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Genetic Analysis of *absR*, a new *abs* locus of *Streptomyces coelicolor*

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Abstract The filamentous soil bacterium *Streptomyces coelicolor* is known to produce four distinct antibiotics. The simultaneous global regulation for the biosynthesis of those four antibiotics was previously confirmed by *absA* and *absB* mutations that blocked all four antibiotics' biosynthesis without influencing their morphological differentiation. To study the complex regulatory cascade that controls the secondary metabolism in *Streptomyces*, a new *abs*-like mutation was characterized, namely *absR*, which is slightly leaky on a complete R2YE medium, yet tight on a minimal medium. A genetic analysis of the *absR* locus indicated that it is located at 10 o'clock on the genetic map, near the site of *absA*. A cloned copy of the *absA* gene that encoded bacterial two-component regulatory kinases did not restore antibiotic biosynthesis to the *absR* mutant. Accordingly, it is proposed that *absR* is another *abs*-type mutation which is less tight than the previously identified *absA* or *absB* mutations in some medium conditions, and can be used to characterize another global regulatory gene for secondary metabolite formation in *S. coelicolor*.

Key words: *Streptomyces coelicolor*, global regulation, secondary metabolism, *absR* mutation

Antibiotics are the most medically beneficial metabolites produced by *Streptomyces* spp. By inhibiting the growth of other microorganisms, antibiotics can help Streptomycetes to retain a relative advantage for better propagation [33].

Various physiological factors including growth rate, responsiveness to catabolite repression, environmental signals and stress may influence antibiotic biosynthesis. Accordingly, many kinds of regulators are expected to be involved in monitoring these various stimuli [8, 35].

Genetic studies on the four antibiotics of *S. coelicolor* have progressed to delineate all of the biosynthetic gene clusters that have been mutationally defined and mapped. Three gene clusters have been cloned; *act* [28], *red* [30], and *mmy* [9]. The 22-kbp *act* gene cluster encodes many open reading frames, expressed in at least six transcripts [29]. The *actII*-ORF4 gene encodes a gene product required for the transcription of the *act* biosynthetic genes [12]. The *red* cluster is also large and complex and includes a gene, *redD*, which is the transcriptional activator [11]. A regulatory protein which only influences a specific pathway is called a pathway-specific regulator. In contrast, where the onset of the biosynthesis of the four antibiotics is coordinately regulated, several types of global regulatory genes involved in several antibiotic syntheses have been isolated in *S. coelicolor* [7, 19].

A mutation in the global regulators, such as the *bld* genes [26, 32], inhibits not only the synthesis of all four antibiotics, but also the production of aerial mycelium and spores. The *bld*-type mutations have been regarded as the evidence that the sporulation and production of the four antibiotics by *S. coelicolor* are subject to a common genetic control.

Alternatively, the *abs*[-] (**a**nti**b**iotic **s**ynthesis deficient) phenotype suggests the existence of a global regulatory mechanism that is specific to antibiotic synthesis and distinct from the control mechanism leading to sporulation [6, 13].

A single mutation (*abs*[-] type) that completely blocks the production of the four *S. coelicolor* antibiotics, and yet allows abundant sporulation, confirms the previously discovered *absA* or *absB* locus [3]. The phenotypes of theses *abs* mutants have been observed very carefully [2]. The *absA* and *absB* mutants are quite tight and stable, accordingly, the concept of global regulators specifically assigned to antibiotic synthesis has been generally accepted [7, 8].

This study includes a less tight *abs* mutant on a R2YE complete medium. This new *abs* mutant, namely *absR*, exhibits conditional leakiness because it is not leaky on any other medium except R2YE. Since the *absR* mutant is believed to be another type of mutant which has a deficiency in the biosynthesis of all four antibiotics, it was

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anticipated that the *absR* mutant would have a mutation(s) in some of its regulatory genes which would control the biosynthesis of the four antibiotics in *S. coelicolor*. This mutant strain will be further studied to characterize a new regulatory gene that produces secondary metabolism in *Streptomyces*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The strains used for the genetic analysis were derivatives of *S. coelicolor* A3(2) (Table 1). The plasmids and the bacteriophage used were the same as those described by Brian *et al.* [4]. The transformation and infection were performed as previously described [17, 25]. The *abs8752* strain was kindly provided by Dr. Wendy Champness at the Michigan State University.

