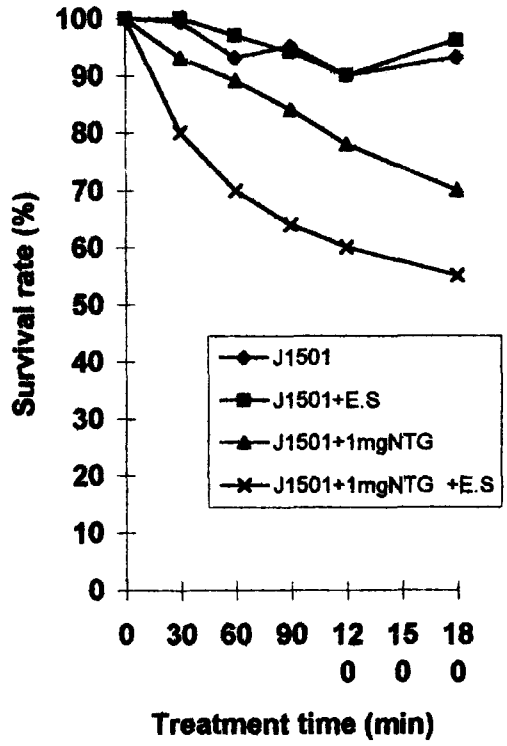

Fig. 1. Comparison of survival ratios between plain chemical mutagenesis (J1501+NTG) and chemical mutagenesis with electric shock. (J1501+NTG+E.S). S. coelicolor J1501 spores were incubated with NTG or NTG with electric shock during the given time. The survival rates were calculated after the treatment which is described in Materials and Methods.

From 20,000 mutagenized colonies, 24 pre candidates were selected on the bases of pigmentation (Table 2). These were further screened and 4 stable candidates were chosen.

The candidates produced significant amounts of pigments that had to have been a mixture of actinorhodin and undecylprodigiosin. We extracted the pigments from the mycelia and obtained two pigment fractions separately according to Horinouchi *et al.* (15). Because the HPLC retention times of the actinorhodin-like material from the

Table 2. The candidates of C120(*abs*B) revertants

Mutant Number	Medium			
	GHUYC[a]		GHUYC+Chloram[b]	
	Pigmentation	Sporulation	Pigmentation	Sporulation
C120 SM1	+++[c]	+++	+++	+++
SM2	++[d]	++	no growth	
SM3	++	++	–	+++
SM4	++	+[e]	++	+++
SM5	++	++	–	+++
SM6	++	++	no growth	
SM7	+++	+++	+++	++
SM8	–[f]	–	–	+++
SM9	+	++	–	+++
SM10	++	++	no growth	
SM11	+	++	–	+++
SM12	–	++	+	+++
SM13	++	++	–	+++
SM14	+	+	–	+++
SM15	–	–	+	+++
SM16	+++	++	no growth	
SM17	+	–	no growth	
SM18	++	++	++	++
SM19	+	+	–	+
SM21	+	+	–	+
SM22	+++	+	+++	+++
SM23	++	++	+	++
SM24	–	+	+	+
C120 (mother strain)	–	++	–	++

[a] Minimal medium added Yeast Extract 0.5~3% and Casaminoacid 0.1~0.01%.
[b] Minimal medium added Yeast Extract 0.5~3%, Casaminoacid 0.1~0.01% and chloramphenicol 10 mg/L.
[c] Overproduction of pogments or spores.
[d] Standard to high amount production of pigments or spores.
[e] Standard to low amount production of pigments or spores.
[f] No production of pogments or spores.
Pre-candidates: 24/20,066=0.12%
Candidates: 4/20,066=0.02% (C120 SM1, 7, 22, 23).

second site mutant strain C120-SM1 (3.28 min) and the strain J1501 (3.2 min) were very similar to each other, we could regard the pigments produced by C120-SM1 and other strains selected in this study as the same pigments produced by the *abs*B$^+$ strains (Fig. 3).

After passage of several generations, we quantitated the amount of pigmented antibiotics produced by the four candidates, C120-SM1, C120-SM 7, C120-SM22 and C120-SM23, respectively. As can be seen in Fig. 4, they all produced the pigments very extensively, but the levels of regained production were not same as that of the original *abs*B$^+$ strain J1501.

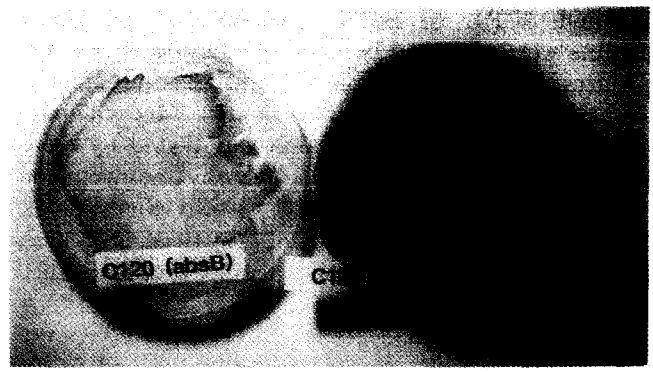

Fig. 2. The comparison between *abs* B strain and *abs* B-suppressor candidate strain (C120 SM1). C120 SM1 produced lots of pigment, and its mother strain C120 did not develop any color. Two strains were streaked on the R_2YE plate and incubated at 30°C for 4 days.

From this, the analyzed strains seem not to be exact revertants of the *abs*B mutant.

All 4 candidates overproduced actinorhodin 3-4 fold compared to *abs*$^+$ J1501 strain. This kind of effect would be expected if the mutated AbsB products have much stronger activating effects than the original AbsB product.

Although, we succeeded in generating new strains with increased actinorhodin production, the effect of the individual new regulators on the overproduction of undecylprodigiosin varied. The amount of undecylprodigiosin produced by C120-SM7 and C120-SM23 was less than that produced by *abs*$^+$ J1501

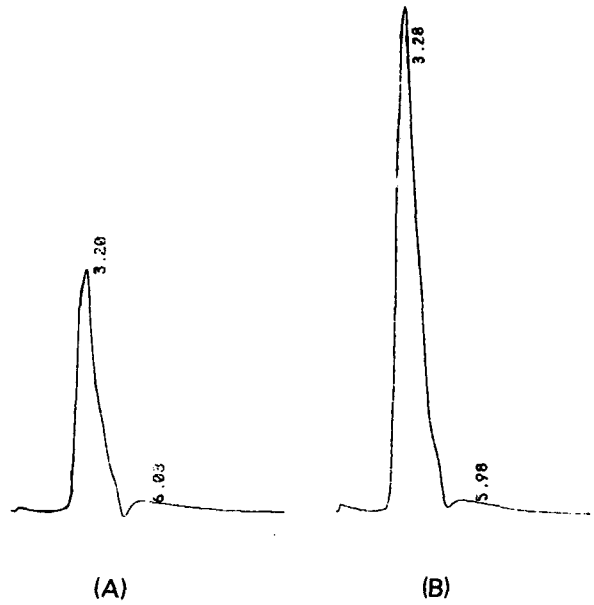

Fig. 3. The HPLC pattern of the actinorhodin like material (A) extracted from C120 SM1 and presumably the actinorhodin extracted from J1501 (*abs*$^+$) strain. Elution conditions are described in Materials and Methods.