Media and Culture Conditions

The minimal plate medium used was previously described by Hopwood *et al.* [17]. The YEG contained 1% yeast extract and 1% glucose; SY contained 0.3% yeast extract and 1% starch [12]. *Streptomyces* strains were maintained on R2YE agar containing the following ingredients in one liter; 103 g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2 \cdot 6H_2O$, 10 g glucose, 0.1 g casamino acid, 5 g yeast extract, 10 ml of 0.5% K_2HPO_4, 80 ml of 3.68% $CaCl_2 \cdot 2H_2O$, 15 ml of 20% L-proline, 100 ml of 5.73% of TES (pH 7.2), 2 ml of trace elements solution, and 2.2% agar [25]. The above described media were supplemented with histidine and uracil at 50 and 7.5 μg/ml, respectively. Thiostrepton (Sigma Chemical Co.) was used at a concentration of 50 μg/ml [23].

Antibiotic Assays

Assays for the detection of actinorhodin, undecylprodigiosin, calcium-dependent antibiotic (CDA), and methylenomycin were performed as previously described [3]. For the methylenomycin assays, a R8752 derivative carrying the methylenomycin-encoding plasmid SCP1, was used. This strain was constructed by crossing SCP1 from the J650 strain. For the actinorhodin and undecylprodigiosin quantitations, the tested strains were streaked onto cellulose-acetate filters on a R2YE medium. After 5 to 6 days, the mycelia were scraped off and weighed. Approximately 20 mg was extracted with 0.5 ml of chloroform for 30 min at room temperature with shaking. Then, 0.5 ml of 1 N NaOH was added, and the tubes were vortexed and spun in a microcentrifuge for 15 s. The aqueous phase contained actinorhodin, which indicated blue at an alkaline pH. The A_{590} of the aqueous phase was determined. The chloroform phase contained the undecylprodigiosin which was yellow. For the absorbance measurements of undecylprodigiosin, the chloroform layer was acidified with HCl, and the A_{530} of the now-red chloroform phase was then determined.

Mutagenesis and Mutant Isolation

Mutagenesis and mutant isolation were carried out as described previously [3, 5]. Spores of *S. coelicolor* J1501 were mutagenized by UV or NTG. The mutagenized spores were then washed twice with a R2YE liquid medium, and finally resuspended in a 20% glycerol solution. The mutagenized spores were serially diluted in the 20% glycerol solution and 0.1 ml from each diluted sample was spread on a R2YE medium. After 4–7 days incubation at 30°C, the candidates for *abs* mutants were selected according to their inability to form pigment on a solid medium, and then further analyzed.

Table 1. List of strains, plasmids and phage used.

Strains and DNAs	Relevant genotypes[a]	Source
S. coelicolor		
R8752	*hisA1 uraA1 strA1 abs8752* $SCP1^-$ $SCP2^-$ Pgl^-	W. Champness
R87521	*hisA1 strA1 abs8752 agaA1* NF	This work
R87522	*proA1 cysA1 strA1 abs8752 agaA1* NF	This work
R87523	*hisA1 uraA1 strA1 abs8752 agaA1* NF	This work
C120	*hisA1 uraA1 strA1 abs120* $SCP1^-$ $SCP2^-$ Pgl^-	W. Champness
C577	*hisA1 uraA1 strA1 abs577* $SCP1^-$ $SCP2^-$ Pgl^-	W. Champness
1514	*proA1 cysA1 argA1 uraA1 nicA7 agaA1* NF $SCP2^+$	K. Chater
J650	*cysD18 mthB2 agaA1* NF $SCP2^+$	K. Chater
J1501	*hisA1 uraA1 strA1* $SCP1^-$ $SCP2^-$ Pgl^-	K. Chater
Plasmids		
pCB620	pIJ922 carrying 3.2-kb *absA* insert	W. Champness
pIJ922	Low copy-number plasmid originated from SCP2	D. A. Hopwood
Bacteriophage		
RS100	KC516 carrying 3.2-kb *Xho*I (*absA*) insert	W. Champness

[a]Abbreviation: SCP1, *S. coelicolor* plasmid 1; SCP2, *S. coelicolor* plasmid 2. NF. SCP1 is integrated into the chromosome at 9 o'clock; Pgl^-, ΦC31 sensitive.

Genetic Mapping Techniques

Crosses and data analyses were done as described previously [2, 6]. The chromosomal recombination was mediated primarily by the SCP1 plasmid, integrated at 9 o'clock on the genetic map to give the NF (Natural Fertility) type [35]. Several phenotypes are associated with the NF state. NF strains are Aga⁻ because they fail to produce agarase for which the gene is deleted when SCP1 integrates at 9 o'clock on the chromosome. Two strains can be easily differentiated by visual observation since Aga⁻ strains sink down into the agar whereas Aga⁻ strains do not. In addition, NF strains are normally methylenomycin producers and are methylenomycin resistant, since the production and resistance genes for this antibiotic are carried by SCP1. In *S. coelicolor* A3(2), the SCP1 plasmid interacted with the chromosome to form various donor strains which yield very high recombinant frequencies in matings with SCP1⁻ (UF: Ultra Fertility) strains. When the SCP1-plasmid integrated into an agarase gene at about a 9 o'clock position, the so-called NF strain can be obtained. In an NF (like J650) × UF (SCP1⁻ like R8752) cross, close to 100% of the progeny will be NF. For mapping the *abs* mutations, biased recombination was avoided in some crosses by using NF Abs⁻ strains derived from primary crosses in subsequent crosses with NF strains carrying standard markers. NF Abs⁻ strains were selected based on their Aga⁻ phenotype.

RESULTS AND DISCUSSION

Isolation and Characterization of Abs8752 Mutants

As previously reported, *absA* and *absB* mutants exhibit a tight Abs⁻ phenotype by producing almost none of the four antibiotics. In the screening process of the *absB* mutants, less tight *abs*-type mutant candidates were also isolated from *S. coelicolor* J1501. Since much efforts were already made in regards to *absA* and *absB* mutants, these mutants were not analyzed further at the time, in Dr. Champness' laboratory. Dr. Champness graciously gave the *abs8752* strain to the authors for the further analysis.

As described in previous reports on *absB* mutants [2], UV mutagenesis and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis were used in the screening process. Usually, *absB* mutants were isolated at about one in 10,000 survivors of mutagenesis. When compared to *absA* and *absB*, one Abs⁻ strain numbered as R8752 developed a little bit of color on the R2YE plate (Fig. 1), which indicated a residual production of the pigmented antibiotics, actinorhodin [29] and undecylprodigiosin [11]. In R2YE liquid culture, R8752 produced 1.5 μg of actinorhodin and 0.22 μg of undecylprodigiosin per ml of culture broth, which corresponds to one tenth and one fifth of those produced by J1501 strain, respectively. However, no color

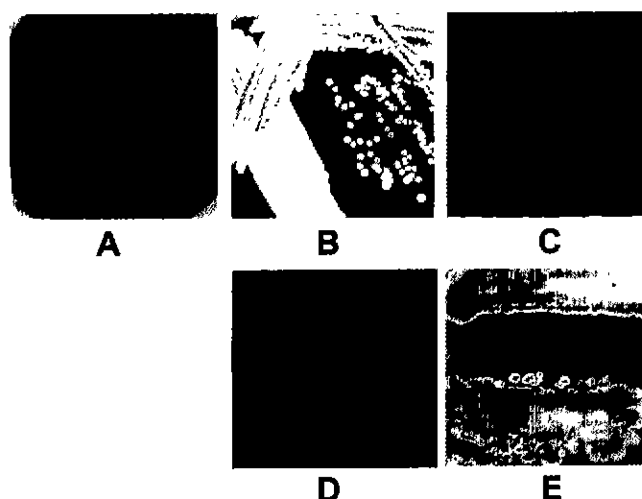

Fig. 1. Photographs of the plates.
Each strain was grown in R2YE medium at 30°C for 7 days. *S. coelicolor* J1501 as a control shows a typical red-pigmented color (A) and C577 with *absA⁻* gives a white color because of the lack of pigmented antibiotics (B). *S. coelicolor* C120 with *absB⁻* (C) and R8752 with *abs8752⁻* (D) show a weak orange color to the same extent because of incomplete blockage for the production of pigments. *S. coelicolor* R87523 with *abs8752⁻* in a clean background (E) gives a weaker color than that of R8752.

was developed on a minimal medium with glucose or maltose and on SY or YEG plates or in liquid culture. High concentrations of sucrose usually induce mass production of pigmented antibiotics in *S. coelicolor* [8]. It also directs actinorhodin and undecylprodigiosin production in *S. lividans* in which the biosynthetic genes are present in a silent state [20]. The exact mechanism for this observation cannot be explained at present, but many factors including metabolic imbalance can induce antibiotic production in *Streptomyces*. The leakiness of the Abs8752 phenotype might be due to the induction of the antibiotics production by the high concentration of sucrose, which would not be completely repressed by the mutation. In fact, it is also known that the *absB⁻* mutant is not a completely blocked mutant for all antibiotic synthesis as in the *absA⁻* mutant [2].