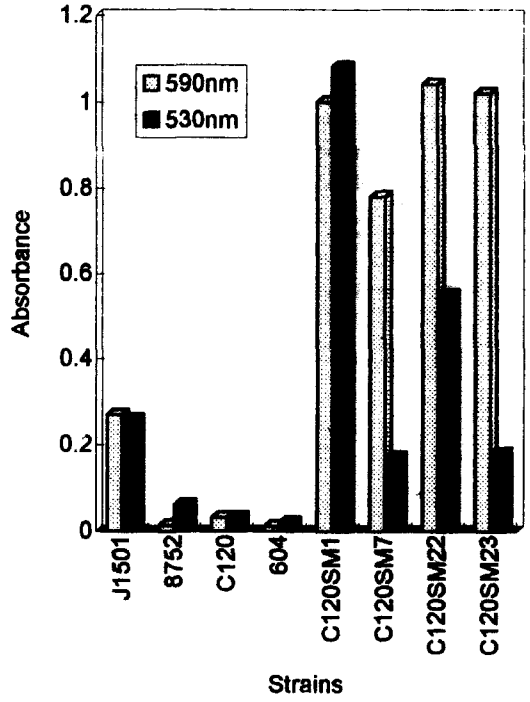

Fig. 4. Visual representation of the production of actinorhodin and undecylprodigiosin in *abs*$^+$ (J1501), *abs*$^-$ (C120, 8752), *act*$^-$*red*$^-$ double mutant (604), and *abs*$^-$-suppressor candidate (C120 SM1, C120 SM7, C120 SM22, C120 SM23) strains. Values represent the average of duplicates, extracted from 20 mg of mycelia grown on R2YE plates. Absorbance at 590 nm was the representative value for the amount of actinorhodin and the absorbance at 530 nm was that of undecylprodigiosin.

(although it was much higher than that by *abs*B mutant). C120-SM1 and C120-SM22 produced 2-3 fold more undecylprodigiosin than *abs*$^+$ J1501.

An explanation if all this might be that the new regulator(s) generated in the C120-SM1 have strong activating effects both on actinorhodin and undecylprodigiosin biosynthesis, while the new regulator(s) in the C120-SM7 or C120-SM23 interact strongly only with the actinorhodin biosynthetic pathway.

Among the four antibiotics produced by *S. coelicolor*, calcium dependent antibiotics(CDA) (18) and methylenomycin (22) do not have any color. A method for quantitating unpigmented antibiotics has not yet been developed. However, the production of unpigmented antibiotics can be qualitatively visualized by the growth inhibition assay. Since, the activities of the unpigmented antibiotics were not detected in the *abs*B, we tested whether the activities of the unpigmented antibiotics produced by the C 120-SM1 and C120-SM7 strains were detectable or not. In the CDA assay we detected quite distinctive halo around the SM1 and SM7 mycelia plugs (Materials and Methods). Such halos represent the growth inhibition of *S. aureus* by the CDA produced by the candidate strains.

In the methylenomycin assay (22), the growth inhibition of UF J1501 strains were observed.

Because in the C120 SM1 and C120 SM7 strains, all the four mutant phenotypes caused by *abs* mutation were restored to wild type phenotype (Table 3) by introducing the second mutation, we can call it a phenotypic suppressor mutation.

In order to analyze this suppression mutation further, we need to isolate the suppressor mutation apart from the original *abs*B mutation. We plan to remove the suppressor mutation to a new *abs*B strain using SCP2* mediated conjugation. Next, we need to prove that the suppressor mutation is a single mutation. We also need to identify its location so that we can tell whether it is in the *abs*B gene or another gene.

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Table 3. Summary of individual antibiotics activies of *abs*$^+$, *abs*$^-$, and *abs*-suppressor strains

Strains \ Antibiotics	Pigmented[a]		Unpigmented	
	Actinorhodin	Undecylprodigiosin	CDA	Methylenomycin[b]
J1501 (*abs*$^+$)	+[c]	+	+	+
C120 (*abs*$^-$)	–	–	–	–
C120 SM1 (*abs*-suppressor)	+++[d]	+++	+	+
C120 SM7 (*abs*-suppressor)	+++	+	+	+
C120 SM23 (*abs*-suppressor)	+++	+	N.D.	N.D.
604 (act$^-$, red$^-$)	–	–	+	+

[a] The amount of pigmented antibiotics was based on the quantitative assay described in Materials and Methods
[b] CDA assay and methylenomycin assay were performed as described in the Material and Method. These assays can not be quantitated. + indicates a positive result and - indicates a negative result.
[c] The amount of antibiotics produced by J1501 (*abs*$^+$, mother strain of C120) which was used as the standard strain.
[d] Overproduction of given antibiotics.

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