An Abs⁻ mutant strain containing the Abs8752 mutation was constructed on a clean background, in order to test just the effect of the *abs8752* locus. After crossing of the R8752 strain with strains 1514 and J650, and backcrossing into J1501 (Fig. 2B), the strain with the Abs8752 mutation was isolated on a clean J1501 background (see next section for details) and named R87523. This strain exhibited less leakiness on the R2YE plate than R8752, yet still showed some leakiness (Fig. 1). Table 2 illustrates the medium dependency of the leakiness of the R8752 and R87523 strains. Actinorhodin and undecylprodigiosin production was quantitated on the R2YE medium (Table 3) as described in Materials and Methods. The phenotype of R8752 strains was leakier than the AbsB⁻ phenotype, however, it produced

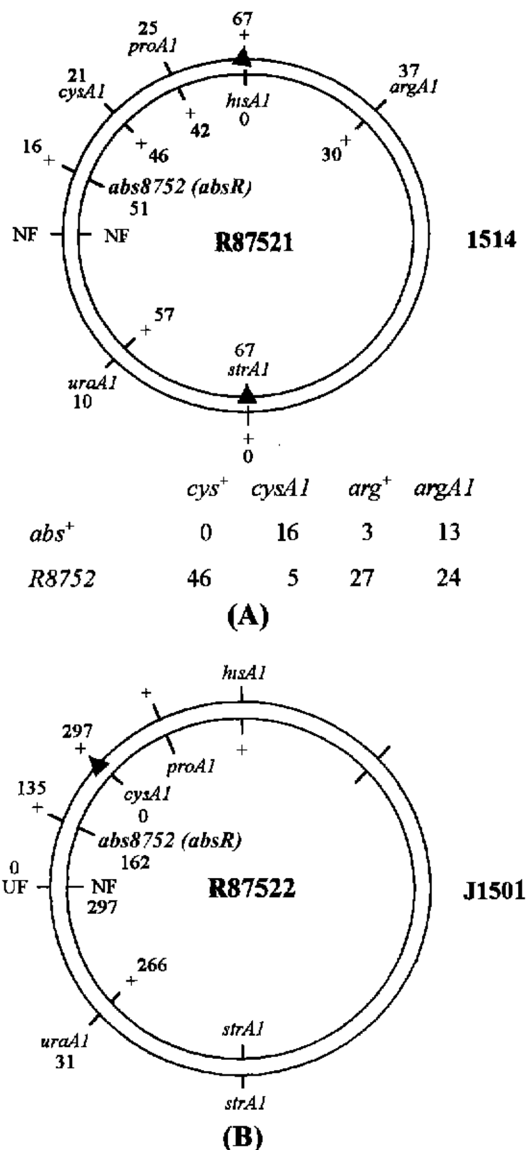

Fig. 2. Genetic maps of *absR* mutants.
The inner and outer circles represent the parental chromosomes involved in each cross. The triangles indicate the alleles used to select the recombinations in one set of selective conditions (A) Crosses between SCP⁻ NF *absR* mutant derivative (R87521) and strain 1514, and the data obtained from the frequency at which Abs⁻ progeny occurred. (B) Crosses between another *absR* mutant derivative (R87522) and J1501 to construct a new *absR* mutant in a clean background. The analysis of the data obtained from the frequency at which Abs⁻ progeny occurred is explained in the text.

much smaller amounts of pigmented antibiotics than the J1501 mother strain. The levels of the unpigmented antibiotics, calcium-dependent antibiotics [18], and methylenomycin [24] were also tested. The amount of the calcium-dependent antibiotics was also greatly reduced in the *abs8752* mutant. No clear differences were found between the *absB* and R8752 mutants in the CDA assay that was applied. Because methylenomycin production and resistance genes are carried on the SCP1 plasmid, SCP1⁺ derivatives of the R8752 mutants (namely R87521) were constructed as described in Material and Methods. Although the effect was not complete, the production of methylenomycin appeared to be greatly reduced, since the effect of growth inhibition was negligible (Table 4).

Genetic Analysis of *Abs8752* (*absR*) Mutants

Crosses in *Streptomyces* influences culture conditions for two different strains on the surface of an agar medium. There is no equivalent of the familiar interrupted mating of *E. coli* genetics. Instead, many rounds of chromosomal DNA exchanges between both the parental strains gives the most reliable mapping data in *Streptomyces*.

In *S. coelicolor* A3(2), when the NF strain (like J650) mates with a UF strain (SCP1⁻ like R8752), the frequency of the recombination, especially in the 9 o'clock region including the SCP1 plasmid, is extremely high. However, this type of crossing cannot provide many mapping data, because the exchange of chromosomal DNA is neither universal nor random. Meaningful mapping data can be obtained from NF × NF crosses.

In the initial crossing between the original *abs* isolates, R8752 (UF) and J650 (NF), the recombinant progeny sorted into two phenotypic groups, Abs⁻ and Abs⁺, suggesting that a single mutant locus is responsible for the *abs*⁻ phenotype. However, the data was not analyzed any further because the cross was UF × NF. For more meaningful data, one SCP1⁺ and an NF strain with an *abs8752* mutation, namely R87521, were isolated from the progeny.

Next, R87521 was crossed with another NF strain 1514. The efficiency of the DNA exchange in the NF × NF cross was quite low, as a selection against the parents was necessary. Section was for histidine prototrophy (*hisA*⁺) as well as streptomycin resistance (*strA1*). The resulting progenies were then scored according to various markers from the parents. The allele ratios at the selected loci had to be 100%:0% and 0%:100%, whereas the allele ratios at non-selected loci could be intermediate values, falling on a continuous gradient in each of the two arcs. As can be seen in Fig. 2A, the known markers like *hisA1*, *proA1*⁺, *cysA1*⁺, *uraA1*⁺, and *strA1* exhibited gradually increasing values. Since 51 Abs8752⁻ progenies were obtained, it seems reasonable to assign *abs8752* between the *cysA1* to *uraA1* interval. However, *abs8752* could be assigned to an alternative position between *argA1*⁺ and *strA1*. The choice between these two alternative positions derived from the frequencies of the particular genotype of the recombinants. Most *abs8752* mutant alleles were found with *cysA*⁺ (see box in Fig. 2A). In other words, the frequency of the cosegregation of *abs8752* and *cysA*⁺ is very high. This suggests that the map location of *abs8752* should be within the *cysA1* to *uraA1* interval.

Table 2. Production of antibiotics by various *abs* mutant strains depending on medium type.

Strains	R2YE	Minimal with glucose	Minimal with maltose	SY[a]	YEG[b]
C577 (with *absA*⁻)	-	-	-	-	-
C120 (with *absB*⁻)	-	-	-	-	-
R8752 (with *abs8752*⁻)	+	-	-	- (poor sporulation)	- (wrinkled sporulation)
R87523 (*abs8752* in clean background)	«+	-	-	-	-

[a]SY: 0.3% yeast extract and 1% starch.
[b]YEG: 1% yeast extract and 1% glucose.
*-, no detectable antibiotic produced; +, small amount produced; «+, smaller amount produced compared to that produced by R8752.

Table 3. Measurements of actinorhodin (A_{590}) and undecylprodigiosin (A_{530}) production in *absR* and *absB* mutants in R2YE medium.

Strains	Color development	
	A_{590}[a]	A_{530}[b]
J1501 (wild-type)	0.45	1.05
C120 (*absB*)	0.03	0.17
R8752 (*abs8752*)	0.05	0.32
R87523 (*abs8752*)	0.04	0.25

[a]The amount of actinorhodin was measured by a spectrophotometric assay (A_{590}) of the alkaline aqueous phase after extraction with chloroform as described in Materials and Methods.
[b]The amount of undecylprodigiosin was measured by a spectrophotometric assay (A_{530}) under acidic conditions of the chloroform layer after extraction as described above.

Apart from the linkage mapping, crosses are useful for strain construction, the preparation of new combinations of chromosomal mutations, or for the cleaning of the background of a mutagenized strain. The R8752 mutant strain was constructed in a relatively clean background as follows: From the cross between R87521 and 1514, another R8752 derivative strain, R87522, was chosen with *cysA1*, *hisA*⁺, *abs8752*, and NF markers. This R87522 strain was then used in a backcross to the UF J1501 strain (Fig. 2B). Advantage was taken to use the characteristic of an NF to UF cross, whereby only a small portion of the DNA near 9 o'clock including the *abs8752* allele was integrated into a new clean J1501 (UF) strain. The result of such a cross is denoted in Fig. 2B. Among the 297 *cysA1*⁺, NF progenies, 162 progeny colonies were Abs⁻. The clockwise portion of the recombinants' chromosomal DNA from the *cysA1* has a very high probability of being derived from J1501. In the recombinant strain with the *uraA1* marker, the counterclockwise portion of chromosomal DNA from *uraA1* was also probably derived from the J1501 strain which provided the *uraA1* marker. Only a small portion of the R87522 DNA, including the *abs8752* and NF markers, seemed to be integrated into the J1501 strain in the proper recombinants. As a result, one NF recombinant including the *hisA1*, *uraA1*, and *abs8752* markers was selected as an *abs8752* mutant in a clean background.

Table 4. Effect of various *abs* mutations on the production of each antibiotic.

Mutations	Antibiotics			
	Undecylprodigiosin[a]	Actinorhodin[a]	CDA[b]	Methylenomycin[b]
AbsA	-	-	-	-
AbsB	-	-	-	-
Abs8752 (In R8752)	+	+	«+	«+
Abs87523 (In R87523)	«+	«+	«+	«+

[a]The amount of pigmented antibiotics was based on the quantitative assay described in Materials and Methods.
[b]CDA and methylenomycin assays were performed as described in Materials and Methods. These assays could not be quantitated.
*Symbols; -, no detectable antibiotic produced; +, small amount produced; «+, smaller amount produced compared to that produced by R8752.

The gradients and linkages which appeared in the backcrossing results suggested that the map location of *abs8752* lies within the interval between NF and *cysA*.

Suppression Test by Cloned *absA*

Since the map position of *abs8752* was very near the *absA* site, it was decided to test whether *abs8752* is in fact the allele of *absA*. A 3.2 kb insert of *absA* was cloned to the low copy plasmid PIJ922 [4] and named pCB620. The plasmid pCB620 [4] was then introduced into the R8752 strain; however, no antibiotic production was observed. This plasmid restored antibiotic production in all previously identified *absA*⁻ mutants. In addition, the R8752 strain was infected by a bacteriophage [27] using the cloned *absA* gene, RS100 [4], and no pigment production was identified. Therefore, it is unlikely that *abs8752* is another allele of *absA*.

From the mapping and segregation experiments, the Abs8752⁻ phenotype behaved as a single mutation blocking the synthesis of the 4 antibiotics. However its map location is far from the *absB* locus, and it is not suppressed by the *absA* gene. Accordingly, it is proposed that Abs8752 be added as a new member of the *abs* mutants, which is less tight than the previously identified *absA* or *absB* mutations. Therefore, the locus for the Abs8752 mutation was named as the *absR* (R standing for regulation).

Many global regulatory genes which control the secondary metabolism or morphogenesis in *S. coelicolor* have been reported, including *bldA*, *afsR/afsK* [15, 31], *afsQ1/afsQ2* [22] and *absA1/absA2* [4], *absB* [2], and *ptpA* [34] which encodes leu-tRNA, ser/thr/tyr-protein kinases, a typical bacterial two component his- and asp-kinases, putative RNase III, and phosphotyrosine protein phosphatase, respectively. All of these regulatory genes were identified using unique mutant strains which blocked antibiotic biosynthesis or cell differentiation. The data from the current study clearly shows that the *absR* mutant exhibits different characteristics from those mutant strains already reported. In addition, the *afsB* gene [20] containing an N-terminal half coding region of the AfsR [21] was able to confer the ability to produce actinorhodin and undecylprodogiosin in the *absR* mutant, yet *act*II-ORF4 [12], a pathway-specific regulatory gene, only induced actinorhodin production (data not shown). These results suggest that the *absR* mutant may have a deficiency in some regulatory gene(s) whose product may be positioned between downstream of AbsA1/A2 and upstream of AfsR in the regulatory hierarchy. The involvement of many eukaryotic-type protein kinases in controlling secondary metabolism and cell differentiation was proved by *in vitro* protein phosphorylation and the *in vivo* effects of protein kinase inhibitors in *S. coelicolor* [14] and *S. griseus* [16]. These observations imply that there are many unidentified genes which play critical roles in the regulatory cascade of *Streptomyces*. Therefore, it is anticipated that a new regulatory gene may be caught by using the *absR* mutant, which will be helpful in studying the complex regulatory cascade that controls secondary metabolism in *S. coelicolor.*

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